In vitro reporter gene assays for assessment of PPAR- and Nrf2-mediated health effects of tomato and its bioactive constituents

Linda Gijsbers

THESIS COMMITTEE

Promotors
Prof. dr. ir. I.M.C.M. Rietjens
Professor of Toxicology
Wageningen University

Prof. dr. ir. J. Keijer
Professor of Human and Animal Physiology
Wageningen University

Co-promotor
Dr. ir. J.M.M.J.G. Aarts
Assistant professor, Sub-department of Toxicology
Wageningen University

Other members
Prof. dr. R.J. Bino, Wageningen University
Prof. dr. A. Bast, Maastricht University
Dr. ir. P.C.H. Hollman, RIKILT, Wageningen UR
Dr. ir. H.T. Besselink, BioDetection Systems, Amsterdam

This research was conducted under the auspices of the Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences).

In vitro reporter gene assays for assessment of PPAR- and Nrf2-mediated health effects of tomato and its bioactive constituents

Linda Gijsbers

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. dr. M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 8 March 2013
at 11 a.m. in the Aula.

Linda Gijsbers

In vitro reporter gene assays for assessment of PPAR- and Nrf2-mediated health effects of tomato and its bioactive constituents

PhD thesis, Wageningen University, Wageningen, NL (2013)

With references, with summaries in Dutch and English

ISBN 978-94-6173-509-6

TABLE OF CONTENTS

1

INTRODUCTION

FUNCTIONAL FOODS AND BIOACTIVE COMPOUNDS

In the last years, the number of functional foods on the market, such as margarines with plant sterols, fruit juice enriched with calcium and cereals with (soluble) fibre, has been growing steadily (*1-3*). The world health organisation (WHO) defines a functional food as "any food claiming to have a health-promoting or disease-preventing property beyond the basic function of supplying nutrients". Using this definition, not only processed food items, but also natural products can be considered as functional foods. Fruits and vegetables are an example of natural foods with additional health-promoting properties; their consumption has been related to many health-promoting effects, including decreased risk for cardiovascular diseases and diabetes mellitus (*4-6*). Therefore, fruits and vegetables can be considered as functional foods (*1, 7*).

The health-promoting effects of functional foods may be (partly) due to the presence of bioactive compounds (*8, 9*). Several definitions of bioactive compounds have been adopted, all sharing the main notion that bioactive compounds are food components that have a beneficial effect on human health (*10, 11*). Many authors exclude nutrients from the definition of bioactive compounds (*11-13*), but some include macronutrients and micronutrients (*10*). As fatty acids and their derivatives also have been related to beneficial health effects (*14*), we choose here to use the more general definition that includes nutrients as possible bioactive compounds. The number of food products with possible additional health benefits, including functional foods, has increased over the past years. In January 2007, the new EU regulation 1924/2006 became effective, stating that nutrition and health claims for functional foods are not allowed unless the claim is approved by the European Commission, based on advice given by the European Food Safety Authority (EFSA)(*15, 16*). In this regulation, two categories of claims are identified: nutrition claims and health claims. A nutrition claim states that a food has a specific beneficial nutritional property, for example that the food is containing calcium or that it is low in sugar. Health claims are defined as "any claim that states, suggests or implies that a relationship exists between a food category, a food or one of its constituents and health" (*15, 16*). Nowadays, the market for functional foods is rapidly growing and given the EU requirements, there is a major interest of food industry to scientifically support beneficial effects of functional foods and their bioactive ingredients. To achieve this, fast and low-cost tools for identification of bio-functional characteristics of food items and food compounds are essential. Until now, research on the health-beneficial effects of food products and food compounds has been mainly based on animal studies, human intervention studies or epidemiologic studies, which are all laborious and expensive. Reporter gene assays are a fast and low-cost alternative to investigate bio-functional characteristics of food compounds and food items. As the activity of various transcription factors has been strongly related to beneficial health effects (*17, 18*), reporter gene assays for such health-related transcription factors may provide first-level support in a tiered approach to support nutrition and health claims for bioactive ingredients.

AIMS OF THE THESIS

The aims of this thesis were:

1. To develop and validate stable reporter cell lines to facilitate high-throughput screening of natural and synthetic compounds for several health-related functions.
2. To show that reporter gene assays provide a tool to link fruits and vegetables to specific health-beneficial pathways, by assessing the activity of tomato extracts in these reporter gene assays.
3. To verify that reporter gene assays are useful tools in selecting health-beneficial traits of crop varieties, again using tomato fruit as a model.

To achieve these aims, three health-related gene expression pathways were chosen; Nrf2/EpRE-mediated gene expression, PPARγ-mediated gene expression and PPARα-mediated gene expression.

SELECTED HEALTH-RELATED GENE EXPRESSION PATHWAYS

Nrf2/EpRE

The nuclear factor E2-related factor (Nrf2) is a transcription factor able to regulate expression of several genes. Normally, Nrf2 is present in the cytoplasm of cells in its inactive form: bound to Kelch-like ECH-associated protein 1 (Keap1). Once the cell is exposed to Nrf2-activating compounds, this leads to the release of Nrf2 from Keap1. Once released from Keap1, Nrf2 migrates into the nucleus where it forms a complex

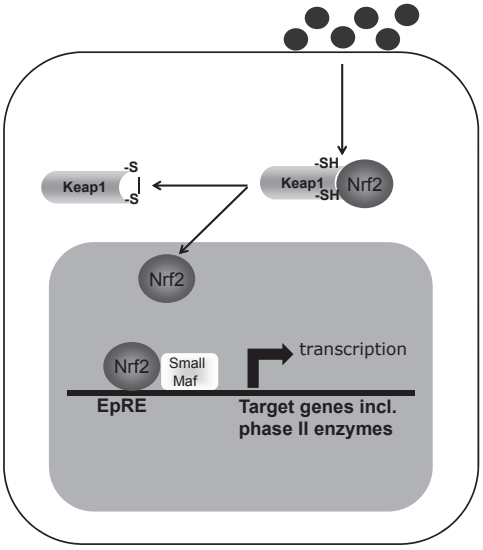

Figure 1. In the presence of Nrf2-activating compounds, Nrf2 is released from Keap1 and moves into the nucleus. In the nucleus, it forms a complex with Small Maf proteins and this complex binds to the EpRE, thereby enhancing transcription of the target genes.

with Small Maf proteins and subsequently binds to the electrophile-responsive element (EpRE) in the regulatory domains of the Nrf2 target genes (figure 1). The expression of these target genes is thereby enhanced. Genes regulated by Nrf2/EpRE include phase II detoxifying enzymes, which catalyse reduction or conjugation of reactive electrophiles. As a result, metabolites are formed which are less toxic and more easily excreted from the body (*17, 19*). In this way, phase II enzymes are able to detoxify harmful (e.g. carcinogenic) metabolites. Therefore, EpRE-mediated gene expression is chosen as one of the health-beneficial endpoints for this thesis. In this thesis, the EpRE-LUX reporter assay, which was earlier described by Boerboom and colleagues, was used to measure Nrf2/EpRE-mediated effects on gene expression (*20*). Nrf2-activating compounds include for example flavonoids such asquercetin and kaempferol (*20-22*) and isothiocyanates (*23*).

PPARs

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear receptor superfamily (*24*). Ligands for PPARs include for example fatty acids and their derivatives, but also synthetic ligands have been produced, mainly for pharmaceutical applications (*25*). Once activated by ligands, PPARs form a complex with retinoid X receptor (RXR) and bind to the peroxisome proliferator-responsive element (PPRE) in the regulatory regions of their target genes (*26*). In this way, activation of PPARs influences the expression of these target genes (figure 2).

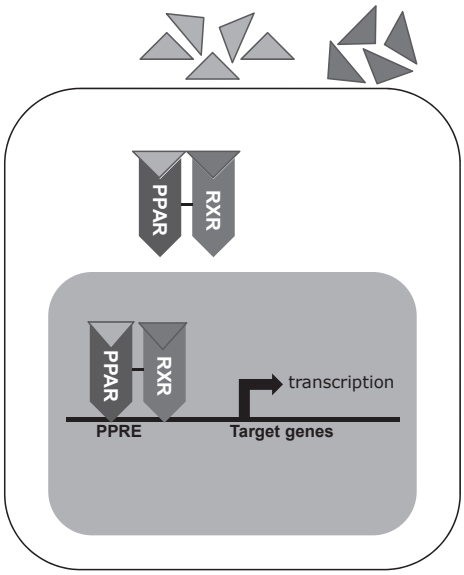

Figure 2. PPAR forms a heterodimer with RXR. Upon activation of the PPAR-RXR complex, it binds to the PPRE and enhances transcription of the target genes.

Three PPAR isoforms are known: PPARα (NR1C1), PPARβ/δ (NR1C2) and PPARγ (NR1C3). These isoforms differ in their tissue distribution and their response to certain ligands. Furthermore, the three isoforms have their own set of target genes, which partially overlap (*27*). By enhancing the transcription of their target genes, PPARs play key roles in important physiological processes. For example, PPARα is known for its role in lipid metabolism (*28, 29*) and PPARγ is essential for adipogenesis (*30, 31*). PPARβ/δ has more pleiotropic effects and plays a role in various processes, for example in lipid and lipoprotein metatolism (*32, 33*), inflammation (*34*), and wound healing (*35, 36*). In the next sections, PPARα and PPARγ, for which reporter gene assays were developed in the present thesis, will be discussed in some more detail.

PPARα

PPARα was the first PPAR isoform discovered and is mainly present in liver, skeletal muscle and brown adipose tissue (*37*). Several fatty acids and their derivatives, including arachidonic acid, linolenic acid, linoleic acid and eicosanoids, function as endogenous ligands for PPARα, while fibrates are a potent class of exogenous ligands (*25, 38*). Fibrates are a class of lipid-lowering drugs including gemfibrozil, clofibrate, fenofibrate, bezafibrate and ciprofibrate.

PPARα is an important factor in lipid metabolism (*28, 29*) and its target genes include many genes involved in uptake and oxidation of fatty acids (*28, 39, 40*). Activation of PPARα by fibrates results in decreased plasma levels of triglycerides and LDL cholesterol, and in increased HDL cholesterol levels (*41-43*). As high levels of triglycerides and LDL cholesterol and low levels of HDL cholesterol are risk factors for atherosclerosis and other cardiovascular diseases (CVDs), fibrates in this way may decrease the risk for developing atherosclerosis or CVDs. Indeed, several authors report a decreased risk for CVDs after treatment with fibrates (*44-46*). Besides its protective role in CVDs, PPARα-mediated gene expression has also been related to improved insulin sensitivity (*42, 47*). An overview of possible health-beneficial effects of PPARα-activation is given in table 1.

PPARγ

PPARγ is mainly expressed in adipose tissue, but is also present in for example colon, liver and kidney (*48*). PPARγ is activated by both endogenous and synthetic ligands. Endogenous ligands include fatty acids and their derivatives, such as 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15d-PGJ2), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)(*25*). Thiazolidinediones (TZDs) are the most well-known synthetic PPARγ agonists and include, amongst others, rosiglitazone, troglitazone and pioglitazone. In humans, PPARγ is present in two isotypes: PPARγ1 and PPARγ2, generated from the same gene by alternative promoter usage and mRNA splicing (*48, 69*). PPARγ1 and PPARγ2 differ in the N-terminus of the protein; PPARγ2 contains 28 extra amino acids at this site. There is also a difference in expression pattern between the isoforms; PPARγ1 is expressed broadly and is present in adipose tissue, spleen, liver, skeletal muscle, heart, large intestine and kidney. PPARγ2 is mainly expressed in adipose tissue and in lower amounts in liver and skeletal muscle (*48, 70, 71*). It is not completely clear to what extent the two subtypes

Table 1. Overview of beneficial health effects mediated by PPARα agonists and corresponding literature references.

Physiological process or disease	References
Cardiovascular diseases (CVD)	
Reduced risk for cardiovascular events	(*44-46*)
Decreased levels of plasma triglycerides	(*42, 49, 50*)
Decreased levels of total cholesterol	(*41, 42, 49*)
Decreased levels of non-HDL cholesterol	(*42, 49*)
Increased levels of HDL cholesterol	(*41, 49, 50*)
Reduced blood pressure	(*41, 51*)
Atherosclerosis	(*52*)
Reduced progression of atherosclerosis	(*53-56*)
Regression of aortic plaques	(*57*)
Inflammation and inflammatory diseases	(*58*)
Reduced cytokine release	(*59-61*)
Decreased inflammatory markers	(*59, 61, 62*)
Insulin sensitivity	(*63*)
Decreased fasting insulin levels	(*49*)
Improved glucose tolerance	(*64*)
Improved insulin sensitivity	(*41, 49, 64*)
Diabetic nephropathy	(*65, 66*)
Reduced progression of albuminuria	(*67*)
Improved glomular filtration rate	(*68*)

display functional differences. Some studies indicate that PPARγ2 is more important in adipogenesis and insulin sensitivity than PPARγ1 (*72-74*).

PPARγ was initially known for its crucial role in adipocyte differentiation and adipogenesis (*30, 31*). Later, it was discovered that insulin-sensitizing drugs from the class of TZDs function as PPARγ agonists (*24*). Several lines of evidence suggest that TZDs exert their insulin-sensitizing effects via PPARγ (*24*). Besides improvements in insulin sensitivity, PPARγ has been related to several other possible beneficial health effects, which are summarized in table 2. PPARγ agonists have been related to decreased risk of developing prostate, breast and colon cancer (*75, 76*). It is, however, not yet known to what extent these anticancer effects are mediated by PPARγ (*77-79*). In addition, activation of PPARγ is considered to have a protective effect on the risk for atherosclerosis and to lower blood pressure (*84, 85, 102, 103*).

TZDs, the best known synthetic PPARγ agonists, are well-known anti-diabetic drugs. Presently, however, pioglitazone is the only TZD still prescribed as insulin-sensitizing drug to patients suffering from type 2 diabetes mellitus (*104, 105*). Troglitazone was taken from the market because of its liver toxicity (*106*), while rosiglitazone is no longer prescribed because of concerns about its cardiovascular safety (*107*). Although TZDs are known for their clinical effects on insulin sensitivity and hyperglycemia, there are also adverse effects known for these drugs, including edema, fluid retention and weight gain (*108, 109*). Selective PPARγ modulators, SPPARMs, may have the same therapeutic effectiveness as TZDs without causing the adverse effects (*110, 111*). Currently known SPPARMs include

Table 2. Overview of beneficial health effects mediated by PPARγ agonists and corresponding literature references.

Physiological process or disease	References
Adipogenesis	
Stimulation of adipocyte differentiation	(*31, 80*)
Increased storage of fatty acids in adipocytes	(*81, 82*)
Reduced serum levels of free fatty acids	(*82, 83*)
Diabetes Mellitus type 2	
Improved insulin sensitivity	(*84, 85*)
Improved glucose tolerance	(*84*)
Lowered fasting insulin levels	(*50, 86*)
Lowered fasting blood glucose levels	(*50, 86*)
Cardiovascular diseases (CVD)	(*87, 88*)
Lowered blood pressure	(*84, 85*)
Increased HDL cholesterol	(*86, 89*)
Slower progression of atherosclerosis	(*50, 90*)
Inflammation and inflammatory diseases	(*91, 92*)
Anti-inflammatory effects	(*91, 92*)
Clinical improvement of asthma	(*93, 94*)
Clinical improvement of inflammatory bowel disease	(*95, 96*)
Cancer	(*75, 76*)
Prostate cancer: inhibition of tumor growth	(*97, 98*)
Liposarcoma: induction of terminal differentiation	(*99*)
Lung cancer: reduced risk / inhibition of growth	(*100, 101*)

for example GW0072, halofenate, linoleic acid and 15d-prostaglandin J2, and the search for new SPPARMs is continuing (*110, 112*).

REPORTER GENE ASSAYS

The aim of this thesis was to develop and validate stable reporter cell lines to facilitate high-throughput screening of bioactive food compounds for several health-related effects on gene expression level. The concept of reporter gene assays is explained in some more detail here.

A reporter gene assay is a cell-based tool which enables measuring gene transcription through a specific signalling pathway. Reporter gene assays make use of cells which are genetically engineered with a reporter construct. This reporter construct contains a reporter gene under transcriptional control of a response element for the transcription factor of interest. When the stimulus is present, the transcription factor binds to its response element, thereby enhancing the transcription of the reporter gene (figure 3). The reporter gene is generally a gene encoding a protein with an enzymatic activity that can be measured easily. In that way, the enzyme activity is 'reporting' transcriptional activation and expression of the reporter gene (*113*). One of the most frequently used reporter genes is the luciferase gene. Transcription of this gene results in the production of the luciferase enzyme, which catalyses the oxidation of luciferin (figure 3). During this

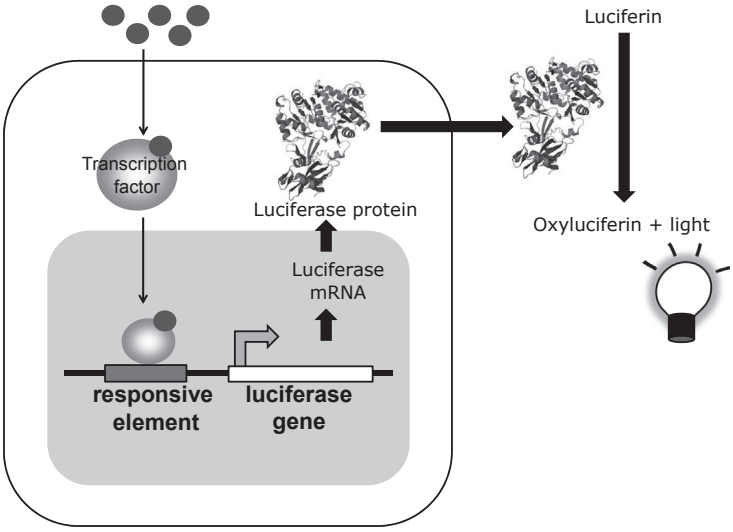

Figure 3. General principle of luciferase-based reporter gene assays.

oxidation reaction, light is produced. This light signal can be measured and indicates the presence of a stimulus capable to induce effects on gene transcription mediated by the regulatory sequence inserted upstream of the reporter gene (*113*).

Reporter gene assays are used to measure potencies of both agonists and antagonists to induce and inhibit transcription factor-mediated expression (*114*). Reporter cell lines, however, contain only one cell type, and therefore miss the context with other cell types as is found in tissues, organs and organ systems when performing *in vivo* studies. This means that results from reporter gene assays cannot be directly translated into *in vivo* effects (*115, 116*). On the other hand, reporter gene assays have the advantage that they are easy to handle, rapid and allowing for many compounds to be screened for their effects on expression mediated by a specific receptor, in a short time frame and at a relatively low cost (*114*).

TOMATO

The present thesis aims at providing proof-of-principle that reporter gene assays can be applied to investigate the effect of (compounds found in) fruits and vegetables on several transcription factor-mediated endpoints. Tomato was chosen as the model fruit, because of its health-beneficial effects (*114*), and because tomato is one of the most consumed vegetables in the world (*117*). In 2009, the average tomato consumption was 20.5 kg per person per year worldwide and 30.6 kg per person per year in Europe (*117*).

The consumption of tomato and tomato products has been related to several beneficial health effects. Epidemiological studies suggest a relation between tomato

consumption and reduced risk of certain forms of cancer, including prostate cancer, lung cancer and colorectal cancer (*118-120*). High intake of tomato has also been related to lower risk of atherosclerosis and cardiovascular diseases (CVDs)(*121, 122*). Furthermore, tomato consumption improves risk factors for CVDs: it leads to increased HDL cholesterol, decreased LDL cholesterol and decreased triglyceride plasma levels (*123*). In addition, treatment with tomato extract induced a decrease in blood pressure in patients suffering from hypertension (*124, 125*). Finally, carotenoids and naringenin, phytochemicals known to be present in tomato, have been shown to improve insulin sensitivity and blood glucose levels (*126, 127*). The beneficial health effects related to tomato consumption may thus be due to the presence of several bioactive phytochemicals present in tomato fruit (*118, 128*), including polyphenols (such as quercetin, kaempferol and naringenin) and carotenoids (such as lycopene and β-carotene)(*129-132*).

Tomato breeding programmes are currently not only focussing on yield, resistance to plant diseases, and taste, but also on nutritional value and the amounts of phytochemicals present in tomato fruits (*133, 134*). For example, high pigment mutants of tomato contain more phytochemicals including lycopene, β-carotene, α-tocopherol, vitamin C, rutin and quercetin (*129, 135*), and purple tomato fruits enriched with anthocyanins were developed as well (*136, 137*). Due to their higher content of phytochemicals, the high pigment tomatoes and purple tomatoes may provide stronger beneficial health effects than wild type tomatoes, but this remains to be investigated.

PHYTOCHEMICALS

Many plants and plant-based food products contain bioactive phytochemicals, such as carotenoids and flavonoids, which are in particular found in fruits and vegetables (*128*). Bioactive phytochemicals are mainly secondary metabolites formed by plants for several functions, such as protection against bacteria and fungi, protection against UV damage, attraction of pollinating insects, etcetera. When (parts of these) plants are eaten by humans, certain bioactive phytochemicals may have beneficial effects on human health (*138, 139*).

In this thesis, we focus mainly on phytochemicals known to be present in tomato fruit, including flavonoids and carotenoids. Many of these compounds are not specific for tomato, and are also present in other fruits and vegetables. Lycopene, for example, is present in tomato, but also in watermelon and pink grapefruit and β-carotene is found in carrots and apricots (*140*). Quercetin is known to be present in onion and apple, while naringenin is found in citrus fruits (*141*).

Carotenoids

Carotenoids are a family of fat-soluble, colorful pigments mainly found in plants. Carotenoids are responsible for the yellow, orange and red colors occurring in plants. Furthermore, carotenoid-chlorophyll complexes give rise to the green colors of leafy vegetables (*7*). Humans are not able to synthesize carotenoids and rely on dietary intake.

In the human diet, fruits and vegetables are the main source of carotenoids (*142*). For example, the main sources of lycopene are tomato and water melon, while carrots and apricots are good sources of β-carotene (*140*). Carotenoids have an isoprenoid structure and consist of a long skeleton containing 40 carbon atoms, as can be clearly recognized in the structure of lycopene (figure 4A). The skeleton is synthesized by tail-to-tail linkage of two C20 geranyl-geranyldiphosphate molecules. The long tail of carotenoids consists of alternating single and double bonds (*143, 144*). There are some variations on the long basic skeleton. For example, some carotenoids have a hydrocarbon ring on one or both ends of the molecule (such as β-carotene, figure 4B). Carotenoids are divided into two subgroups: carotenes and xanthophylls. Carotenes contain only carbon and hydrogen atoms, while xanthophylls, such as violaxanthin (figure 4C), also contain oxygen atoms (*143, 144*).

The major dietary carotenoids are lycopene, β-carotene, α-carotene, cryptoxanthin, and lutein, which are especially present in deeply pigmented fruits and vegetables such as apricots, tomato fruits and carrots, and in green leafy vegetables such as spinach and kale (*145*). Carotenoids have been related to several health-promoting properties. First of all, some carotenoids, including β-carotene, function as precursors of vitamin A (*144*). These carotenoids can be metabolized in the enterocytes into retinoids by cleaving the carotenoid C40 molecule into two C20 retinal molecules. Retinal is then further metabolized into retinoic acid and into retinol, the active form of vitamin A (*146, 147*). Vitamin A is known to play an important role in vision, and carotenoid intake has been related to protection from cataract and from oxidative damage of the retina (*148, 149*). In addition, it has been suggested that dietary intake of carotenoids is related to reduced risk of chronic diseases including cardiovascular diseases and certain forms of cancer (*145, 150*).

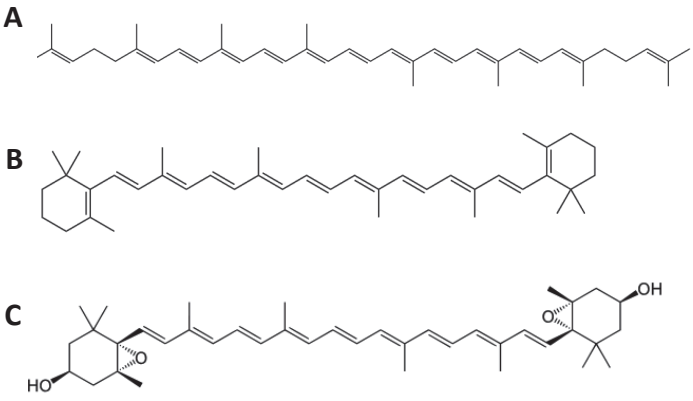

Figure 4. Structures of some carotenoids. A) Lycopene has a C40 carboskeleton, which is the standard structure of carotenoids; B) β-carotene has hydrocarbon rings on both ends of the molecule; C) Violaxanthin contains oxygen atoms and is therefore a xanthophyll.

Flavonoids

Flavonoids belong to the group of polyphenols and are found in many fruits and vegetables, but also in plant-derived products such as tea and chocolate (*151, 152*). Important food sources of flavonoids include natural food products such as soy, citrus fruits, grapes, berries and onions, but also plant-derived food products such as red wine, tea and chocolate (*153, 154*). Flavonoids consist of 15 carbon atoms arranged in three aromatic rings (C6 – C3 – C6) and can be divided into many subclasses, including flavonols (figure 5A), flavones (figure 5B), isoflavones (figure 5C) and anthocyanidins (figure 5D) (*151, 152, 155*).

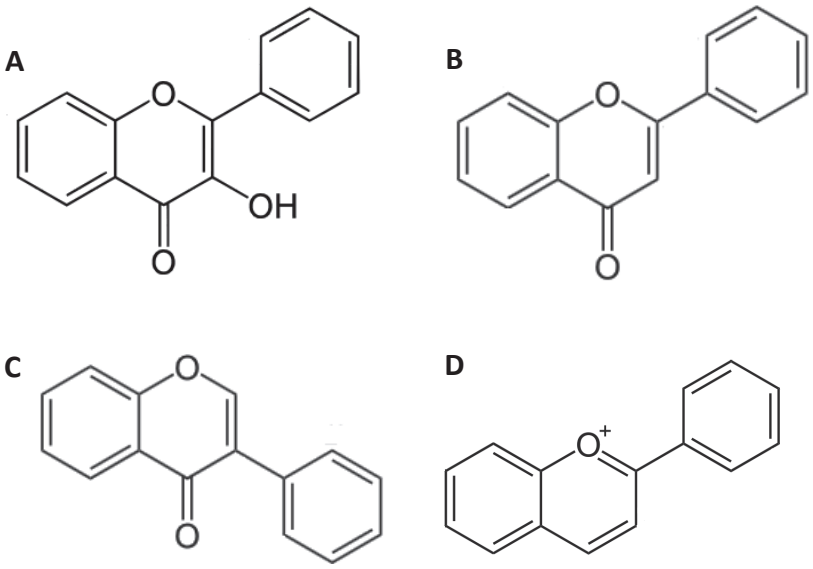

Figure 5. Basic structures of subclasses of flavonoids. A) flavonols; B) flavones; C) isoflavones; D) anthocyanidins.

Many flavonoids have been related to various beneficial health effects. For example, flavonoids have been linked to reduced overall risk of atherosclerosis and other cardiovascular diseases (*156*), and have been shown to improve specific risk factors for cardiovascular diseases, such as hypertension and high levels of LDL cholesterol (*157*). In addition, intake of flavonoids has been related to protective effects against cancer (*158*). Several *in vitro* experiments have shown that flavonoids have antioxidant properties (*152*). Although it was previously thought that the health-beneficial effects of flavonoids were due to their antioxidant properties, nowadays the relevance of the antioxidant properties of flavonoids *in vivo* is questioned and beneficial effects of flavonoids may rather be due to their effects on transcriptional gene regulation (*159, 160*).

OUTLINE OF THE THESIS

In order to scientifically support and facilitate the development of nutrition and health claims for functional foods or their bioactive ingredients, reporter gene assays may be fast and useful tools. Therefore, the aim of this thesis was to develop and validate stable reporter cell lines to facilitate high-throughput screening of bioactive food compounds for several health-related functions. **Chapter 1**, the present chapter, defines the aim of the studies and provides some background information on the topics of the thesis and the three health-beneficial endpoints addressed in this thesis.

In order to test whole fruits or vegetables for their effects on transcription factor-mediated expression, methods are needed to prepare extracts of fruits and vegetables which can be tested in reporter gene assays. **Chapter 2** describes methods for the preparation of extracts, using tomato fruit as a model for fruits and vegetables. These tomato extracts, as well as individual tomato constituents, were tested for their potency to enhance EpRE-mediated gene expression using the EpRE-LUX reporter cells.

In chapters 3, 4 and 5, new reporter gene assays for additional health-related functions were developed and subsequently used to investigate the capacity of individual tomato compounds and tomato extracts to influence the related gene expression pathways. Newly developed reporter gene assays for PPARγ1- and PPARγ2-mediated gene expression are presented in **chapter 3**. The two new cell lines, named PPARγ1 CALUX and PPARγ2 CALUX, provide *in vitro* tools to test known PPARγ ligands, chemicals, and food compounds for their potency to activate PPARγ-mediated gene expression. In **chapter 4**, the PPARγ2 CALUX cell line was used to investigate the capacity of tomato extracts and individual tomato components, including polyphenols, isoprenoids and fatty acids, to activate PPARγ-mediated gene expression. The development and validation of a stable reporter gene assay for PPARα-mediated gene expression, called the PPARα CALUX assay, was described in **chapter 5**. Furthermore, capacity of individual tomato compounds and tomato extracts to influence PPARα-mediated gene expression, was characterized using this newly developed reporter gene assay.

In **chapter 6**, it was verified that reporter gene assays are useful tools in measuring differences between tomato varieties regarding their capacity to induce EpRE-mediated gene expression and in selecting varieties which show the highest potency. To that end, extracts of 97 different tomato accessions were prepared and screened for their capacity to induce EPRE-mediated expression using the EpRE-LUX reporter cells. Next, metabolomic profiles of these extracts were generated in order to identify phytochemicals responsible for the differences in potency of the 97 tomato varieties to induce EpRE-mediated expression. This study provided a proof of principle of how to identify specific ingredients contributing to the activity of the whole tomato extracts using metabolic profiling.

The thesis concludes with an overall discussion and future perspectives in **chapter 7**. In the future perspectives it is discussed, for example, if and how reporter gene assays can be useful in a tiered approach to support nutrition and health claims for functional foods.

REFERENCES

1. Katan, M. B.; Roos, N. M., Promises and Problems of Functional Foods. *Critical Reviews in Food Science and Nutrition* **2004,** *44*, 369-377.
2. Law, M., Plant sterol and stanol margarines and health. *BMJ* **2000,** *320*, 861-864.
3. Menrad, K., Market and marketing of functional food in Europe. *Journal of Food Engineering* **2003,** *56*, 181-188.
4. Bazzano, L.; Serdula, M.; Liu, S., Dietary intake of fruits and vegetables and risk of cardiovascular disease. *Current Atherosclerosis Reports* **2003,** *5*, 492-499.
5. Harding A, W. N. J. B. S. A.; et al., Plasma vitamin c level, fruit and vegetable consumption, and the risk of new-onset type 2 diabetes mellitus: The european prospective investigation of cancer–norfolk prospective study. *Archives of Internal Medicine* **2008,** *168*, 1493-1499.
6. Nöthlings, U.; Schulze, M. B.; Weikert, C.; Boeing, H.; van der Schouw, Y. T.; Bamia, C.; Benetou, V.; Lagiou, P.; Krogh, V.; Beulens, J. W. J.; Peeters, P. H. M.; Halkjær, J.; Tjønneland, A.; Tumino, R.; Panico, S.; Masala, G.; Clavel-Chapelon, F.; de Lauzon, B.; Boutron-Ruault, M.-C.; Vercambre, M.-N.; Kaaks, R.; Linseisen, J.; Overvad, K.; Arriola, L.; Ardanaz, E.; Gonzalez, C. A.; Tormo, M.-J.; Bingham, S.; Khaw, K.-T.; Key, T. J. A.; Vineis, P.; Riboli, E.; Ferrari, P.; Boffetta, P.; Bueno-de-Mesquita, H. B.; van der A, D. L.; Berglund, G.; Wirfält, E.; Hallmans, G.; Johansson, I.; Lund, E.; Trichopoulo, A., Intake of Vegetables, Legumes, and Fruit, and Risk for All-Cause, Cardiovascular, and Cancer Mortality in a European Diabetic Population. *The Journal of Nutrition* **2008,** *138*, 775-781.
7. Khoo, H.-E.; Prasad, K. N.; Kong, K.-W.; Jiang, Y.; Ismail, A., Carotenoids and Their Isomers: Color Pigments in Fruits and Vegetables. *Molecules* **2011,** *16*, 1710-1738.
8. Arvanitoyannis, I. S.; Van Houwelingen-Koukaliaroglou, M., Functional Foods: A Survey of Health Claims, Pros and Cons, and Current Legislation. *Critical Reviews in Food Science and Nutrition* **2005,** *45*, 385-404.
9. Hasler, C. M., Functional Foods: Benefits, Concerns and Challenges—A Position Paper from the American Council on Science and Health. *The Journal of Nutrition* **2002,** *132*, 3772-3781.
10. Biesalski, H.-K.; Dragsted, L. O.; Elmadfa, I.; Grossklaus, R.; Müller, M.; Schrenk, D.; Walter, P.; Weber, P., Bioactive compounds: Definition and assessment of activity. *Nutrition* **2009,** *25*, 1202-1205.
11. Kris-Etherton, P. M.; Hecker, K. D.; Bonanome, A.; Coval, S. M.; Binkoski, A. E.; Hilpert, K. F.; Griel, A. E.; Etherton, T. D., Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. *The American Journal of Medicine* **2002,** *113*, 71-88.
12. Kris-Etherton, P. M.; Lefevre, M.; Beecher, G. R.; Gross, M. D.; Keen, C. L.; Etherton, T. D., Bioactive compounds in nutrition and health-research methodologies for establishing biological function: the antioxidant and anti-inflammatory effects of flavonoids on atherosclerosis. *Annual review of nutrition* **2004,** *24*, 511-538.
13. Saura-Calixto, F.; Goñi, I., Definition of the Mediterranean Diet Based on Bioactive Compounds. *Critical Reviews in Food Science and Nutrition* **2008,** *49*, 145-152.
14. Chapkin, R. S.; McMurray, D. N.; Davidson, L. A.; Patil, B. S.; Fan, Y.-Y.; Lupton, J. R., Bioactive dietary long-chain fatty acids: emerging mechanisms of action. *British Journal of Nutrition* **2008,** *100*, 1152-1157.
15. Asp, N.-G.; Bryngelsson, S., Health Claims in Europe: New Legislation and PASSCLAIM for Substantiation. *The Journal of Nutrition* **2008,** *138*, 1210S-1215S.
16. Verhagen, H.; Vos, E.; Francl, S.; Heinonen, M.; van Loveren, H., Status of nutrition and health claims in Europe. *Archives of Biochemistry and Biophysics* **2010,** *501*, 6-15.
17. Giudice, A.; Montella, M., Activation of the Nrf2–ARE signaling pathway: a promising strategy in cancer prevention. *Bioessays* **2006,** *28*, 169-181.
18. Kersten, S.; Desvergne, B.; Wahli, W., Roles of PPARs in health and disease. **2000,** *405*, 421-424.
19. Talalay, P.; Dinkova-Kostova, A. T.; Holtzclaw, W. D., Importance of phase 2 gene regulation in protection against electrophile and reactive oxygen toxicity and carcinogenesis.

Advances in Enzyme Regulation **2003,** *43*, 121-134.

20. Boerboom, A.-M. J. F.; Vermeulen, M.; van der Woude, H.; Bremer, B. I.; Lee-Hilz, Y. Y.; Kampman, E.; van Bladeren, P. J.; Rietjens, I. M. C. M.; Aarts, J. M. M. J. G., Newly constructed stable reporter cell lines for mechanistic studies on electrophile-responsive element-mediated gene expression reveal a role for flavonoid planarity. *Biochemical Pharmacology* **2006,** *72*, 217-226.
21. Lee-Hilz, Y. Y.; Boerboom, A.-M. J. F.; Westphal, A. H.; van Berkel, W. J. H.; Aarts, J. M. M. J. G.; Rietjens, I. M. C. M., Pro-Oxidant Activity of Flavonoids Induces EpRE-Mediated Gene Expression. *Chemical Research in Toxicology* **2006,** *19*, 1499-1505.
22. Uda, Y.; Price, K. R.; Williamson, G.; Rhodes, M. J. C., Induction of the anticarcinogenic marker enzyme, quinone reductase, in murine hepatoma cells *in vitro* by flavonoids. *Cancer Letters* **1997,** *120*, 213-216.
23. Vermeulen, M.; Boerboom, A.-M. M. J. F.; Blankvoort, B. M. G.; Aarts, J. M. M. J. G.; Rietjens, I. M. C. M.; van Bladeren, P. J.; Vaes, W. H. J., Potency of isothiocyanates to induce luciferase reporter gene expression via the electrophile-responsive element from murine glutathione S-transferase Ya. *Toxicology in Vitro* **2009,** *23*, 617-621.
24. Tontonoz, P.; Spiegelman, B. M., Fat and Beyond: The Diverse Biology of PPARγ. *Annual Review of Biochemistry* **2008,** *77*, 289-312.
25. Krey, G.; Braissant, O.; L'Horset, F.; Kalkhoven, E.; Perroud, M.; Parker, M. G.; Wahli, W., Fatty Acids, Eicosanoids, and Hypolipidemic Agents Identified as Ligands of Peroxisome Proliferator-Activated Receptors by Coactivator-Dependent Receptor Ligand Assay. *Mol Endocrinol* **1997,** *11*, 779-791.
26. Berger, J.; Moller, D. E., The mechanisms of action of PPARs. *Annual Review of Medicine* **2002,** *53*, 409-435.
27. Michalik, L.; Wahli, W., Peroxisome proliferator-activated receptors: three isotypes for a multitude of functions. *Current Opinion in Biotechnology* **1999,** *10*, 564-570.
28. Kersten, S.; Seydoux, J.; Peters, J. M.; Gonzalez, F. J.; Desvergne, B.; Wahli, W., Peroxisome proliferator–activated receptor α mediates the adaptive response to fasting. *The Journal of Clinical Investigation* **1999,** *103*, 10.
29. Reddy, J. K.; Hashimoto, T., Peroxisomal beta-oxidation and peroxisome proliferator-activated receptor alpha: an adaptive metabolic system. *Annual review of nutrition* **2001,** *21*, 193-230.
30. Barak, Y.; Nelson, M. C.; Ong, E. S.; Jones, Y. Z.; Ruiz-Lozano, P.; Chien, K. R.; Koder, A.; Evans, R. M., PPAR[gamma] Is Required for Placental, Cardiac, and Adipose Tissue Development. *Molecular Cell* **1999,** *4*, 585-595.
31. Tontonoz, P.; Graves, R. A.; Budavari, A. I.; Erdjument-Bromage, H.; Lui, M.; Hu, E.; Tempst, P.; Spiegelman, B. M., Adipocyte-specific transcription factor ARF6 is a heterodimeric complex of two nuclear hormone receptors, PPARγ and RXRa. *Nucleic Acids Research* **1994,** *22*, 5628-5634.
32. Grimaldi, P. A., Regulatory functions of PPARβ in metabolism: implications for the treatment of metabolic syndrome. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **2007,** *1771*, 983-990.
33. Michalik, L.; Desvergne, B.; Wahli, W., Peroxisome proliferator-activated receptors [beta]/[delta]: emerging roles for a previously neglected third family member. *Current Opinion in Lipidology* **2003,** *14*, 129-135.
34. Bishop-Bailey, D.; Bystrom, J., Emerging roles of peroxisome proliferator-activated receptor-β/δ in inflammation. *Pharmacology & Therapeutics* **2009,** *124*, 141-150.
35. Michalik, L.; Desvergne, B.; Tan, N. S.; Basu-Modak, S.; Escher, P.; Rieusset, J.; Peters, J. M.; Kaya, G.; Gonzalez, F. J.; Zakany, J.; Metzger, D.; Chambon, P.; Duboule, D.; Wahli, W., Impaired skin wound healing in peroxisome proliferator–activated receptor (PPAR)α and PPARβ mutant mice. *The Journal of Cell Biology* **2001,** *154*, 799-814.
36. Tan, N. S.; Michalik, L.; Di-Poi, N.; Desvergne, B.; Wahli, W., Critical roles of the nuclear receptor PPARbeta (peroxisome-proliferator-activated receptor beta) in skin wound healing. *Biochemical Society transactions* **2004,** *32*, 97-102.

37. Issemann, I.; Green, S., Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature* **1990,** *347*, 645-650.

38. Kliewer, S. A.; Sundseth, S. S.; Jones, S. A.; Brown, P. J.; Wisely, G. B.; Koble, C. S.; Devchand, P.; Wahli, W.; Willson, T. M.; Lenhard, J. M.; Lehmann, J. r. M., Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors α and γ. *Proceedings of the National Academy of Sciences of the United States of America* **1997,** *94*, 4318-4323.

39. Aoyama, T.; Peters, J. M.; Iritani, N.; Nakajima, T.; Furihata, K.; Hashimoto, T.; Gonzalez, F. J., Altered Constitutive Expression of Fatty Acid-metabolizing Enzymes in Mice Lacking the Peroxisome Proliferator-activated Receptor α (PPARα). *Journal of Biological Chemistry* **1998,** *273*, 5678-5684.

40. Mandard, S.; Müller, M.; Kersten, S., Peroxisome proliferator-activated receptor a target genes. *Cellular and Molecular Life Sciences* **2004,** *61*, 393-416.

41. Idzior-Walus, B.; Sieradzki, J.; Rostworowski, W.; Zdzienicka, A.; Kawalec, E.; Wójcik, J.; Żarnecki, A.; Blane, G., Effects of comicronised fenofibrate on lipid and insulin sensitivity in patients with polymetabolic syndrome X. *European Journal of Clinical Investigation* **2000,** *30*, 871-878.

42. Koh, K. K.; Han, S. H.; Quon, M. J.; Yeal Ahn, J.; Shin, E. K., Beneficial Effects of Fenofibrate to Improve Endothelial Dysfunction and Raise Adiponectin Levels in Patients With Primary Hypertriglyceridemia. *Diabetes Care* **2005,** *28*, 1419-1424.

43. Remick, J.; Weintraub, H.; Setton, R.; Offenbacher, J.; Fisher, E.; Schwartzbard, A., Fibrate Therapy: An Update. *Cardiology in Review* **2008,** *16*, 129-141 10.1097/CRD.0b013e31816b43d3.

44. Burgess, D. C.; Hunt, D.; Li, L.; Zannino, D.; Williamson, E.; Davis, T. M. E.; Laakso, M.; Kesäniemi, Y. A.; Zhang, J.; Sy, R. W.; Lehto, S.; Mann, S.; Keech, A. C., Incidence and predictors of silent myocardial infarction in type 2 diabetes and the effect of fenofibrate: an analysis from the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study. *European Heart Journal* **2010,** *31*, 92-99.

45. Keech, A.; Simes, R. J.; Barter, P.; Best, J.; Scott, R.; Taskinen, M. R.; Forder, P.; Pillai, A.; Davis, T.; Glasziou, P.; Drury, P.; Kesäniemi, Y. A.; Sullivan, D.; Hunt, D.; Colman, P.; d'Emden, M.; Whiting, M.; Ehnholm, C.; Laakso, M.; null, Effects of long-term fenofibrate therapy on cardiovascular events in 9795 people with type 2 diabetes mellitus (the FIELD study): randomised controlled trial. *Lancet* **2005,** *366*, 1849-1861.

46. Lee, M.; Saver, J. L.; Towfighi, A.; Chow, J.; Ovbiagele, B., Efficacy of fibrates for cardiovascular risk reduction in persons with atherogenic dyslipidemia: A meta-analysis. *Atherosclerosis* **2011,** *217*, 492-498.

47. Guerre-Millo, M.; Gervois, P.; Raspé, E.; Madsen, L.; Poulain, P.; Derudas, B.; Herbert, J.-M.; Winegar, D. A.; Willson, T. M.; Fruchart, J.-C.; Berge, R. K.; Staels, B., Peroxisome Proliferator-activated Receptor α Activators Improve Insulin Sensitivity and Reduce Adiposity. *Journal of Biological Chemistry* **2000,** *275*, 16638-16642.

48. Fajas, L.; Auboeuf, D.; RaspÃ©, E.; Schoonjans, K.; Lefebvre, A.-M.; Saladin, R.; Najib, J.; Laville, M.; Fruchart, J.-C.; Deeb, S.; Vidal-Puig, A.; Flier, J.; Briggs, M. R.; Staels, B.; Vidal, H.; Auwerx, J., The Organization, Promoter Analysis, and Expression of the Human PPARγ Gene. *Journal of Biological Chemistry* **1997,** *272*, 18779-18789.

49. Koh, K. K.; Quon, M. J.; Shin, K.-C.; Lim, S.; Lee, Y.; Sakuma, I.; Lee, K.; Han, S. H.; Shin, E. K., Significant differential effects of omega-3 fatty acids and fenofibrate in patients with hypertriglyceridemia. *Atherosclerosis* **2012,** *220*, 537-544.

50. Nissen Se, N. S. J. W. K.; et al., Effects of a potent and selective ppar-α agonist in patients with atherogenic dyslipidemia or hypercholesterolemia: Two randomized controlled trials. *JAMA: The Journal of the American Medical Association* **2007,** *297*, 1362-1373.

51. Russell, M.; Silverman, A.; Fleg, J. L.; Lee, E. T.; Mete, M.; Weir, M.; Wilson, C.; Yeh, F.; Howard, B. V.; Howard, W. J., Achieving lipid targets in adults with type 2 diabetes: The Stop Atherosclerosis in Native Diabetics Study. *Journal of Clinical Lipidology* **2010,** *4*, 435-443.

52. Robillard, R.; Fontaine, C.; Chinetti, G.; Fruchart, J. C.; Staels, B.; Eckardstein, A.,

Fibrates Atherosclerosis: Diet and Drugs. In Springer Berlin Heidelberg: 2005; Vol. 170, pp 389-406.

53. Ericsson, C. G.; de Faire, U.; Grip, L.; Svane, B.; Hamsten, A.; Nilsson, J., Angiographic assessment of effects of bezafibrate on progression of coronary artery disease in young male postinfarction patients. *The Lancet* **1996,** *347*, 849-853.
54. Ericsson, C. G.; Nilsson, J.; Grip, L.; Svane, B.; Hamsten, A., Effect of bezafibrate treatment over five years on coronary plaques causing 20% to 50% diameter narrowing (The Bezafibrate Coronary Atherosclerosis Intervention Trial [BECAIT]). *The American journal of cardiology* **1997,** *80*, 1125-1129.
55. Frick, M. H.; Syvänne, M.; Nieminen, M. S.; Kauma, H.; Majahalme, S.; Virtanen, V.; Kesäniemi, Y. A.; Pasternack, A.; Taskinen, M.-R.; Group, f. t. L. C. A. T. S., Prevention of the Angiographic Progression of Coronary and Vein-Graft Atherosclerosis by Gemfibrozil After Coronary Bypass Surgery in Men With Low Levels of HDL Cholesterol. *Circulation* **1997,** *96*, 2137-2143.
56. Steiner, Effect of fenofibrate on progression of coronary-artery disease in type 2 diabetes: the Diabetes Atherosclerosis Intervention Study, a randomised study. *The Lancet* **2001,** *357*, 905-910.
57. Ayaori, M.; Momiyama, Y.; Fayad, Z. A.; Yonemura, A.; Ohmori, R.; Kihara, T.; Tanaka, N.; Nakaya, K.; Ogura, M.; Sawada, S.; Taniguchi, H.; Kusuhara, M.; Nagata, M.; Nakamura, H.; Ohsuzu, F., Effect of bezafibrate therapy on atherosclerotic aortic plaques detected by MRI in dyslipidemic patients with hypertriglyceridemia. *Atherosclerosis* **2008,** *196*, 425-433.
58. Staels, B.; Koenig, W.; Habib, A.; Merval, R.; Lebret, M.; Torra, I. P.; Delerive, P.; Fadel, A.; Chinetti, G.; Fruchart, J.-C.; Najib, J.; Maclouf, J.; Tedgui, A., Activation of human aortic smooth-muscle cells is inhibited by PPAR[alpha] but not by PPAR[gamma] activators. **1998,** *393*, 790-793.
59. Krysiak, R.; Gdula-Dymek, A.; Okopien, B., The effect of bezafibrate and omega-3 fatty acids on lymphocyte cytokine release and systemic inflammation in patients with isolated hypertriglyceridemia. *European Journal of Clinical Pharmacology* **2011,** *67*, 1109-1117.
60. Okopień, B.; Krysiak, R.; Herman, Z. S., Effects of Short-Term Fenofibrate Treatment on Circulating Markers of Inflammation and Hemostasis in Patients with Impaired Glucose Tolerance. *Journal of Clinical Endocrinology & Metabolism* **2006,** *91*, 1770-1778.
61. Wägner, A. M.; Sánchez-Quesada, J. L.; Benítez, S.; Bancells, C.; Ordóñez-Llanos, J.; Pérez, A., Effect of statin and fibrate treatment on inflammation in type 2 diabetes. A randomized, cross-over study. *Diabetes Research and Clinical Practice* **2011,** *93*, e25-e28.
62. Muhlestein, J. B.; May, H. T.; Jensen, J. R.; Horne, B. D.; Lanman, R. B.; Lavasani, F.; Wolfert, R. L.; Pearson, R. R.; Yannicelli, H. D.; Anderson, J. L., The Reduction of Inflammatory Biomarkers by Statin, Fibrate, and Combination Therapy Among Diabetic Patients With Mixed DyslipidemiaThe DIACOR (Diabetes and Combined Lipid Therapy Regimen) Study. *Journal of the American College of Cardiology* **2006,** *48*, 396-401.
63. Krysiak, R.; Stachura-Kulach, A.; Okopien, B., Metabolic and monocyte-suppressing actions of fenofibrate in patients with mixed dyslipidemia and early glucose metabolism disturbances. *Pharmacol Rep* **2010,** *62*, 120-30.
64. Li, X. M.; Li, Y.; Zhang, N. N.; Xie, Y. H.; Shi, Y. Q., Combination Therapy with Metformin and Fenofibrate for Insulin Resistance in Obesity. *The Journal of International Medical Research* **2011,** *39*, 1876-1882.
65. Balakumar, P.; Kadian, S.; Mahadevan, N., Are PPAR alpha agonists a rational therapeutic strategy for preventing abnormalities of the diabetic kidney? *Pharmacological Research* **2012,** *65*, 430-436.
66. Kouroumichakis, I.; Papanas, N.; Zarogoulidis, P.; Liakopoulos, V.; Maltezos, E.; Mikhailidis, D. P., Fibrates: Therapeutic potential for diabetic nephropathy? *European Journal of Internal Medicine* **2012,** *23*, 309-316.
67. Smulders, Y. M.; Van Eeden, A. E.; Stehouwer, C. D. A.; Weijers, R. N. M.; Slaats, E. H.; Silberbusch, J., Can reduction in hypertriglyceridaemia slow progression of microalbuminuria in patients with non-insulin-dependent diabetes mellitus?

European Journal of Clinical Investigation **1997,** *27*, 997-1002.

68. Davis, T.; Ting, R.; Best, J.; Donoghoe, M.; Drury, P.; Sullivan, D.; Jenkins, A.; O'Connell, R.; Whiting, M.; Glasziou, P.; Simes, R.; Kesäniemi, Y.; Gebski, V.; Scott, R.; Keech, A.; investigators, o. b. o. t. F. S., Effects of fenofibrate on renal function in patients with type 2 diabetes mellitus: the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) Study. *Diabetologia* **2011,** *54*, 280-290.
69. Elbrecht, A.; Chen, Y.; Cullinan, C. A.; Hayes, N.; Leibowitz, M. D.; Moller, D. E.; Berger, J., Molecular Cloning, Expression and Characterization of Human Peroxisome Proliferator Activated Receptors [gamma]1 and [gamma]2. *Biochemical and Biophysical Research Communications* **1996,** *224*, 431-437.
70. Vidal-Puig, A.; Jimenez-Linan, M.; Lowell, B. B.; Hamann, A.; Hu, E.; Spiegelman, B.; Flier, J. S.; Moller, D. E., Regulation of PPAR gamma gene expression by nutrition and obesity in rodents. *The journal of Clinical Investigation* **1996,** *97*, 2553-61.
71. Vidal-Puig, A. J.; Considine, R. V.; Jimenez-Liñan, M.; Werman, A.; Pories, W. J.; Caro, J. F.; Flier, J. S., Peroxisome proliferator-activated receptor gene expression in human tissues. Effects of obesity, weight loss, and regulation by insulin and glucocorticoids. *The journal of Clinical Investigation* **1997,** *99*, 2416-2422.
72. Medina-Gomez, G.; Virtue, S.; Lelliott, C.; Boiani, R.; Campbell, M.; Christodoulides, C.; Perrin, C.; Jimenez-Linan, M.; Blount, M.; Dixon, J.; Zahn, D.; Thresher, R. R.; Aparicio, S.; Carlton, M.; Colledge, W. H.; Kettunen, M. I.; Seppänen-Laakso, T.; Sethi, J. K.; O'Rahilly, S.; Brindle, K.; Cinti, S.; Orešič, M.; Burcelin, R.; Vidal-Puig, A., The Link Between Nutritional Status and Insulin Sensitivity Is Dependent on the Adipocyte-Specific Peroxisome Proliferator-Activated Receptor-γ2 Isoform. *Diabetes* **2005,** *54*, 1706-1716.
73. Mueller, E.; Drori, S.; Aiyer, A.; Yie, J.; Sarraf, P.; Chen, H.; Hauser, S.; Rosen, E. D.; Ge, K.; Roeder, R. G.; Spiegelman, B. M., Genetic Analysis of Adipogenesis through Peroxisome Proliferator-activated Receptor γ Isoforms. *Journal of Biological Chemistry* **2002,** *277*, 41925-41930.
74. Ren, D.; Collingwood, T. N.; Rebar, E. J.; Wolffe, A. P.; Camp, H. S., PPARγ knockdown by engineered transcription factors: exogenous PPARγ2 but not PPARγ1 reactivates adipogenesis. *Genes & Development* **2002,** *16*, 27-32.
75. Grommes, C.; Landreth, G. E.; Heneka, M. T., Antineoplastic effects of peroxisome proliferatoractivated receptor γ agonists. *The Lancet Oncology* **2004,** *5*, 419-429.
76. Michalik, L.; Desvergne, B.; Wahli, W., Peroxisome-proliferator-activated receptors and cancers: complex stories. *Nat Rev Cancer* **2004,** *4*, 61-70.
77. Chen, G. G.; Xu, H.; Lee, J. F. Y.; Subramaniam, M.; Leung, K. L.; Wang, S. H.; Chan, U. P. F.; Spelsberg, T. C., 15-hydroxy-eicosatetraenoic acid arrests growth of colorectal cancer cells via a peroxisome proliferator-activated receptor gamma-dependent pathway. *Int. J. Cancer* **2003,** *107*, 837-843.
78. Girnun, G. D.; Naseri, E.; Vafai, S. B.; Qu, L.; Szwaya, J. D.; Bronson, R.; Alberta, J. A.; Spiegelman, B. M., Synergy between PPARγ Ligands and Platinum-Based Drugs in Cancer. *Cancer Cell* **2007,** *11*, 395-406.
79. Yang, C.-C.; Ku, C.-Y.; Wei, S.; Shiau, C.-W.; Chen, C.-S.; Pinzone, J. J.; Ringel, M. D.; Chen, C.-S., Peroxisome Proliferator-Activated Receptor γ-Independent Repression of Prostate-Specific Antigen Expression by Thiazolidinediones in Prostate Cancer Cells. *Molecular Pharmacology* **2006,** *69*, 1564-1570.
80. Rosen, E. D.; Sarraf, P.; Troy, A. E.; Bradwin, G.; Moore, K.; Milstone, D. S.; Spiegelman, B. M.; Mortensen, R. M., PPARγ Is Required for the Differentiation of Adipose Tissue *In Vivo* and *In Vitro*. *Molecular Cell* **1999,** *4*, 611-617.
81. Guan, H.-P.; Li, Y.; Jensen, M. V.; Newgard, C. B.; Steppan, C. M.; Lazar, M. A., A futile metabolic cycle activated in adipocytes by antidiabetic agents. **2002,** *8*, 1122-1128.
82. Yamauchi, T.; Kamon, J.; Waki, H.; Murakami, K.; Motojima, K.; Komeda, K.; Ide, T.; Kubota, N.; Terauchi, Y.; Tobe, K.; Miki, H.; Tsuchida, A.; Akanuma, Y.; Nagai, R.; Kimura, S.; Kadowaki, T., The Mechanisms by Which Both Heterozygous Peroxisome Proliferator-activated Receptor γ (PPARγ) Deficiency and PPARγ Agonist Improve Insulin Resistance. *Journal of Biological Chemistry* **2001,** *276*, 41245-41254.

83. Boden, G.; Homko, C.; Mozzoli, M.; Showe, L. C.; Nichols, C.; Cheung, P., Thiazolidinediones Upregulate Fatty Acid Uptake and Oxidation in Adipose Tissue of Diabetic Patients. *Diabetes* **2005,** *54*, 880-885.

84. Bennett, S. M. A.; Agrawal, A.; Elasha, H.; Heise, M.; Jones, N. P.; Walker, M.; Wilding, J. P. H., Rosiglitazone improves insulin sensitivity, glucose tolerance and ambulatory blood pressure in subjects with impaired glucose tolerance. *Diabetic Medicine* **2004,** *21*, 415-422.

85. Raji, A.; Seely, E. W.; Bekins, S. A.; Williams, G. H.; Simonson, D. C., Rosiglitazone Improves Insulin Sensitivity and Lowers Blood Pressure in Hypertensive Patients. *Diabetes Care* **2003,** *26*, 172-178.

86. Goldberg, R. B.; Kendall, D. M.; Deeg, M. A.; Buse, J. B.; Zagar, A. J.; Pinaire, J. A.; Tan, M. H.; Khan, M. A.; Perez, A. T.; Jacober, S. J., A Comparison of Lipid and Glycemic Effects of Pioglitazone and Rosiglitazone in Patients With Type 2 Diabetes and Dyslipidemia. *Diabetes Care* **2005,** *28*, 1547-1554.

87. Dormandy, J. A.; Charbonnel, B.; Eckland, D. J. A.; Erdmann, E.; Massi-Benedetti, M.; Moules, I. K.; Skene, A. M.; Tan, M. H.; Lefèbvre, P. J.; Murray, G. D.; Standl, E.; Wilcox, R. G.; Wilhelmsen, L.; Betteridge, J.; Birkeland, K.; Golay, A.; Heine, R. J.; Korányi, L.; Laakso, M.; Mokáň, M.; Norkus, A.; Pirags, V.; Podar, T.; Scheen, A.; Scherbaum, W.; Schernthaner, G.; Schmitz, O.; Škrha, J.; Smith, U.; Tatoň, J., Secondary prevention of macrovascular events in patients with type 2 diabetes in the PROactive Study (PROspective pioglitAzone Clinical Trial In macroVascular Events): a randomised controlled trial. *The Lancet* **2005,** *366*, 1279-1289.

88. Lincoff, A. M.; Wolski, K.; Nicholls, S. J.; Nissen, S. E., Pioglitazone and Risk of Cardiovascular Events in Patients With Type 2 Diabetes Mellitus. *JAMA: The Journal of the American Medical Association* **2007,** *298*, 1180-1188.

89. Davidson, M.; Meyer, P. M.; Haffner, S.; Feinstein, S.; D'Agostino, R.; Kondos, G. T.; Perez, A.; Chen, Z.; Mazzone, T., Increased High-Density Lipoprotein Cholesterol Predicts the Pioglitazone-Mediated Reduction of Carotid Intima-Media Thickness Progression in Patients With Type 2 Diabetes Mellitus. *Circulation* **2008,** *117*, 2123-2130.

90. Mazzone, T.; Meyer, P. M.; Feinstein, S. B.; Davidson, M. H.; Kondos, G. T.; D'Agostino, R. B.; Perez, A.; Provost, J.-C.; Haffner, S. M., Effect of Pioglitazone Compared With Glimepiride on Carotid Intima-Media Thickness in Type 2 Diabetes. *JAMA: The Journal of the American Medical Association* **2006,** *296*, 2572-2581.

91. Martin, H., Role of PPAR-gamma in inflammation. Prospects for therapeutic intervention by food components. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **2010,** *690*, 57-63.

92. Széles, L.; Töröcsik, D.; Nagy, L., PPARγ in immunity and inflammation: cell types and diseases. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **2007,** *1771*, 1014-1030.

93. Hashimoto, Y.; Nakahara, K., Improvement of Asthma After Administration of Pioglitazone. *Diabetes Care* **2002,** *25*, 401.

94. Spears, M.; Donnelly, I.; Jolly, L.; Brannigan, M.; Ito, K.; McSharry, C.; Lafferty, J.; Chaudhuri, R.; Braganza, G.; Bareille, P.; Sweeney, L.; Adcock, I. M.; Barnes, P. J.; Wood, S.; Thomson, N. C., Bronchodilatory Effect of the PPAR-[gamma] Agonist Rosiglitazone in Smokers With Asthma. *Clin Pharmacol Ther* **2009,** *86*, 49-53.

95. Lewis, J. D.; Lichtenstein, G. R.; Deren, J. J.; Sands, B. E.; Hanauer, S. B.; Katz, J. A.; Lashner, B.; Present, D. H.; Chuai, S.; Ellenberg, J. H.; Nessel, L.; Wu, G. D., Rosiglitazone for Active Ulcerative Colitis: A Randomized Placebo-Controlled Trial. *Gastroenterology* **2008,** *134*, 688-695.

96. Liang, H.-L.; Ouyang, Q., A clinical trial of combined use of rosiglitazone and 5-aminosalicylate for ulcerative colitis. *World journal of gastroenteroloty* **2008,** *14*.

97. Kubota, T.; Koshizuka, K.; Williamson, E. A.; Asou, H.; Said, J. W.; Holden, S.; Miyoshi, I.; Phillip Koeffler, H., Ligand for Peroxisome Proliferator-activated Receptor γ (Troglitazone) Has Potent Antitumor Effect against Human Prostate Cancer Both in Vitro and in Vivo. *Cancer Research* **1998,** *58*, 3344-3352.

98. Mueller, E.; Smith, M.; Sarraf, P.; Kroll, T.; Aiyer, A.; Kaufman, D. S.; Oh, W.; Demetri, G.; Figg,

W. D.; Zhou, X.-P.; Eng, C.; Spiegelman, B. M.; Kantoff, P. W., Effects of ligand activation of peroxisome proliferator-activated receptor γ in human prostate cancer. *Proceedings of the National Academy of Sciences* **2000,** *97*, 10990-10995.

99. Demetri, G. D.; Fletcher, C. D. M.; Mueller, E.; Sarraf, P.; Naujoks, R.; Campbell, N.; Spiegelman, B. M.; Singer, S., Induction of solid tumor differentiation by the peroxisome proliferator-activated receptor-γ ligand troglitazone in patients with liposarcoma. *Proceedings of the National Academy of Sciences* **1999,** *96*, 3951-3956.
100. Govindarajan, R.; Ratnasinghe, L.; Simmons, D. L.; Siegel, E. R.; Midathada, M. V.; Kim, L.; Kim, P. J.; Owens, R. J.; Lang, N. P., Thiazolidinediones and the Risk of Lung, Prostate, and Colon Cancer in Patients With Diabetes. *Journal of Clinical Oncology* **2007,** *25*, 1476-1481.
101. Keshamouni, V. G.; Reddy, R. C.; Arenberg, D. A.; Joel, B.; Thannickal, V. J.; Kalemkerian, G. P.; Standiford, T. J., Peroxisome proliferator-activated receptor-[gamma] activation inhibits tumor progression in non-small-cell lung cancer. **2004,** *23*, 100-108.
102. Hamblin, M.; Chang, L.; Fan, Y.; Zhang, J.; Chen, Y. E., PPARs and the Cardiovascular System. *Antioxidants & Redox Signaling* **2009,** *11*, 1415-1452.
103. Walczak, R.; Tontonoz, P., PPARadigms and PPARadoxes: expanding roles for PPARγ in the control of lipid metabolism. *Journal of Lipid Research* **2002,** *43*, 177-186.
104. Derosa, G., Efficacy and Tolerability of Pioglitazone in Patients with Type 2 Diabetes Mellitus: Comparison with Other Oral Antihyperglycaemic Agents. *Drugs* **2010,** *70*, 1945-1961.
105. Forst, T.; Hanefeld, M.; Pfützner, A., Review of approved pioglitazone combinations for type 2 diabetes. *Expert Opinion on Pharmacotherapy* **2011,** *12*, 1571-1584.
106. Gale, E. A. M., Lessons from the glitazones: a story of drug development. *The Lancet* **2001,** *357*, 1870-1875.
107. Krentz, A. J., Rosiglitazone: Trials, Tribulations and Termination. *Drugs* **2011,** *71*, 123-130.
108. Berlie, H. D.; Kalus, J. S.; Jaber, L. A., Thiazolidinediones and the risk of edema: A meta-analysis. *Diabetes Research and Clinical Practice* **2007,** *76*, 279-289.
109. Yamanouchi, T., Concomitant therapy with pioglitazone and insulin for the treatment of type 2 diabetes. *Vascular health and risk management* **2010,** *6*, 189-97.
110. Higgins, L. S.; DePaoli, A. M., Selective peroxisome proliferator-activated receptor γ (PPARγ) modulation as a strategy for safer therapeutic PPARγ activation. *The American Journal of Clinical Nutrition* **2010,** *91*, 267S-272S.
111. Zhang, F.; Lavan, B. E.; Gregoire, F. M., Selective Modulators of PPAR-γ Activity: Molecular Aspects Related to Obesity and Side-Effects. *PPAR Research* **2007,** *2007*.
112. Lecka-Czernik, B.; Moerman, E. J.; Grant, D. F.; Lehmann, J. M.; Manolagas, S. C.; Jilka, R. L., Divergent Effects of Selective Peroxisome Proliferator-Activated Receptor-γ2 Ligands on Adipocyte Versus Osteoblast Differentiation. *Endocrinology* **2002,** *143*, 2376-2384.
113. New, D. C.; Miller-Martini, D. M.; Wong, Y. H., Reporter gene assays and their applications to bioassays of natural products. *Phytotherapy Research* **2003,** *17*, 439-448.
114. Hill, S. J.; Baker, J. G.; Rees, S., Reporter-gene systems for the study of G-protein-coupled receptors. *Current Opinion in Pharmacology* **2001,** *1*, 526-532.
115. Harry, G. J.; Billingsley, M.; Bruinink, A.; Campbell, I. L.; Classen, W.; Dorman, D. C.; Galli, C.; Ray, D.; Smith, R. A.; Tilson, H. A., In Vitro Techniques for the Assessment of Neurotoxicity. *Environmental Health Perspectives* **1998,** *106*, 131-158.
116. Harvey, A. L.; Cree, I. A., High-Throughput Screening of Natural Products for Cancer Therapy. *Planta Med* **2010,** *76*, 1080-1086.
117. Faostat, Food Balance Sheet. *accessed 15-10-2012* **2009**.
118. Canene-Adams, K.; Campbell, J. K.; Zaripheh, S.; Jeffery, E. H.; Erdman, J. W., The Tomato As a Functional Food. *The Journal of Nutrition* **2005,** *135*, 1226-1230.
119. Etminan, M.; Takkouche, B.; Caamaño-Isorna, F., The Role of Tomato Products and Lycopene in the Prevention of Prostate Cancer: A Meta-Analysis of Observational Studies. *Cancer Epidemiology Biomarkers & Prevention* **2004,** *13*, 340-345.

120. Giovannucci, E., Tomatoes, Tomato-Based Products, Lycopene, and Cancer: Review of the Epidemiologic Literature. *Journal of the National Cancer Institute* **1999,** *91*, 317-331.

121. Sesso, H. D.; Liu, S.; Gaziano, J. M.; Buring, J. E., Dietary Lycopene, Tomato-Based Food Products and Cardiovascular Disease in Women. *The Journal of Nutrition* **2003,** *133*, 2336-2341.

122. Visioli, F.; Riso, P.; Grande, S.; Galli, C.; Porrini, M., Protective activity of tomato products on in vivo markers of lipid oxidation. *European Journal of Nutrition* **2003,** *42*, 201-206.

123. Shen, Y.-C.; Chen, S.-L.; Wang, C.-K., Contribution of Tomato Phenolics to Antioxidation and Down-regulation of Blood Lipids. *Journal of Agricultural and Food Chemistry* **2007,** *55*, 6475-6481.

124. Engelhard, Y. N.; Gazer, B.; Paran, E., Natural antioxidants from tomato extract reduce blood pressure in patients with grade-1 hypertension: A double-blind, placebo-controlled pilot study. *American Heart Journal* **2006,** *151*, 100.e6-100.e1.

125. Paran, E.; Novack, V.; Engelhard, Y.; Hazan-Halevy, I., The Effects of Natural Antioxidants from Tomato Extract in Treated but Uncontrolled Hypertensive Patients. *Cardiovascular Drugs and Therapy* **2009,** *23*, 145-151.

126. Mulvihill, E. E.; Allister, E. M.; Sutherland, B. G.; Telford, D. E.; Sawyez, C. G.; Edwards, J. Y.; Markle, J. M.; Hegele, R. A.; Huff, M. W., Naringenin Prevents Dyslipidemia, Apolipoprotein B Overproduction, and Hyperinsulinemia in LDL Receptor–Null Mice With Diet-Induced Insulin Resistance. *Diabetes* **2009,** *58*, 2198-2210.

127. Ylönen, K.; Alfthan, G.; Groop, L.; Saloranta, C.; Aro, A.; Virtanen, S. M.; Group, t. B. R., Dietary intakes and plasma concentrations of carotenoids and tocopherols in relation to glucose metabolism in subjects at high risk of type 2 diabetes: the Botnia Dietary Study. *The American Journal of Clinical Nutrition* **2003,** *77*, 1434-1441.

128. Liu, R. H., Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. *The American Journal of Clinical Nutrition* **2003,** *78*, 517S-520S.

129. Bino, R. J.; De Vos, C. H. R.; Lieberman, M.; Hall, R. D.; Bovy, A.; Jonker, H. H.; Tikunov, Y.; Lommen, A.; Moco, S.; Levin, I., The light-hyperresponsive high pigment-2dg mutation of tomato: alterations in the fruit metabolome. *New Phytologist* **2005,** *166*, 427-438.

130. Fraser, P. D.; Truesdale, M. R.; Bird, C. R.; Schuch, W.; Bramley, P. M., Carotenoid Biosynthesis during Tomato Fruit Development (Evidence for Tissue-Specific Gene Expression). *Plant Physiology* **1994,** *105*, 405-413.

131. Torres, C. A.; Davies, N. M.; Yanez, J. A.; Andrews, P. K., Disposition of Selected Flavonoids in Fruit Tissues of Various Tomato (Lycopersicon esculentum Mill.) Genotypes. *Journal of Agricultural and Food Chemistry* **2005,** *53*, 9536-9543.

132. Slimestad, R.; Verheul, M. J., Seasonal Variations in the Level of Plant Constituents in Greenhouse Production of Cherry Tomatoes. *Journal of Agricultural and Food Chemistry* **2005,** *53*, 3114-3119.

133. Dorais, M.; Ehret, D.; Papadopoulos, A., Tomato (Solanum lycopersicum) health components: from the seed to the consumer. *Phytochemistry Reviews* **2008,** *7*, 231-250.

134. Foolad, M. R., Genome Mapping and Molecular Breeding of Tomato. *International Journal of Plant Genomics* **2007,** *2007*.

135. Levin, I.; de Vos, C.; Tadmor, Y.; Bovy, A.; Lieberman, M.; Oren-Shamir, M.; Segev, O.; Kolotilin, I.; Keller, M.; Ovadia, R.; Meir, A.; Bino, R., High pigment tomato mutants—more than just lycopene (a review). *Israel Journal of Plant Sciences* **2006,** *54*, 179-190.

136. Butelli, E.; Titta, L.; Giorgio, M.; Mock, H.-P.; Matros, A.; Peterek, S.; Schijlen, E. G. W. M.; Hall, R. D.; Bovy, A. G.; Luo, J.; Martin, C., Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. **2008,** *26*, 1301-1308.

137. Gonzali, S.; Mazzucato, A.; Perata, P., Purple as a tomato: towards high anthocyanin tomatoes. *Trends in Plant Science* **2009,** *14*, 237-241.

138. Kennedy, D. O.; Wightman, E. L., Herbal Extracts and Phytochemicals: Plant Secondary Metabolites and the Enhancement of Human Brain Function. *Advances in Nutrition: An International Review Journal* **2011,** *2*, 32-50.

139. Murakami, A.; Ohnishi, K., Target molecules of food phytochemicals: Food science bound for the next dimension. *Food & Function* **2012,** *3*, 462-476.

140. Voutilainen, S.; Nurmi, T.; Mursu, J.; Rissanen, T. H., Carotenoids and cardiovascular health. *The American Journal of Clinical Nutrition* **2006,** *83*, 1265-1271.

141. Erlund, I., Review of the flavonoids quercetin, hesperetin, and naringenin. Dietary sources, bioactivities, bioavailability, and epidemiology. *Nutrition Research* **2004,** *24*, 851-874.

142. Maiani, G.; Periago Castón, M. J.; Catasta, G.; Toti, E.; Cambrodón, I. G.; Bysted, A.; Granado-Lorencio, F.; Olmedilla-Alonso, B.; Knuthsen, P.; Valoti, M.; Böhm, V.; Mayer-Miebach, E.; Behsnilian, D.; Schlemmer, U., Carotenoids: Actual knowledge on food sources, intakes, stability and bioavailability and their protective role in humans. *Mol. Nutr. Food Res.* **2009,** *53*, S194-S218.

143. Britton, G., Structure and properties of carotenoids in relation to function. *The FASEB Journal* **1995,** *9*, 1551-8.

144. Dutta, D.; Chaudhuri, U.; Chakraborty, R., Structure, health benefits, antioxidant property and processing and storage of carotenoids. *African Journal of Biotechnology* **2005,** *4*, 1510-1520.

145. Rao, A. V.; Rao, L. G., Carotenoids and human health. *Pharmacological Research* **2007,** *55*, 207-216.

146. Nagao, A., Oxidative Conversion of Carotenoids to Retinoids and Other Products. *The Journal of Nutrition* **2004,** *134*, 237S-240S.

147. von Lintig, J., Metabolism of Carotenoids and Retinoids Related to Vision. *Journal of Biological Chemistry* **2012,** *287*, 1627-1634.

148. Jacques, P. F.; Chylack, L. T., Epidemiologic evidence of a role for the antioxidant vitamins and carotenoids in cataract prevention. *The American Journal of Clinical Nutrition* **1991,** *53*, 352S-355S.

149. Snodderly, D. M., Evidence for protection against age-related macular degeneration by carotenoids and antioxidant vitamins. *The American Journal of Clinical Nutrition* **1995,** *62*, 1448S-1461S.

150. Tapiero, H.; Townsend, D. M.; Tew, K. D., The role of carotenoids in the prevention of human pathologies. *Biomedicine & Pharmacotherapy* **2004,** *58*, 100-110.

151. Cook, N. C.; Samman, S., Flavonoids—Chemistry, metabolism, cardioprotective effects, and dietary sources. *The Journal of Nutritional Biochemistry* **1996,** *7*, 66-76.

152. Pietta, P.-G., Flavonoids as Antioxidants. *Journal of Natural Products* **2000,** *63*, 1035-1042.

153. Manach, C.; Scalbert, A.; Morand, C.; Rémésy, C.; Jiménez, L., Polyphenols: food sources and bioavailability. *The American Journal of Clinical Nutrition* **2004,** *79*, 727-747.

154. Scalbert, A.; Williamson, G., Dietary Intake and Bioavailability of Polyphenols. *The Journal of Nutrition* **2000,** *130*, 2073S-2085S.

155. Crozier, A.; Jaganath, I. B.; Clifford, M. N., Dietary phenolics: chemistry, bioavailability and effects on health. *Natural Product Reports* **2009,** *26*, 1001-1043.

156. Hooper, L.; Kroon, P. A.; Rimm, E. B.; Cohn, J. S.; Harvey, I.; Le Cornu, K. A.; Ryder, J. J.; Hall, W. L.; Cassidy, A., Flavonoids, flavonoid-rich foods, and cardiovascular risk: a meta-analysis of randomized controlled trials. *The American Journal of Clinical Nutrition* **2008,** *88*, 38-50.

157. Perez-Vizcaino, F.; Duarte, J., Flavonols and cardiovascular disease. *Molecular Aspects of Medicine* **2010,** *31*, 478-494.

158. Le Marchand, L., Cancer preventive effects of flavonoids—a review. *Biomedicine & Pharmacotherapy* **2002,** *56*, 296-301.

159. Hollman, P. C. H.; Cassidy, A.; Comte, B.; Heinonen, M.; Richelle, M.; Richling, E.; Serafini, M.; Scalbert, A.; Sies, H.; Vidry, S., The Biological Relevance of Direct Antioxidant Effects of Polyphenols for Cardiovascular Health in Humans Is Not Established. *The Journal of Nutrition* **2011,** *141*, 989S-1009S.

160. Sies, H.; Hollman, P. C. H.; Grune, T.; Stahl, W.; Biesalski, H. K.; Williamson, G., Protection by Flavanol-Rich Foods Against Vascular Dysfunction and Oxidative Damage: 27th Hohenheim Consensus Conference. *Advances in Nutrition: An International Review Journal* **2012,** *3*, 217-221.

2

INDUCTION OF ELECTROPHILE-RESPONSIVE ELEMENT (EpRE)-MEDIATED GENE EXPRESSION BY TOMATO EXTRACTS *IN VITRO*

Linda Gijsbers, Henriëtte D.L.M. van Eekelen, Thuy H. Nguyen, Laura H.J. De Haan, Bart van der Burg, Jac M.M.J.G. Aarts, Ivonne M.C.M Rietjens, Arnaud G. Bovy

Based on: Food Chemistry 2012, volume 135, issue 3, pages 1166-1172

ABSTRACT

The market for food products with additional health benefits is increasing rapidly and tools for identification of bio-functional characteristics of food items are essential. To facilitate the detection of beneficial effects of tomato on gene expression, methods to prepare tomato extracts suitable to test in the EpRE-LUX assay and other cell-based reporter gene assays for health-related bioactivity mechanisms, were developed. An isoprenoid-containing chloroform extract of tomato fruit and most individual isoprenoids did not induce electrophile-responsive element (EpRE)-mediated gene expression. A semi-polar extract of tomato fruits, enzymatically hydrolyzed to remove the glycosyl residues from the phenolic ingredients, was able to induce EpRE-mediated luciferase expression at both mRNA and protein level, which might be partly due to the presence of quercetin, kaempferol, naringenin and naringenin chalcone. It was concluded that induction of EpRE-regulated genes, such as detoxifying phase II and antioxidant enzymes, may contribute to the beneficial health effects of tomato.

KEY WORDS

Tomato, *Solanum lycopersicum*, extracts, electrophile-responsive element (EpRE), phenolic compounds, isoprenoids.

INTRODUCTION

Recently, there has been increased interest to obtain scientific support for possible beneficial health effects of phytochemicals and other bioactive food compounds. For example, both flavonoids (*1, 2*) and carotenoids (*3*) have been proposed to protect against cancer and cardiovascular diseases (CVD).

Epidemiological studies indicate that a diet rich in tomato or tomato products reduces the risk of certain chronic diseases, including coronary heart disease and certain forms of cancer (*4-6*). Although the cancer-protective properties of tomato and its products are often related to lycopene, the most abundant carotenoid present in these fruits (*7*), tomatoes contain many other potentially health-beneficial phytochemicals (*8*). These include for example considerable levels of phenolic compounds like naringenin chalcone, quercetin and kaempferol (*9, 10*), which have also been related to anti-carcinogenic effects (*11, 12*). Some of the beneficial effects of these bioactive phytochemicals have been attributed to their capacity to activate Nrf2 and EpRE-mediated changes in gene expression (*13*).

The nuclear factor E_2-related factor 2 (Nrf2) is a transcription factor that can form a complex with Small Maf proteins and subsequently bind to the electrophile-responsive element (EpRE) in the regulatory region of its target genes, thereby enhancing the expression of these genes (*14*). The target genes of Nrf2 include phase II detoxifying enzymes, such as NAD(P)H:quinone oxidoreductase 1 (NQO1) and glutathione S-transferases (GSTs)(*14*). Phase II detoxifying enzymes are able to catalyze reduction or conjugation of reactive electrophiles, resulting in metabolites that are less toxic and more easily excreted from the body (*15*). Phytochemicals able to induce this EpRE-mediated gene expression include the phenolic compounds quercetin and kaempferol (*16, 17*) which are present in tomato fruit (*10*).

In the last years, reporter gene assays were developed with the goal to link bioactive compounds found in food products to beneficial health effects (*16, 18, 19*). So far these assays have been mainly used to screen isolated pure compounds, but the assays have not yet been used to test extracts from fruits and vegetables to get an integral view of their potential effects on gene regulatory pathways. A method to test fruits and vegetables in reporter gene assays would provide a tool to link certain fruits or vegetables to specific health-beneficial pathways. It would also provide a tool to test different varieties from breeding programs and/or different preparation technologies in food processing practice, in order to select the variety or processing strategy that results in the largest possible content of active ingredients producing a selected beneficial biological effect. Such an *in vitro* assay would also minimize the need for animal testing at early stages of breeding programs or processing strategies. Therefore, the aim of the present study was to develop a method to test extracts of fruits and vegetables in reporter gene assays. The present study will focus on tomato fruit as a model for fruit and vegetables and on the EpRE-LUX reporter cell line (*16*) as a model reporter gene assay.

MATERIALS AND METHODS

Chemicals and reagents

Kaempferol, kaempferol-3-O-rutinoside, delphinidin chloride, cyanidin chloride, and lutein were purchased from Extrasynthese (Genay, France). Trifluoroacetic acid (TFA), β-carotene, lycopene, α-tocopherol, (+)-δ-tocopherol, (+)-γ-tocopherol, quercetin, chlorogenic acid, caffeic acid, ascorbic acid, *tert*-butylhydroquinone (tBHQ), tetrahydrofuran (THF) containing 0.025% butylated hydroxytoluene (BHT), β-glucuronidase from *Helix pomatia (H. pomatia)* type HP2 and Viscozyme L were purchased from Sigma-Aldrich (St. Louis, USA). Phytoene, phytofluene, neoxanthin, violaxanthin, neurosporene, δ-carotene and γ-carotene were obtained from CaroteNature GmbH (Lupsingen, Switzerland). Naringenin and a second preparation of β-carotene (which was stored for several years at -20°C) were obtained from ICN Biomedicals Inc. (Aurora, USA). Dimethyl sulfoxide (DMSO) and rutin were obtained from Acros Organics (Geel, Belgium). Methanol and chloroform were purchased from Biosolve (Valkenswaard, The Netherlands).

Minimum Essential Medium alpha (further referred to as α-MEM), Minimum Essential Medium alpha without phenol red, the 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (further referred to as DMEM/F12), DMEM/F12 without phenol red, fetal calf serum (FCS), Hank's balanced salt solution (HBSS), trypsin, nonessential amino acids (further referred to as NEAA), gentamicin, penicillin/streptomycin and G418 were purchased from Invitrogen Corporation (Breda, The Netherlands). Dextran-coated charcoal-stripped FCS (DCC-FCS) was purchased from Thermo Scientific (Waltham, USA).

Naringenin chalcone was not commercially available as a pure compound. Naringenin chalcone was obtained from Apin Chemicals (Abingdon, UK) as a mixture with naringenin and the percentage of naringenin chalcone in the mixture was determined to be approximately 40% by HPLC analysis.

Tomato samples

The model tomato sample was described earlier (*20*). In short, the sample was prepared by pooling fruits from several genotypes of beef, cherry, and round tomatoes in fully red stage of ripening. The fruit material was snap frozen in liquid nitrogen and ground in an analytical mill. The tomato powder was stored at -80°C until further use, and in this article will be further referred to as: tomato mix.

Another tomato sample was prepared from roma tomatoes that were purchased at a local supermarket in Wageningen (The Netherlands). These fruits were also pooled and snap frozen in liquid nitrogen and ground in an analytical mill. The tomato powder was stored at -80°C until further use, and in this article will be further referred to as: roma tomato mix.

Preparation of tomato extracts containing semi-polar metabolites

Semi-polar metabolites such as flavonoids and phenolic acids and their glycosides, were extracted using methods described earlier (*21*). Briefly, nonhydrolyzed tomato extracts containing phenolic compounds were prepared by adding 3.4 ml of methanol

to 0.6 g of tomato mix followed by sonication for 10 minutes and centrifugation at 1000 g for 10-15 minutes. The supernatant of the samples was filtered using 0.2 µm polytetrafluoroethylene (PTFE)-filters (M-filter, Tiel, The Netherlands) and freeze-dried. Prior to analysis in the EpRE-LUX assay these tomato extracts, further referred to as nonhydrolyzed tomato extracts, were redissolved in DMSO:α-MEM (1:2 v/v).

Preparation of enzymatically hydrolyzed tomato extracts containing semi-polar metabolites

Enzymatically hydrolyzed tomato extracts containing phenolic compounds were prepared by adding 300 µl of 0.1 M sodium acetate (pH 4.8) and 100 µl of Viscozyme L or β-glucuronidase from *H. pomatia* type HP2 to 0.6 g tomato mix, followed by 1 hour incubation in a water bath at 37°C. Then, samples were put on ice and 3.0 ml of methanol were added, followed by 10 minutes sonication and 15 minutes centrifugation at 1000 g. The supernatant was filtered (0.2 µm PTFE-filters) and 2.0 ml of filtered methanol supernatant were dried under a stream of nitrogen. The remaining solution was finally freeze-dried and stored at -80°C until further analysis. Prior to analysis in the EpRE-LUX assay these extracts, further referred to as enzymatically hydrolyzed tomato extracts, were redissolved in DMSO:α-MEM (1:4 v/v).

Preparation of tomato extracts containing isoprenoid derivatives

Isoprenoids such as carotenoids and tocopherols, were extracted using methods described earlier (*21*). In short, 4.5 ml of methanol/chloroform (2.5/2.0 v/v) was added to 0.5 g roma tomato mix, and the sample was mixed and put on ice for 10 minutes. Subsequently, to each sample 2.5 ml of cold 50 mM Tris-HCl buffer (pH 7.4) were added, samples were mixed and then centrifuged for 10 minutes at 1000 g. The chloroform phase was transferred to a new tube. The methanol/Tris sample was re-extracted twice by adding 1 ml of chloroform, mixing and centrifugation of the tubes. The three chloroform extraction fractions were combined and dried under a stream of nitrogen. Prior to analysis in the EpRE-LUX assay these extracts, further referred to as isoprenoid-containing tomato extracts, were redissolved in THF:α-MEM (1:1 v/v).

Separation and detection of semi-polar metabolites by HPLC-PDA

The separation, detection and quantification of semi-polar metabolites such as flavonoids and phenolic acids was performed using high-performance liquid chromatography with photodiode array detection (HPLC-PDA) according to a modified method described before (*21*). Briefly, the freeze-dried samples were dissolved in 2 ml methanol and analyzed using a Waters e2695 separation module (Waters, Milford, USA) connected to a Waters 996 photodiode array detector. The system was equipped with a Luna C_{18} (3 µm, 150 mm x 4.60 mm)(Phenomenex, Torrance, USA) column coupled to a C_{18} guard column (Phenomenex, Torrance, USA). Column temperature was maintained at 40°C by a Pharmacia oven. Separation was performed using a 5 - 35% acetonitrile linear gradient in 0.1% TFA for 45 minutes, followed by 35% - 75% acetonitrile linear gradient in 0.1% TFA for 2 minutes. Hereafter, the column was washed for 5 minutes at 75% acetonitrile

in 0.1% TFA and in 2 minutes brought back to initial concentrations and equilibrated for 15 minutes before the next injection. This was all performed at a flow rate of 1 ml/min. The samples were monitored continuously from 200 to 600 nm by the Waters 996 photodiode array detector. Data were collected and analyzed using Waters Empower 2 software. HPLC chromatograms were recorded at 360 nm.

Cell culture

The EpRE-LUX cells, which are Hepa-1c1c7 mouse hepatoma cells stably transfected with a reporter construct carrying a luciferase reporter gene under transcriptional control of an EpRE enhancer element in conjunction with a minimal promoter and an initiator (*16*), were cultured in α-MEM medium supplemented with 10% fetal calf serum (FCS), 50 µg/mL gentamicin and 0.5 mg/mL G418. The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

The Cytotox CALUX cells are human osteosarcoma U2OS cells stably transfected with a reporter construct carrying a luciferase reporter gene under transcriptional control of a constitutive promoter. The reporter construct was generated by inserting the luciferase gene into the multiple cloning site of the pSG5-neo vector (*22*). These cells have an invariant luciferase expression and were originally designed to discover cytotoxicity (*23*). The Cytotox CALUX cells were cultured in DMEM/F12 supplemented with 7.5% FCS, NEAA, and penicillin/streptomycin (final concentrations 10 U/ml and 10 µg/ml, respectively). Once per week, 200 µg/ml G418 was added to the culture medium in order to keep the selection pressure.

EpRE-LUX assay

The ability of tomato extracts and individual tomato compounds to induce EpRE-mediated luciferase expression at protein level was tested by measuring luciferase activity in the EpRE-LUX reporter cells. EpRE-LUX cells were seeded in the 60 inner wells of a white 96-wells view plate at a density of 20,000 cells per well in 100 µL culture medium. In the outer wells 100 µl Hanks' Balanced Salt Solution (HBSS) was added to maintain physical homogeneity throughout the plate. The seeded cells were incubated for 24 hours to allow them to attach and form a confluent monolayer. Subsequently, the cells were exposed for 24 hours to α-MEM without FCS and antibiotics containing individual compounds or tomato extracts. The freeze-dried extracts were dissolved in DMSO: α-MEM (1:2 v/v), DMSO:α-MEM (1:4 v/v) or THF:α-MEM (1:1 v/v) as described above. The pure phenolic compounds were dissolved in DMSO and the pure isoprenoids were dissolved in THF. The final concentration of solvent in the exposure medium was 0.5% for studies with all individual compounds, 1.5% for studies with enzymatically hydrolyzed tomato extract, and 1.0% for studies with isoprenoid-containing tomato extract. On each plate, 30 µM tBHQ, a well-known inducer of EpRE-controlled gene transcription (*16, 24, 25*), was included as a positive control. Experiments with isoprenoids were conducted under reduced light conditions (exclusion of light with a wavelength <550nm).

After 24 hours of exposure, medium was removed, cells were lysed by adding low salt lysis buffer (*16*) and incubated overnight at -80°C. Luciferase activity in the lysate was

measured using a luminometer (Luminoscan Ascent, Thermo Scientific, Waltham, USA) and flash mix (*16*). Luciferase activity per well was measured in relative light units (RLU).

Compounds giving less than twofold induction at the maximum concentration (100 µM or the highest concentration that could be tested without cytotoxicity) were considered unable to induce EpRE-mediated luciferase expression at protein level. A viable cell count using trypan blue exclusion was performed as described elsewhere (*26*) in order to ascertain that the individual compounds and the various tomato extracts did not cause cytotoxicity at the concentrations used (data not shown).

Quantitative PCR

The ability of the enzymatically hydrolyzed tomato extract and individual tomato compounds to induce EpRE-mediated luciferase expression at mRNA level was tested by measuring luciferase mRNA in EpRE-LUX cells by quantitative PCR. Since exposure time curves with tBHQ showed that 6 hour exposure is optimal for luciferase mRNA induction (data not shown), the exposure time for qPCR experiments was 6 hours compared to 24 hour exposure in luciferase activity experiments. EpRE-LUX cells were plated in 6-well plates. The next day, the EpRE-LUX cells were exposed for 6 hours to the individual compounds or to the enzymatically hydrolyzed tomato extract and then total RNA was extracted using a RNeasy kit (Qiagen, Hilden, Germany). The extracted RNA was reverse transcribed into cDNA using iScript RT supermix (Biorad, Hercules, USA). Quantitative PCR was performed using SYBR Green (Biorad, Hercules, USA), an iCycler Real Time PCR machine (Biorad, Hercules, USA), and specific primers for luciferase (forward 5'-CGGGCGCGGTCGGTAAAGTT-3'; reverse 5'-AAGGCGTTGGTCGCTTCCGG-3') and for mouse succinate dehydrogenase complex, subunit A (*Sdha*) as a reference gene (forward 5'-CTTGAATGAGGCTGACTGTG -3'; reverse 5'- ATCACATAAGCTGGTCCTGT-3').

Cytotox CALUX assay

The Cytotox CALUX cells have an invariant luciferase expression and were originally designed to discover cytotoxicity (*23*). These cells, however, can also be used to investigate whether stabilization of the luciferase enzyme is occurring during the exposure to compounds or extracts, a phenomenon that would result in increased luciferase activity without underlying increased expression of the gene (*27*). The Cytotox CALUX cells were seeded in the 60 inner wells of a white 96-well view plate at a density of 10,000 cells per well in 100 µl assay medium: phenol red-free DMEM/F12 medium supplemented with 5% dextran-coated charcoal-stripped FCS (DCC-FCS), NEAA and penicillin/streptomycin (final concentrations 10 U/ml and 10 µg/ml, respectively). The outer wells were filled with HBSS. The next day, the cells were exposed to assay medium (200 µl per well) containing the test compounds. After 24 hours of exposure, the cells were lysed by adding low salt lysis buffer (*16*) and overnight incubation at -80°C. Luciferase activity in the lysate was measured using a luminometer and flash mix (*16*). Luciferase activity per well was measured in relative light units (RLU).

Data processing and statistical analysis

For each experiment, at least 2 independent repetitions were performed, of which one representative curve is presented. Data were analyzed using GraphPad Prism version 5.04 and Microsoft Excel 2010. Data are expressed as fold induction over the solvent control and are presented as mean values ± standard error of the mean (SEM). Each data point was measured (at least) in triplicate. Statistical significance was assessed using the one-sided Student's t-test and a cut-off value of $p \leq 0.05$.

RESULTS

Induction of EpRE-mediated luciferase expression at protein level by isoprenoid-containing tomato extracts

The EpRE-LUX assay was used to determine whether tomato extracts are capable of inducing EpRE-mediated luciferase expression at the protein level. A chloroform extract containing isoprenoids was prepared from the roma tomato mix. After 24 hours of exposure, the isoprenoid-containing tomato extract was not able to induce EpRE-mediated luciferase protein expression at concentrations up to 100 gram fresh weight per liter (g FW/L)(data not shown). This is in line with our findings that of the individual isoprenoids lycopene, lutein, α-tocopherol, δ-tocopherol, γ-tocopherol, β-carotene, δ-carotene, γ-carotene, phytoene, phytofluene, neoxanthin, violaxanthin and neurosporene, only the latter was able to induce EpRE-mediated luciferase protein expression at concentrations higher than 20 μM (figure 1).

Altogether, these results indicate that isoprenoids, either as individual compounds or as a constituent in tomato, do not play an important role as inducers of EpRE-regulated genes.

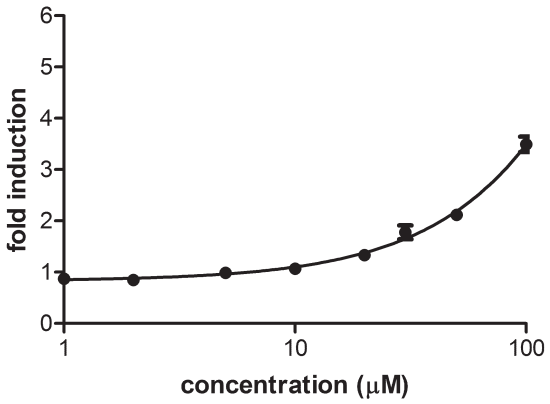

Figure 1. Luciferase activity induced in the EpRE-LUX reporter cells by 24 hour exposure to neurosporene. Luciferase activity is expressed as fold induction compared to solvent control and data are presented as mean ± SEM of six replicates.

Induction of EpRE-mediated luciferase expression at protein level by tomato extracts containing phenolic compounds

In order to determine whether phenolic compounds in tomato extracts are capable of activating EpRE-mediated luciferase protein expression, a nonhydrolyzed methanol extract containing phenolic compounds was prepared from the tomato mix. This extract, tested in the EpRE-LUX cells in concentrations up to 15 g FW/L, was unable to induce EpRE-mediated luciferase protein expression after 24 hours of exposure (data not shown). Qualitative HPLC analysis showed that quercetin and kaempferol were only present in these extracts in the form of glycosides, mainly quercetin-rutinoside and kaempferol-rutinoside (figure 2A). Unlike flavonoid aglycones (*16, 17, 24*), flavonoid glycosides were not able to activate EpRE-mediated luciferase protein expression (table 1). In order to deglycosylate these flavonoids, a method for hydrolysis of the extract containing phenolic compounds had to be developed. To avoid generation of non-natural by-products of the hydrolysis reaction, enzymatic hydrolysis was used instead of acid hydrolysis.

Table 1. Capacity of individual compounds known to be present in ripe tomato, to induce EpRE-mediated luciferase expression. In order to rank the compounds, the fold inductions at 20 µM and at the highest concentration that could be tested without cytotoxicity, were compared. Fold inductions are given as the average value of at least three independent experiments ± SEM.

Compound	Fold induction at 20 µM	Maximum fold induction at the concentration indicated between parentheses
Quercetin	4.2 ± 0.3	4.9 ± 0.4 (30 µM)
Kaempferol	3.8 ± 0.4	3.5 ± 0.4 (20 µM)
Naringenin	0.9 ± 0.1	2.5 ± 0.4 (100 µM)
Naringenin Chalcone	1.0 ± 0.1	3.2 ± 0.3 (100 µM)
Isoquercetin	inactive	inactive
Rutin	inactive	inactive
Kaempferol-3O-rutinoside	inactive	inactive
Chlorogenic acid	inactive	inactive
Caffeic acid	inactive	inactive
Ferulic acid	inactive	inactive
Cyanidin chloride	inactive	inactive
Delphinidin chloride	inactive	inactive

Induction of EpRE-mediated luciferase expression at protein and mRNA level by enzymatically hydrolyzed tomato extracts

To enzymatically hydrolyze flavonoid glycosides present in tomato mix, two commercially available enzyme preparations were used; Viscozyme L and *H. pomatia* type-2 β-glucuronidase. The optimal incubation time was determined by following the hydrolysis of rutin and kaempferol-3-O-rutinoside into their corresponding aglycones in time using HPLC. Even after 15 hours of incubation with *H. pomatia* type-2 β-glucuronidase, the

rutin present in the tomato mix was not completely hydrolyzed (figure 2B). After only one hour incubation with Viscozyme L, neither rutin nor kaempferol-3-O-rutinoside could be detected anymore and a corresponding increase in the concentrations of quercetin and kaempferol was observed (figure 2C), indicating that hydrolysis of the glycosides was complete. In subsequent experiments it was demonstrated that tomato extract generated with this method, further referred to as enzymatically hydrolyzed tomato extract, was able to significantly induce EpRE-mediated luciferase expression in a dose dependent manner, both at protein level (figure 3) and at mRNA level (figure 4). In addition, it was shown that exposure to the enzymatically hydrolyzed tomato extract had hardly any effect on the luciferase activity measured in the Cytotox CALUX cells (figure 3), indicating that stabilization of the luciferase enzyme is not playing a major role in the induction of luciferase activity.

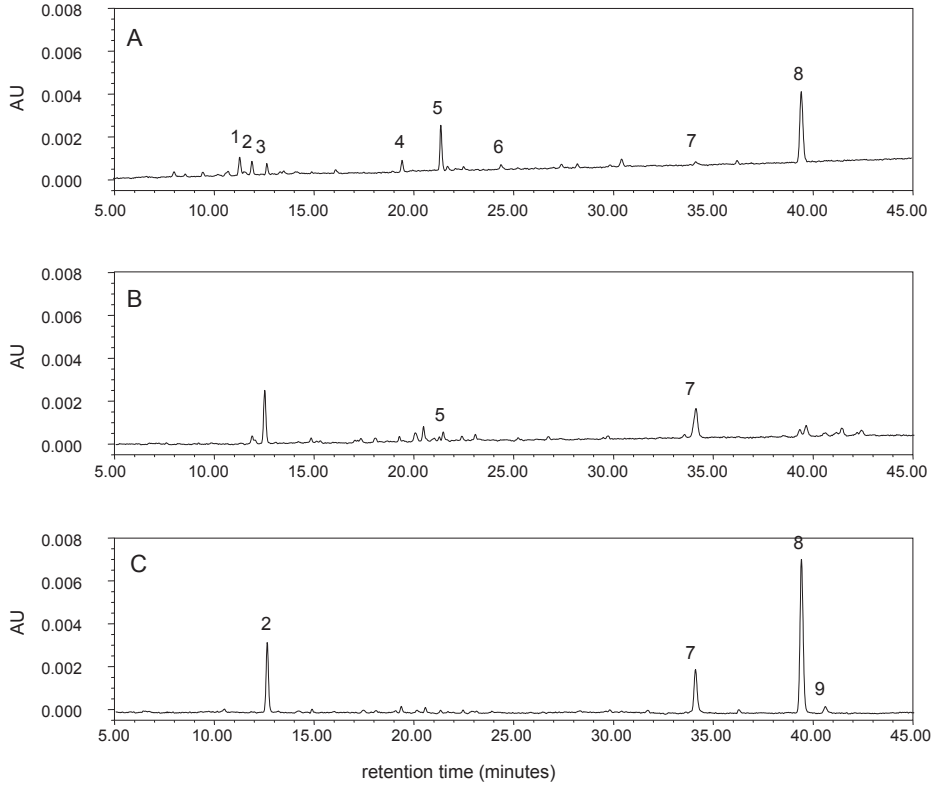

Figure 2. HPLC chromatograms of tomato extracts recorded at 360 nm. A) nonhydrolyzed tomato extract; B) tomato extract hydrolyzed for 15 hours with H. pomatia type-2 β-glucuronidase; and C) tomato extract hydrolyzed for 1 hour with Viscozyme L. 1: chlorogenic acid; 2: caffeic acid; 3: ferulic acid; 4: quercetin-3-O-glycoside 5: rutin; 6: kaempferol-3-O-rutinoside; 7: quercetin; 8: naringenin chalcone; 9: kaempferol.

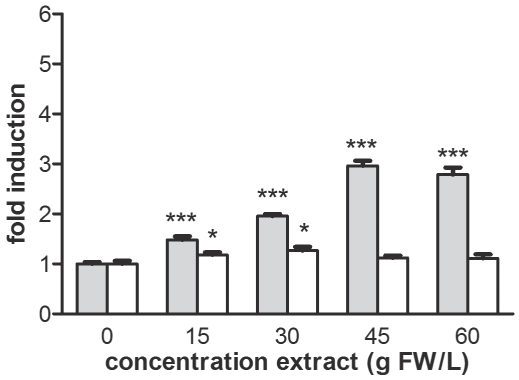

Figure 3. Luciferase activity induced in EpRE-LUX reporter cells (grey bars) and in Cytotox CALUX cells (white bars) by 24 hour exposure to enzymatically hydrolyzed tomato extract. Concentrations of the extract are expressed as gram fresh weight per liter (g FW/L). Luciferase activity is expressed as fold induction compared to solvent control and data are presented as mean ± SEM of six replicates. Asterisks indicate a significant difference from the corresponding solvent control: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$.

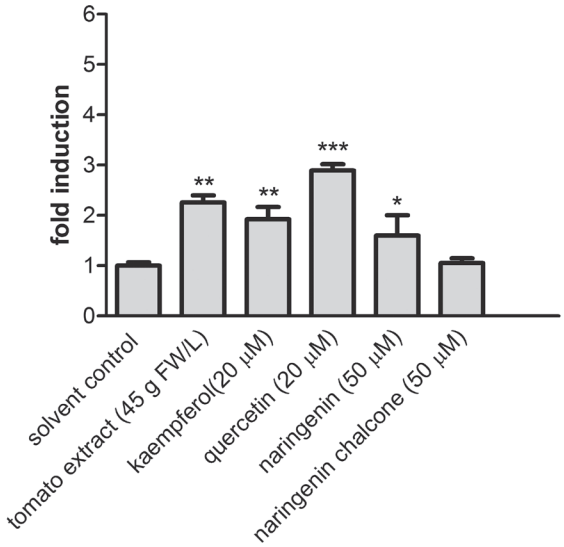

Figure 4. Luciferase gene expression measured as luciferase mRNA levels in EpRE-LUX cells after 6 hour exposure to enzymatically hydrolyzed tomato extract (45 g FW/L) or to individual phenolic compounds. Luciferase mRNA levels were measured by qPCR and are expressed as fold induction compared to solvent control. Data are presented as mean ± SEM of three (tomato extract) or four replicates (individual compounds). Asterisks indicate a significant difference from the solvent control: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$.

Activation of EpRE-mediated luciferase expression by individual compounds present in enzymatically hydrolyzed tomato extracts

The induction of EpRE-mediated luciferase expression found after exposure of EpRE-LUX cells to the enzymatically hydrolyzed tomato extract raises the question which compounds in this extract are responsible for this effect. In order to investigate this, EpRE-LUX reporter cells were exposed for 24 hours to increasing concentrations of the following compounds, known to be important phenolic ingredients in tomato fruits (*9, 10, 28*)**:** quercetin, isoquercetin, rutin, naringenin, naringenin chalcone, kaempferol, kaempferol-3-O-rutinoside, chlorogenic acid, ferulic acid, caffeic acid, delphinidin chloride, and cyanidin chloride. Quercetin, kaempferol, naringenin and naringenin chalcone were able to induce EpRE-mediated luciferase protein expression in EpRE-LUX cells in a dose-dependent manner (figure 5), while all other compounds tested were not able to induce EpRE-mediated protein expression in concentrations up to 100 µM (data not shown). Quercetin was slightly more potent than kaempferol. Naringenin and naringenin chalcone were equally potent, but both were less potent than quercetin and kaempferol (table 1 / figure 5). Quercetin, kaempferol and naringenin were also able to induce EpRE-mediated expression at the mRNA level, although naringenin chalcone was not (figure 4).

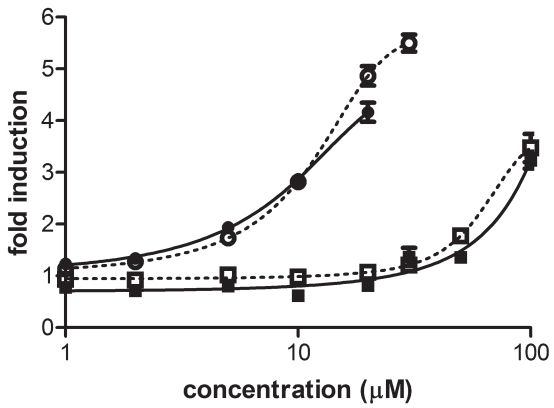

Figure 5. Luciferase activity induced in the EpRE-LUX reporter cells by 24 hour exposure to individual phenolic compounds; kaempferol (●), quercetin (O), naringenin (■), and naringenin chalcone (□). Luciferase activity is expressed as fold induction compared to solvent control and data are presented as mean ± SEM of six replicates.

DISCUSSION

In this study, a robust method was developed to prepare crude extracts of ripe tomato fruits that are compatible with cell-physiological and cell culture conditions and thus enable testing of these extracts in the cell-based EpRE-LUX reporter assay and other cell-based assays. This method may also be used to test other fruits and vegetables for their

EpRE-mediated effects on gene expression and to test fruit and vegetable extracts in other cell-based bioassays. It may also provide a tool to test different varieties and/or food processing strategies, in order to select the variety or processing strategy resulting in the most optimal content of bioactive compounds producing the selected beneficial biological effect. The method developed might therefore provide a tool to link fruits or vegetables to beneficial gene expression profiles without the need for animal testing.

An isoprenoid-containing tomato extract was prepared by chloroform extraction and an enzymatically hydrolyzed tomato extract containing flavonoid aglycones was prepared by enzymatic hydrolysis for 1 hour with Viscozyme L followed by methanol extraction. Both the isoprenoid-containing tomato extract and the enzymatically hydrolyzed tomato extract were shown to be biocompatible with the EpRE-LUX reporter cells. It is shown that the isoprenoid-containing tomato extract was not able to induce EpRE-mediated luciferase protein expression, while the enzymatically hydrolyzed tomato extract was able to induce a dose-dependent induction of EpRE-mediated luciferase expression as detected both at the protein and the mRNA level.

In line with our findings that the isoprenoid-containing tomato extract was not able to induce EpRE-mediated luciferase, most individual isoprenoids also showed no EpRE-mediated luciferase induction. Most isoprenoids lack an electrophilic moiety and are therefore unable to react with thiol-groups of Keap1 and to facilitate release of Nrf2 from the Keap1-Nrf2 complex, which is necessary to activate EpRE-mediated expression (*25*). Previously, however, Linnewiel and coworkers showed that not the carotenoid parent compounds, but rather their oxidation products are activators of EpRE-mediated gene expression (*25*). This is in line with our observation that an old and partially discolored batch of β-carotene was able to induce luciferase activity in the EpRE-LUX cells (data not shown), whereas the newly purchased batch of β-carotene was not.

The nonhydrolyzed tomato extract and the flavonoid glycosides rutin and kaempferol-3-O-rutinoside were not able to induce EpRE-mediated luciferase protein expression. This lack response is likely due to the fact that these glycosides are not able to enter the cells. The tomato mix was therefore enzymatically hydrolyzed in order to convert the flavonoid glycosides into their aglycones. This enzymatic hydrolysis of flavonoid glycosides also occurs in humans in the small intestine before uptake (*29, 30*). The enzymatically hydrolyzed tomato extract made of ripe tomato fruit was able to induce EpRE-mediated luciferase expression at the mRNA level and at the protein level. These results indicate that phytochemicals present in tomato may induce transcription of phase II detoxifying enzymes, which are often under control of an EpRE. Induction of EpRE-mediated gene expression has frequently been suggested to play a role in the protection against cancer (*14, 15*). Quercetin, kaempferol, naringenin and naringenin chalcone are present in enzymatically hydrolyzed tomato fruit extracts (*9, 10*) and were able to activate EpRE-mediated gene expression when tested as pure compounds. These findings are in line with previous *in vitro* studies showing that quercetin and kaempferol are able to induce EpRE-mediated gene expression (*16, 17, 24*). It is likely that these four compounds are at least partly responsible for the induction of EpRE-mediated luciferase expression by enzymatically hydrolyzed tomato extracts.

It is important to note that some chemicals can bind to and stabilize the luciferase enzyme produced in the reporter gene cells, resulting in high bioluminescence values reflecting luciferase stabilization rather than increased expression of the luciferase gene (*27*). In the present study, exposure to enzymatically hydrolyzed tomato extract (45 g FW/L) induced luciferase expression at both protein level and at mRNA level in the same order of magnitude. This indicates that stabilization of the luciferase enzyme is not playing an important role when testing tomato extracts in the EpRE-LUX assay. This was corroborated by the observation that exposure to enzymatically hydrolyzed tomato extract had hardly any effect on the luciferase activity in the Cytotox CALUX cells. In case the enzymatically hydrolyzed tomato extract would lead to stabilization of the luciferase enzyme, a dose-dependent increase in luciferase activity in the Cytotox CALUX cells would be expected. This was clearly not observed. Thus, based on the mRNA data and the results of the Cytotox CALUX assay it was firmly established that the luciferase induction by the tomato constituents and the enzymatically hydrolyzed tomato extract was not due to an effect on the luciferase enzyme itself.

Although the results of the present study indicate that tomato may increase EpRE-mediated expression of phase II enzymes and other EpRE-regulated genes, this result needs to be confirmed and validated in *in vivo* studies. From *in vivo* studies it is known that in mice, a combination of tomato and garlic leads to increased activity of the phase II enzyme GST and to reduced frequencies of chemically-induced genotoxicity (*31*).

In conclusion, the current study has developed an extraction and enzymatic deglycosylation method that enables testing of fruits and vegetables in *in vitro* bioassays for beneficial health effects. The developed method may allow the selection of optimal breeding and processing technologies and the definition of specific health claims without the need for animal testing. The utility of the developed method for these purposes should be further explored. The results obtained show that enzymatically hydrolyzed tomato extracts and phytochemicals present in these tomato extracts are able to induce EpRE-mediated changes in gene expression and may thereby increase the levels of phase II detoxifying enzymes, which may play a role in protection against cancer.

ACKNOWLEDGEMENT

This work was financially supported by the Food and Nutrition Delta (FND07007).

REFERENCES

1. Perez-Vizcaino, F.; Duarte, J., Flavonols and cardiovascular disease. *Molecular Aspects of Medicine* **2010,** *31*, 478-494.
2. Le Marchand, L., Cancer preventive effects of flavonoids—a review. *Biomedicine & Pharmacotherapy* **2002,** *56*, 296-301.
3. Rao, A. V.; Rao, L. G., Carotenoids and human health. *Pharmacological Research* **2007,** *55*, 207-216.
4. Agudo, A.; Esteve, M. G.; Pallarés, C.; Martínez-Ballarín, I.; Fabregat, X.; Malats, N.; Machengs, I.; Badia, A.; González, C. A., Vegetable and fruit intake and the risk of lung cancer in women in Barcelona, Spain. *European Journal of Cancer* **1997,** *33*, 1256-1261.
5. Campbell, J. K.; Canene-Adams, K.; Lindshield, B. L.; Boileau, T. W.-M.; Clinton, S. K.; Erdman, J. W., Tomato Phytochemicals and Prostate Cancer Risk. *The Journal of Nutrition* **2004,** *134*, 3486S-3492S.
6. Sesso, H. D.; Liu, S.; Gaziano, J. M.; Buring, J. E., Dietary Lycopene, Tomato-Based Food Products and Cardiovascular Disease in Women. *The Journal of Nutrition* **2003,** *133*, 2336-2341.
7. Giovannucci, E., Tomatoes, Tomato-Based Products, Lycopene, and Cancer: Review of the Epidemiologic Literature. *Journal of the National Cancer Institute* **1999,** *91*, 317-331.
8. Levin, I.; de Vos, C.; Tadmor, Y.; Bovy, A.; Lieberman, M.; Oren-Shamir, M.; Segev, O.; Kolotilin, I.; Keller, M.; Ovadia, R.; Meir, A.; Bino, R., High pigment tomato mutants—more than just lycopene (a review). *Israel Journal of Plant Sciences* **2006,** *54*, 179-190.
9. Slimestad, R.; Verheul, M. J., Seasonal Variations in the Level of Plant Constituents in Greenhouse Production of Cherry Tomatoes. *Journal of Agricultural and Food Chemistry* **2005,** *53*, 3114-3119.
10. Torres, C. A.; Davies, N. M.; Yanez, J. A.; Andrews, P. K., Disposition of Selected Flavonoids in Fruit Tissues of Various Tomato (Lycopersicon esculentum Mill.) Genotypes. *Journal of Agricultural and Food Chemistry* **2005,** *53*, 9536-9543.
11. Cui, Y.; Morgenstern, H.; Greenland, S.; Tashkin, D. P.; Mao, J. T.; Cai, L.; Cozen, W.; Mack, T. M.; Lu, Q.-Y.; Zhang, Z.-F., Dietary flavonoid intake and lung cancer—A population-based case-control study. *Cancer* **2008,** *112*, 2241-2248.
12. Knekt, P.; Kumpulainen, J.; Järvinen, R.; Rissanen, H.; Heliövaara, M.; Reunanen, A.; Hakulinen, T.; Aromaa, A., Flavonoid intake and risk of chronic diseases. *The American Journal of Clinical Nutrition* **2002,** *76*, 560-568.
13. Talalay, P.; Fahey, J. W., Phytochemicals from Cruciferous Plants Protect against Cancer by Modulating Carcinogen Metabolism. *The Journal of Nutrition* **2001,** *131*, 3027S-3033S.
14. Talalay, P.; Dinkova-Kostova, A. T.; Holtzclaw, W. D., Importance of phase 2 gene regulation in protection against electrophile and reactive oxygen toxicity and carcinogenesis. *Advances in Enzyme Regulation* **2003,** *43*, 121-134.
15. Giudice, A.; Montella, M., Activation of the Nrf2–ARE signaling pathway: a promising strategy in cancer prevention. *Bioessays* **2006,** *28*, 169-181.
16. Boerboom, A.-M. J. F.; Vermeulen, M.; van der Woude, H.; Bremer, B. I.; Lee-Hilz, Y. Y.; Kampman, E.; van Bladeren, P. J.; Rietjens, I. M. C. M.; Aarts, J. M. M. J. G., Newly constructed stable reporter cell lines for mechanistic studies on electrophile-responsive element-mediated gene expression reveal a role for flavonoid planarity. *Biochemical Pharmacology* **2006,** *72*, 217-226.
17. Uda, Y.; Price, K. R.; Williamson, G.; Rhodes, M. J. C., Induction of the anticarcinogenic marker enzyme, quinone reductase, in murine hepatoma cells in vitro by flavonoids. *Cancer Letters* **1997,** *120*, 213-216.
18. Gijsbers, L.; Man, H.-Y.; Kloet, S. K.; de Haan, L. H. J.; Keijer, J.; Rietjens, I. M. C. M.; van der Burg, B.; Aarts, J. M. M. J. G., Stable reporter cell lines for peroxisome proliferator-activated receptor γ (PPARγ)-mediated modulation of gene expression. *Analytical Biochemistry* **2011,** *414*, 77-83.
19. Shih, H.; Pickwell, G. V.; Quattrochi, L. C., Differential Effects of Flavonoid Compounds on Tumor Promoter-Induced Activation of the Human CYP1A2 Enhancer. *Archives*

of Biochemistry and Biophysics **2000,** *373*, 287-294.

20. Tikunov, Y.; Lommen, A.; de Vos, C. H. R.; Verhoeven, H. A.; Bino, R. J.; Hall, R. D.; Bovy, A. G., A Novel Approach for Nontargeted Data Analysis for Metabolomics. Large-Scale Profiling of Tomato Fruit Volatiles. *Plant Physiology* **2005,** *139*, 1125-1137.
21. Bino, R. J.; De Vos, C. H. R.; Lieberman, M.; Hall, R. D.; Bovy, A.; Jonker, H. H.; Tikunov, Y.; Lommen, A.; Moco, S.; Levin, I., The light-hyperresponsive high pigment-2dg mutation of tomato: alterations in the fruit metabolome. *New Phytologist* **2005,** *166*, 427-438.
22. Sonneveld, E.; van den Brink, C.; van der Leede, B.; Schulkes, R.; Petkovich, M.; van der Burg, B.; van der Saag, P., Human retinoic acid (RA) 4-hydroxylase (CYP26) is highly specific for all-trans-RA and can be induced through RA receptors in human breast and colon carcinoma cells. *Cell Growth Differ* **1998,** *9*, 629-637.
23. Van der Linden, S. C.; von Bergh, A. R. M.; Van Vugt-Lussenburg, B.; Jonker, L.; Brouwer, A.; Teunis, M.; Krul, C. A. M.; Van der Burg, B., Development of a panel of high throughput reporter gene assays to detect genotoxicity and oxidative stress. **Submitted for publication**.
24. Lee-Hilz, Y. Y.; Boerboom, A.-M. J. F.; Westphal, A. H.; van Berkel, W. J. H.; Aarts, J. M. M. J. G.; Rietjens, I. M. C. M., Pro-Oxidant Activity of Flavonoids Induces EpRE-Mediated Gene Expression. *Chemical Research in Toxicology* **2006,** *19*, 1499-1505.
25. Linnewiel, K.; Ernst, H.; Caris-Veyrat, C.; Ben-Dor, A.; Kampf, A.; Salman, H.; Danilenko, M.; Levy, J.; Sharoni, Y., Structure activity relationship of carotenoid derivatives in activation of the electrophile/antioxidant response element transcription system. *Free Radical Biology and Medicine* **2009,** *47*, 659-667.
26. Weil, B. R.; Markel, T. A.; Herrmann, J. L.; Abarbanell, A. M.; Meldrum, D. R., Mesenchymal stem cells enhance the viability and proliferation of human fetal intestinal epithelial cells following hypoxic injury via paracrine mechanisms. *Surgery* **2009,** *146*, 190-197.
27. Sotoca, A. M.; Bovee, T. F. H.; Brand, W.; Velikova, N.; Boeren, S.; Murk, A. J.; Vervoort, J.; Rietjens, I. M. C. M., Superinduction of estrogen receptor mediated gene expression in luciferase based reporter gene assays is mediated by a post-transcriptional mechanism. *The Journal of Steroid Biochemistry and Molecular Biology* **2010,** *122*, 204-211.
28. Luthria, D. L.; Mukhopadhyay, S.; Krizek, D. T., Content of total phenolics and phenolic acids in tomato (Lycopersicon esculentum Mill.) fruits as influenced by cultivar and solar UV radiation. *Journal of Food Composition and Analysis* **2006,** *19*, 771-777.
29. Day, A. J.; DuPont, M. S.; Ridley, S.; Rhodes, M.; Rhodes, M. J. C.; Morgan, M. R. A.; Williamson, G., Deglycosylation of flavonoid and isoflavonoid glycosides by human small intestine and liver β-glucosidase activity. *FEBS Letters* **1998,** *436*, 71-75.
30. Walle, T.; Otake, Y.; Walle, U. K.; Wilson, F. A., Quercetin Glucosides Are Completely Hydrolyzed in Ileostomy Patients before Absorption. *The Journal of Nutrition* **2000,** *130*, 2658-2661.
31. Kumaraguruparan, R.; Chandra Mohan, K. V. P.; Abraham, S. K.; Nagini, S., Attenuation of N-methyl-N'-nitro-N-nitrosoguanidine induced genotoxicity and oxidative stress by tomato and garlic combination. *Life Sciences* **2005,** *76*, 2247-2255.

3

STABLE REPORTER CELL LINES FOR PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR γ (PPARγ)-MEDIATED MODULATION OF GENE EXPRESSION

Linda Gijsbers, Hai-Yen Man, Samantha K. Kloet, Laura H.J. de Haan, Jaap Keijer, Ivonne M.C.M. Rietjens, Bart van der Burg, Jac M.M.J.G. Aarts

Based on: Analytical Biochemistry 2011, volume 414, pages 77-83

ABSTRACT

Activation of peroxisome proliferator-activated receptor γ (PPARγ) by ligands is associated with beneficial health effects including anti-inflammatory and insulin-sensitizing effects. The aim of the present study was to develop luciferase reporter gene assays to enable fast and low-cost measurement of PPARγ agonist and antagonist activity.

Two reporter gene assays, PPARγ1 CALUX and PPARγ2 CALUX, were developed by stable transfection of U2OS cells with an expression vector for PPARγ1 or PPARγ2, and a pGL3-3xPPRE-tata-luc or pGL4-3xPPRE-tata-luc reporter construct, respectively. PPARγ1 CALUX and PPARγ2 CALUX cells showed similar concentration-dependent luciferase induction upon exposure to the PPARγ agonists rosiglitazone, troglitazone, pioglitazone, ciglitazone, netoglitazone and 15-deoxγ-$\Delta^{12,14}$-prostaglandin J_2. The potency to induce luciferase decreased in the following order: rosiglitazone > troglitazone = pioglitazone > netoglitazone > ciglitazone. A concentration-dependent decrease in the response to 50 nM rosiglitazone was observed upon addition of PPARγ antagonist GW9662 or T0070907, both in PPARγ1 CALUX and PPARγ2 CALUX cells. The PPARα agonists WY14643 and fenofibrate failed to induce luciferase activity, confirming the specificity of these cell lines for PPARγ agonists.

In conclusion, PPARγ1 CALUX and PPARγ2 CALUX cells provide a reliable and useful tool to screen (bio-)chemicals for PPARγ agonist or antagonist activity.

Keywords: peroxisome proliferator-activated receptor γ (PPARγ), peroxisome proliferator-responsive element (PPRE), stable luciferase reporter cell line, human U2OS cells, thiazolidinediones (TZDs)

INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are transcription factors which are activated by ligands (*1*). Ligand-activated PPARs can bind to a peroxisome proliferator-responsive element (PPRE) in the regulatory region of target genes and thereby influence the expression of those genes. At present, three PPAR isoforms are known: PPARα (NR1C1), PPARβ/δ (NR1C2) and PPARγ (NR1C3). Each isoform has its own set of target genes, which partially overlap, and the isoforms show differential activation levels in response to certain ligands (*2*). The target genes include genes considered to play a role in health preservation and health promotion. Especially PPARγ has been associated with several beneficial health effects.

PPARγ was initially known for its role in adipogenesis (*3-5*) and lipid metabolism in adipose tissue (*6*). Nowadays, PPARγ agonists are especially renowned for their function in the regulation of insulin sensitivity: thiazolidinediones (TZDs) are a group of synthetic PPARγ agonists showing insulin-sensitizing activity in animal models (*7, 8*). Of the TZDs, pioglitazone is presently used as insulin-sensitizing drug in patients suffering from type 2 diabetes mellitus (*9-11*). PPARγ-mediated gene expression also results in anti-inflammatory effects (*12-15*). Therefore, ligands for PPARγ are assumed to exert health-promoting effects on several inflammation-related diseases, including atherosclerosis (*16, 17*), inflammatory bowel disease (*17*), Alzheimer's disease (*16, 18*) and airway inflammation (including asthma)(*17, 19*). Furthermore, activation of PPARγ has been reported to promote health by lowering blood pressure (*20-23*) and by inhibiting the development of certain cancers (*24-27*).

PPARγ is present in humans in two isotypes: PPARγ1 and PPARγ2. The two isoforms are generated from the same gene by alternative promoter usage. Human PPARγ2 has 28 extra amino acids at the N-terminus of the protein (*28, 29*) and is found mainly in adipose tissue and in lower amounts in liver and skeletal muscle (*29-31*). PPARγ1 is expressed more broadly and is found in adipose tissue, spleen, liver, skeletal muscle, heart, large intestine and kidney (*29-31*). The functional differences between PPARγ1 and PPARγ2 are not completely clarified yet. There are some indications that PPARγ2 plays a more important role than PPARγ1 in adipogenesis (*32, 33*), insulin sensitivity (*34*) and in the response to nutritional conditions (*30, 34*).

Given the possible beneficial health effects mediated by PPARγ activation, the aim of the present study was to develop and validate stable luciferase reporter cell lines to facilitate high-throughput screening of bioactive food compounds for PPARγ agonist and antagonist activity. In order to achieve this, a DNA construct containing multiple PPREs and a minimal promoter controlling the transcription of a luciferase reporter gene was created and co-transfected into U2OS cells together with an expression plasmid for human PPARγ1 or human PPARγ2 respectively. Since U2OS cells have very low endogenous PPAR levels (*35, 36*), the co-transfection of the human PPARγ1 or human PPARγ2 receptor will make the system specific for detecting PPARγ1-mediated or PPARγ2-mediated effects on gene expression.

MATERIAL AND METHODS

Chemicals

Rosiglitazone (CAS no: 122320-73-4), 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ2; CAS no: 89886-60-2), netoglitazone (also known as MCC-555, CAS no: 161600-01-7), GW9662 (CAS no: 22978-25-2), T0070907 (CAS no: 313516-66-4), ciglitazone (CAS no: 74772-77-3), pioglitazone (CAS no: 111025-46-8), troglitazone (CAS no: 97322-87-7) and GW501516 (CAS no: 317318-70-0) were all obtained from Cayman Chemical (Ann Arbor, USA). All chemicals were dissolved in dimethylsulphoxide (DMSO, 99.9%)(Acros, Geel, Belgium) and stored at -20°C. G418-disulfate (CAS no: 108321-42-2) was purchased from Duchefa Biochemie (Haarlem, The Netherlands).

DNA constructs

Expression vectors

A 2 kb NotI fragment from pCMV6-hPPARγ1 and a 1.6 kb NotI fragment from pCMV6-hPPARγ2 (both purchased from Origene, Rockville, USA) were inserted into the unique NotI site in the multiple cloning site of pSG5-neo (*37*) containing the neomycin resistance gene. This resulted in the expression plasmids pSG5-neo-PPARγ1 and pSG5-neo-PPARγ2, respectively.

Reporter constructs

To generate the reporter construct, a DNA fragment containing three copies of the PPRE (consisting of the nucleotide sequence 5'-GTCGACAGGGGACCAGGACAAAGGTCA CGTTCGGGAGTCGAC-3' repeated three times in tandem, consensus PPRE underlined (*38*)) was designed and synthesized at GeneArt (Regensburg, Germany), and cloned in vector pMA (GeneArt, Regensburg, Germany). The KpnI/XbaI fragment of pMA-3xPPRE was ligated into a TATA box-containing pGL3 vector partially digested with KpnI/XbaI to generate pGL3-3xPPRE-tata-luc. The pGL4-3xPPRE-tata-luc was generated by cloning the 3xPPRE-tata containing DNA fragment from pGL3-3xPPRE-tata-luc into a pGL4.10[luc] vector (Promega, Madison, USA).

Cell culture

Human osteoblastic osteosarcoma U2OS cells (American Type Culture Collection, ATCC) were cultured as described before in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DF)(Invitrogen, Breda, The Netherlands) supplemented with 7.5% fetal calf serum (FCS)(Invitrogen), non-essential amino acids (NEAA)(Invitrogen) and penicillin/streptomycin (Invitrogen)(final concentrations 10 U/ml and 10 µg/ml, respectively)(*39*). Stably transfected PPARγ1 CALUX cells and PPARγ2 CALUX cells were cultured in DF medium supplemented with 7.5% FCS, NEAA, and penicillin/streptomycin. Once per week 200 µg/ml G418 was added to the culture medium.

Generation of stable cell lines

Stable transfectants were generated essentially as described before (*39*). U2OS cells were plated in 12-well tissue culture plates. After culturing for one day, cells were transfected with reporter plasmid (pGL3-3xPPRE-tata-luc to generate PPARγ1 CALUX, and pGL4-3xPPRE-tata-luc for PPARγ2 CALUX), and an expression plasmid for PPARγ (pSG5-neo-

PPARγ1 or pSG5-neo-PPARγ2, respectively), using a calcium phosphate co-precipitation method (*40*). G418-resistant clones were tested for their response to 1 µM rosiglitazone, fifteen responsive clones were selected and tested to generate full dose-response curves. From these, the clone repeatedly showing the best response was selected based on the obtained fold induction and the level of luciferase expression.

Reporter gene assays

Assays were performed as described before (*39*). In short, PPARγ1 CALUX cells and PPARγ2 CALUX cells were plated in 96-well plates with phenol red-free DF medium supplemented with 5% dextran-coated charcoal-stripped FCS (DCC-FCS). The next day, the cells were incubated in triplicate wells with the test compounds added to the culture medium from a 1000 times concentrated stock solution in DMSO. After 24 hours, the cells were checked visually for cytotoxicity, the medium was removed and the cells were lysed. Luciferase activity in the lysate was measured using a luminometer (Anthos, Eugendorf, Austria) and 100 µl of Glow Mix (20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM dithiothreitol, 270 µM Co-enzyme A, 470 µM D-Luciferine, 530 µM ATP) or 100 µl flash mix (20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 2.0 mM dithiothreitol, 470 µM D-luciferine, 5.0 mM ATP) per well. Luciferase activity per well was measured as relative light units (RLUs). For each test compound, at least three independent experiments were performed, of which one representative curve is presented. Data are presented as mean values ± standard error of the mean (SEM) with each experimental point measured in triplicate.

Data analysis

Dose-response curves were fitted to the equation of a sigmoidal curve $y = a_0 + a_1/(1 + \exp(-(x-a_2)/a_3))$ using GraphPad Prism (version 4.00 for Windows, GraphPad Software, San Diego, CA), which determines the fitting coefficients by an iterative process minimizing the c2 merit function (least squares criterion).

RESULTS

Construction of the PPARγ1 CALUX and PPARγ2 CALUX reporter cell lines

Responsiveness of the luciferase constructs to the PPARγ agonist rosiglitazone was confirmed by transient transfection experiments in U2OS cells. U2OS cells were co-transfected with pGL3-3xPPRE-tata-luc and with one of three pSG5-neo vectors: a pSG5-neo vector without any PPARγ-encoding sequence, a pSG5-neo-PPARγ1 vector containing a constitutive human PPARγ1 expression cassette, or a pSG5-neo-PPARγ2 vector containing a constitutive human PPARγ2 expression cassette. U2OS cells co-transfected with pSG5-neo-PPARγ1 or pSG5-neo-PPARγ2 showed induction of luciferase activity up to 27-fold when exposed to rosiglitazone (figure 1). In cells co-transfected with pSG5-neo (figure 1), only a 2-fold induction in luciferase activity was seen after 24 hours of exposure to a high (1000 nM) concentration of rosiglitazone, indicating that luciferase expression due to endogenous PPARγ in U2OS cells is relatively low. From these results it can be concluded that co-transfection with pSG5-neo-PPARγ1 or pSG5-neo-PPARγ2 largely

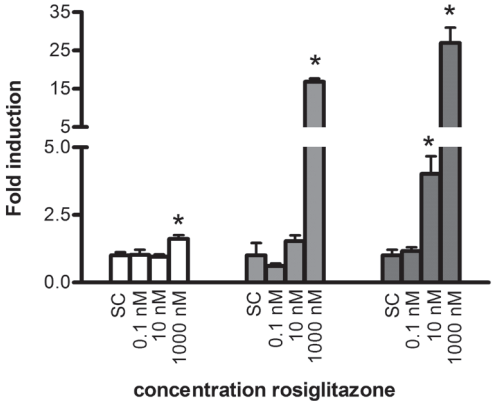

Figure 1. Luciferase induction in U2OS cells that were transiently co-transfected with pGL3-3xPPRE-tata-luc construct and a pSG5-neo vector (pSG5-neo (white bars); pSG5-neo-PPARγ1 (light grey bars); or pSG5-neo-PPARγ2 (dark grey bars)) and subsequently exposed for 24 hours to three concentrations of rosiglitazone or solvent control (SC). Fold induction of luciferase activity was calculated from measured RLUs. Data are expressed as mean ± SEM. An asterisk (*) indicates a significant ($p<0.05$) difference compared to solvent control.

increased the level of the corresponding PPARγ subtype in U2OS cells, leading to much higher fold inductions of the luciferase activity upon exposure to rosiglitazone.

In the next step, two stably transfected cell lines were created. The first cell line was generated by co-transfecting pGL3-3xPPRE-tata-luc and pSG5-neo-PPARγ1 into U2OS cells. From the transfected cell population, single cell clones were isolated and tested for responsiveness to rosiglitazone. Clone 141 was selected for its high luciferase values and fold inductions upon exposure to rosiglitazone. This clone was designated PPARγ1 CALUX. The second cell line was created by co-transfecting pGL4-3xPPRE-tata-luc and pSG5-neo-PPARγ2 into U2OS cells and cloning of the transfected cell population. Clone 54 was selected for its high luciferase values and fold inductions after exposure to rosiglitazone and was designated PPARγ2 CALUX.

Characterization of PPARγ1 CALUX and PPARγ2 CALUX reporter cell lines

PPARγ1 CALUX reporter cells and PPARγ2 CALUX reporter cells were exposed to increasing concentrations of six known PPARγ agonists (rosiglitazone, troglitazone, pioglitazone, ciglitazone, netoglitazone (MCC-555) and 15d-PGJ2 (*41-43*)) and of two PPARγ antagonists (GW9662 (*44*) and T0070907 (*45*)) in the presence of rosiglitazone. Upon exposure to rosiglitazone, troglitazone, pioglitazone, ciglitazone, netoglitazone and 15d-PGJ2, both PPARγ1 CALUX and PPARγ2 CALUX cells showed a concentration-dependent luciferase induction (figure 2A-F). The level of luciferase induction differs greatly among the various agonists tested. In the concentration range that could be tested without inducing cytotoxicity, most agonists (including troglitazone and ciglitazone) did not reach their maximum induction level; dose-response curves of these agonists were not yet leveling off at the highest concentration that could be tested. EC_{50} values could therefore not be

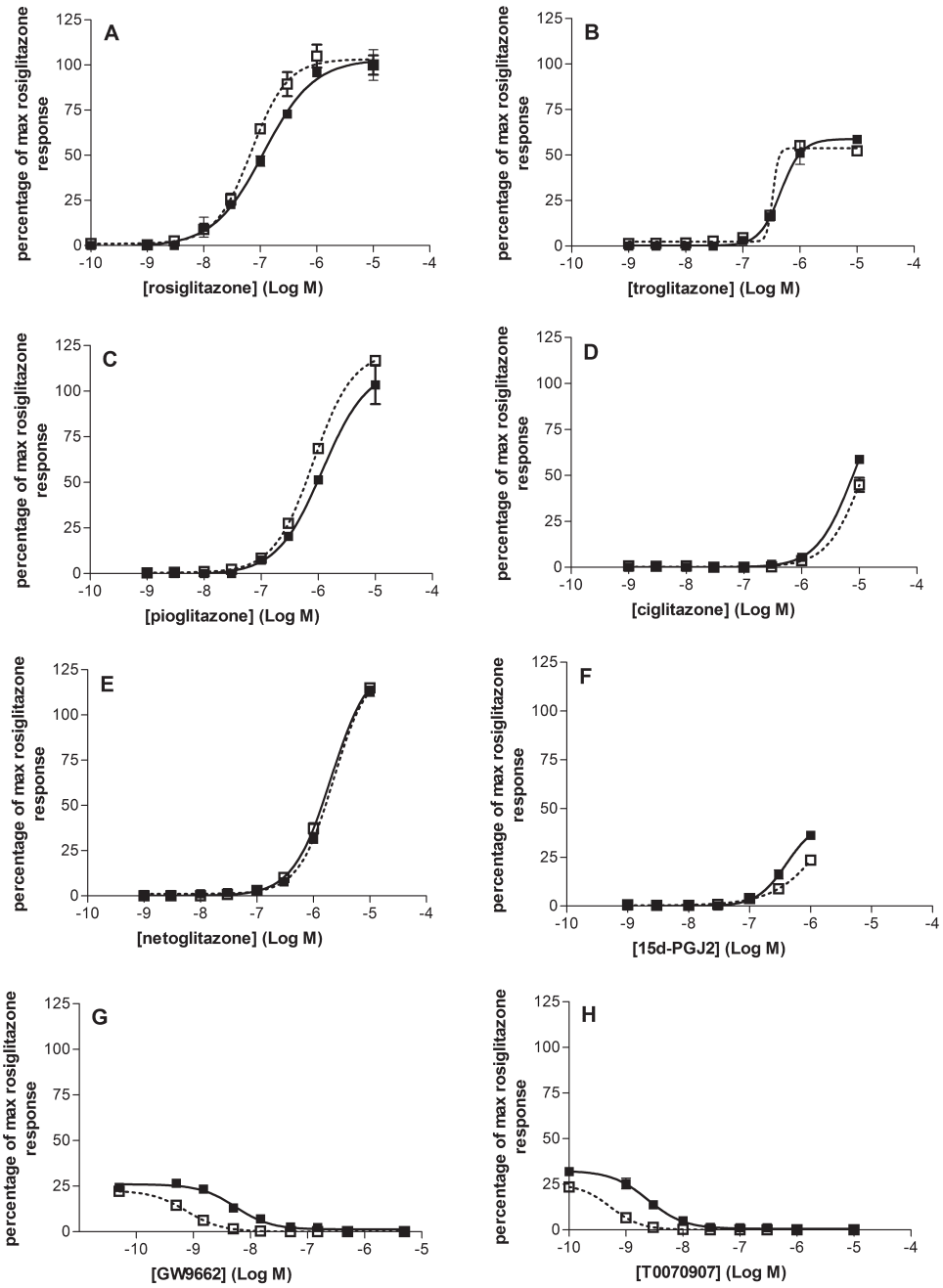

Figure 2. Luciferase activity induced in the two PPARγ CALUX cell lines. PPARγ1 CALUX cells (continuous lines) and PPARγ2 CALUX cells (dotted lines) were exposed to agonists (A-F) or antagonists (G and H), the latter in the presence of 50 nM rosiglitazone. Luciferase activity is expressed as percentage of the maximum rosiglitazone response. Data are corrected for solvent control luciferase values and are expressed as mean ± SEM (n=3). A: rosiglitazone. B: troglitazone. C: pioglitazone. D: ciglitazone. E: netoglitazone. F: 15d-PGJ2. G: GW9662. H: T0070907.

determined reliably for these agonists. Consequently, we chose to determine the relative potency of all compounds based on the concentration that induced 10% and 50% of the maximum response level attained with rosiglitazone (table 1). This analysis showed that in both PPARγ1 CALUX and PPARγ2 CALUX cells rosiglitazone is the most potent TZD followed by troglitazone/pioglitazone, netoglitazone and ciglitazone. Troglitazone and pioglitazone showed comparable potencies. Since 15d-PGJ2 could not be tested in concentrations higher than 1 µM, there is not enough information to include 15d-PGJ2 in this potency ranking.

For testing antagonist activity, the PPARγ1 CALUX cells and PPARγ2 CALUX cells were exposed to increasing concentrations of the antagonist in the presence of 50 nM rosiglitazone. Upon exposure of PPARγ1 CALUX cells and PPARγ2 CALUX cells to both PPARγ antagonists, the luciferase induction by 50 nM rosiglitazone was inhibited in a concentration-dependent manner (figure 2G and H). The observed IC_{50} values are presented in table 2.

In order to facilitate their comparison, the responses in the PPARγ1 and PPARγ2 CALUX cells were plotted in the same graph (figure 2). Comparison of the dose-response curves reveals that PPARγ1 CALUX cells and PPARγ2 CALUX cells respond in the same way to each of the tested agonists: induction of the luciferase activity starts at the same concentration in both PPARγ1 CALUX cells and PPARγ2 CALUX cells and increases with comparable dose-

Table 1. Concentrations (in µM) of PPARγ agonists inducing 10% or 50% of the maximum response level attained with rosiglitazone, both for PPARγ1 CALUX cells and for PPARγ2 CALUX cells.

compound	PPARγ1 10% (µM)	PPARγ2 10% (µM)	PPARγ1 50% (µM)	PPARγ2 50% (µM)	EC_{50} (µM) in literature	
rosiglitazone	0.01	0.01	0.11	0.07	0.04	(*52*)
					0.06	(*43*)
					0.09	(*49*)
					0.21	(*50*)
					0.30	(*51*)
troglitazone	0.23	0.28	0.95	0.47	0.54	(*49*)
					0.55	(*52*)
pioglitazone	0.14	0.11	0.95	0.60	0.10	(*65*)
					0.26	(*48*)
					0.58	(*52*)
					0.59	(*49*)
					0.69	(*43*)
					0.90	(*51*)
ciglitazone	1.55	2.34	7.76	X[a]	3.0	(*43*)
					4.0	(*65*)
					23	(*49*)
netoglitazone (MCC-555)	0.30	0.37	1.41	1.62	0.76	(*63*)
					8.0	(*41*)
15d-PGJ2	0.20	0.34	X[a]	X[a]	0.82	(*67*)
					2.0	(*68*)
					3.0	(*65*)
					20	(*69*)

[a]X indicates that the 50% of the maximum response level attained with rosiglitazone was not reached.

Table 2. IC_{50} values of PPARγ antagonists found in PPARγ1 CALUX cells, PPARγ2 CALUX cells and in literature.

Compound	IC_{50} (nM)[a] PPARγ1 CALUX	IC_{50} (nM)[a] PPARγ2 CALUX	IC_{50} (nM) in literature	
GW9662	9.1 ± 5.3	1.2 ± 0.3	3.8 7.6	(*48*) (*44*)
T0070907	3.2 ± 0.8	0.9 ± 0.3	nM range 4100	(*45*) (*66*)

[a]Mean IC_{50} values from three independent experiments ± SEM.

dependency. For the two antagonists, GW9662 and T0070907, PPARγ2 CALUX cells seem to be somewhat more sensitive than PPARγ1 CALUX cells (figure 2G-H), although the differences were not statistically significant (p>0.05).

In order to test specificity of the PPARγ CALUX cells for PPARγ agonists, the response to one PPARβ/δ agonist and two PPARα agonists was investigated as well. Exposure of PPARγ1 CALUX cells and PPARγ2 CALUX cells to the PPARα agonists WY14643 (*46*) and fenofibrate (*47*) showed no increase in luciferase activity (figure 3), indicating that both PPARγ1 CALUX cells and PPARγ2 CALUX cells do not respond to agonists for PPARα. GW501516 is known as PPARβ/δ agonist, but can also activate PPARγ at high concentrations (*48*). Both PPARγ1 CALUX cells and PPARγ2 CALUX cells showed an increase in luciferase activity upon exposure to PPARβ/δ agonist GW501516, but only at concentrations of 1 µM or higher (figure 3).

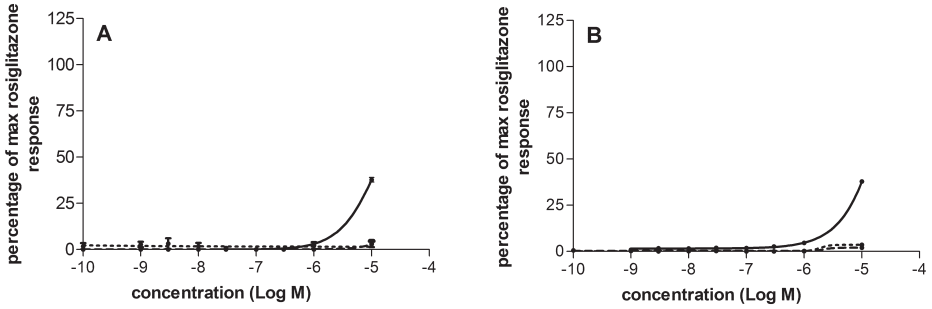

Figure 3. Luciferase activity of PPARγ1 CALUX cells (A) and PPARγ2 CALUX cells (B) upon exposure to agonists for PPARα (fenofibrate (dotted line) and WY14643 (dashed line)) and PPARβ/δ (GW501516, continuous line), expressed as percentage of the maximum rosiglitazone response. Data are corrected for solvent control values and are expressed as mean ± SEM (n=3).

DISCUSSION

In the present study, two stable human luciferase reporter cell lines specifically responding to PPARγ1 or PPARγ2 agonist and antagonist activity were developed and designated PPARγ1 CALUX and PPARγ2 CALUX. The PPARγ1 CALUX and PPARγ2 CALUX

cell lines enable high-throughput *in vitro* screening of chemicals for their potential PPARγ activating capacity, and they are faster and less costly than the conventional *in vivo* test methods. The PPARγ CALUX cell lines can contribute to the 3Rs (Replacement, Refinement and Reduction) of *in vivo* experimental animal testing, since these *in vitro* tools provide an alternative for animal testing in the initial stages of compound screening. The PPARγ CALUX cell lines provide a first level support to health claims of functional food ingredients in which activation of PPARγ-mediated gene expression is known to play a role. In addition, the cell lines may provide an important tool to identify endogenous ligands for PPARγ. Finally, these cell lines express the complete human PPARγ receptor, potentially allowing more faithful translation to effects in humans than the presently available systems based on a chimeric PPARγ-GAL4 receptor (*43, 49-52*).

To generate highly PPARγ-selective CALUX reporter cell lines reliably reflecting the PPARγ-mediated activation of gene expression in humans, a cell line of human origin with low background levels of other PPAR isotypes was preferred. The U2OS cell line fulfills these criteria, is relatively easy to transfect and was therefore selected. Besides low levels of PPARα and PPARβ/δ, the selected cell line has also low endogenous PPARγ expression. This allowed generation of PPARγ1- and PPARγ2-specific reporter gene assays by co-transfecting an expression plasmid for the PPARγ1 gene or the PPARγ2 gene into the U2OS cells. Specificity to PPARγ agonists was confirmed by exposing PPARγ1 CALUX cells and PPARγ2 CALUX cells to the PPARα agonists WY14643 and fenofibrate, which failed to induce luciferase activity. In addition, upon exposure of PPARγ1 CALUX and PPARγ2 CALUX cells to the PPARβ/δ agonist GW501516, luciferase induction in both PPARγ1 CALUX and PPARγ2 CALUX cells was only seen at concentrations of 1 μM and higher. The EC_{50} value of GW501516 for activation of human PPARβ/δ is around 1-2 nM (*53-56*), while luciferase induction in PPARγ CALUX cells is only seen at concentrations higher than 1 μM. This is consistent with earlier findings showing that GW501516 is 1000-fold selective for PPARβ/δ over the other subtypes (*48, 53, 55*). It was therefore concluded that both PPARγ1 CALUX cells and PPARγ2 CALUX cells specifically respond to PPARγ agonists and not to agonists for other PPAR isotypes.

A large difference in absolute RLU levels between the PPARγ1 CALUX and PPARγ2 CALUX cell lines was observed. The PPARγ2 CALUX cell line, which is transfected with pGL4-3xPPRE-tata-luc, showed the highest absolute luciferase levels. This reporter construct was based on the pGL4 vector containing Luc2 as the reporter gene, while the pGL3-3xPPRE-tata-luc construct present in the PPARγ1 CALUX cells was based on the pGL3 vector containing Luc+ as the reporter gene. According to the manufacturer the pGL4 vector shows increased reporter gene expression compared to the pGL3 vector (*57*). This may partially explain the higher absolute luciferase activity levels found in the PPARγ2 CALUX cell line compared to the PPARγ1 CALUX cell line. The difference in absolute luciferase activity levels may also be due to a difference in the integration site or a difference in the number of copies integrated in the genome. Although absolute RLU levels differed greatly between the PPARγ1 CALUX and PPARγ2 CALUX cell lines, the relative response to all PPARγ agonists tested was similar.

Compared with PPARγ1, PPARγ2 contains 28 additional amino acids at the N-terminus of the protein (*28, 29*). These additional amino acids are part of the A/B domain, which comprises an activation function called AF-1 (*58-60*). The observation that AF-1 acts as ligand-independent activation domain (*61*), suggests that the extra amino acid stretch in PPARγ2 would not affect the ligand-dependent transcription activation by this receptor. Results of the present research indicate that this may indeed be the case; human PPARγ1 and PPARγ2 are activated with similar dose-dependency by all PPARγ agonists tested sofar. This is in line with results found by Elbrecht and colleagues who report similar binding affinities and EC_{50} values for rosiglitazone and troglitazone for both isoforms of human PPARγ (*28*). In addition, Lehmann *et al.* found that troglitazone, pioglitazone and ciglitazone showed no differences in potency for murine PPARγ1 and PPARγ2 (*62*). They report, however, that rosiglitazone is a more potent activator for murine PPARγ1 than for murine PPARγ2 (*62*), which may indicate a difference between the human and the murine transcription factor.

Since EC_{50} values for most PPARγ agonists tested could not be determined reliably, the concentrations inducing 10% and, if possible, 50% of the maximum response level attained by rosiglitazone were determined for each agonist. Although this approach differs from the conventional method to determine EC_{50} values, the absolute concentrations of rosiglitazone, troglitazone, pioglitazone and netoglitazone inducing 50% of the maximum rosiglitazone response level are comparable to the conventional EC_{50} values for PPARγ-mediated reporter activity for these compounds reported in literature (table 1).

Based on the concentrations of PPARγ agonists inducing 10% and 50% of the maximum response level attained with rosiglitazone, the potency of each PPARγ agonist was determined. The PPARγ agonists were ranked based on their potency to induce PPARγ-mediated luciferase activity: rosiglitazone > troglitazone = pioglitazone > netoglitazone > ciglitazone. This ranking is in line with results found by Willson *et al.* (*52*), Lehmann *et al.* (*62*), Giaginis *et al.* (*63*) and Henke *et al.* (*49*), but differs from the results found by Young *et al.* (*64*) and Awais *et al.* (*65*) who report that troglitazone is less potent than pioglitazone and ciglitazone.

Based on IC_{50} values, T0070907 was shown to be slightly more potent as an antagonist than GW9662. This is in line with results reported by Burton *et al.* who found lower IC_{50} values for T0070907 than for GW9662 for antiproliferative effects in several cancer cell lines (*66*).

In conclusion, the present study describes the development, characterization and validation of the stable reporter gene cell lines PPARγ1 CALUX and PPARγ2 CALUX. These new assays provide *in vitro* tools to test (mixtures of) chemicals, endogenous ligands and (food) compounds for their ability to activate PPARγ1-mediated and PPARγ2-mediated gene expression.

ACKNOWLEDGMENTS

This work was financially supported by the Food and Nutrition Delta (grant number FND07007).

REFERENCES

1. Berger, J.; Moller, D. E., The mechanisms of action of PPARs. *Annual Review of Medicine* **2002,** *53*, 409-435.
2. Michalik, L.; Wahli, W., Peroxisome proliferator-activated receptors: three isotypes for a multitude of functions. *Current Opinion in Biotechnology* **1999,** *10*, 564-570.
3. Barak, Y.; Nelson, M. C.; Ong, E. S.; Jones, Y. Z.; Ruiz-Lozano, P.; Chien, K. R.; Koder, A.; Evans, R. M., PPAR[gamma] Is Required for Placental, Cardiac, and Adipose Tissue Development. *Molecular Cell* **1999,** *4*, 585-595.
4. Tontonoz, P.; Hu, E.; Graves, R. A.; Budavari, A. I.; Spiegelman, B. M., mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. *Genes & Development* **1994,** *8*, 1224-1234.
5. Tontonoz, P.; Hu, E.; Spiegelman, B. M., Stimulation of adipogenesis in fibroblasts by PPARγ2, a lipid-activated transcription factor. **1994,** *79*, 1147-1156.
6. Kersten, S.; Desvergne, B.; Wahli, W., Roles of PPARs in health and disease. *Nature* **2000,** *405*, 421-424.
7. Imran, M.; Ilyas, B.; Deepanjali; Khan, S. A., Recent thiazolidinediones as antidiabetics. *Journal of scientific and industrial research* **2007,** *66*, 99-109.
8. Wang, M.; Tafuri, S., Modulation of PPARgamma activity with pharmaceutical agents: Treatment of insulin resistance and atherosclerosis. *Journal of Cellular Biochemistry* **2003,** *89*, 38-47.
9. O'Moore-Sullivan, T. M.; Prins, J. B., Thiazolidinediones and type 2 diabetes: new drugs for an old disease. *The Medical Journal of Australia* **2002,** *176*, 381-386.
10. Quinn, C. E.; Hamilton, P. K.; Lockhart, C. J.; McVeigh, G. E., Thiazolidinediones: effects on insulin resistance and the cardiovascular system. *British Journal of Pharmacology* **2008,** *153*, 636-645.
11. Yki-Jarvinen, H., Thiazolidinediones. *N Engl J Med* **2004,** *351*, 1106-1118.
12. Devchand, P. R.; Keller, H.; Peters, J. M.; Vazquez, M.; Gonzalez, F. J.; Wahli, W., The PPAR[alpha]-leukotriene B4 pathway to inflammation control. *Nature* **1996,** *384*, 39-43.
13. Jiang, C.; Ting, A. T.; Seed, B., PPAR-[gamma] agonists inhibit production of monocyte inflammatory cytokines. *Nature* **1998,** *391*, 82-86.
14. Ricote, M.; Li, A. C.; Willson, T. M.; Kelly, C. J.; Glass, C. K., The peroxisome proliferator-activated receptor-[gamma] is a negative regulator of macrophage activation. *Nature* **1998,** *391*, 79-82.
15. Staels, B.; Koenig, W.; Habib, A.; Merval, R.; Lebret, M.; Torra, I. P.; Delerive, P.; Fadel, A.; Chinetti, G.; Fruchart, J.-C.; Najib, J.; Maclouf, J.; Tedgui, A., Activation of human aortic smooth-muscle cells is inhibited by PPAR[alpha] but not by PPAR[gamma] activators. *Nature* **1998,** *393*, 790-793.
16. Chung, J. H.; Seo, A. Y.; Chung, S. W.; Kim, M. K.; Leeuwenburgh, C.; Yu, B. P.; Chung, H. Y., Molecular mechanism of PPAR in the regulation of age-related inflammation. *Ageing Research Reviews* **2008,** *7*, 126-136.
17. Szanto, A.; Nagy, L., The many faces of PPAR[gamma]: Anti-inflammatory by any means? *Immunobiology* **2008,** *213*, 789-803.
18. Landreth, G.; Jiang, Q.; Mandrekar, S.; Heneka, M., PPAR[gamma] Agonists as Therapeutics for the Treatment of Alzheimer's Disease. *Neurotherapeutics* **2008,** *5*, 481-489.
19. Belvisi, M. G.; Hele, D. J.; Birrell, M. A., Peroxisome proliferator-activated receptor gamma agonists as therapy for chronic airway inflammation. *European Journal of Pharmacology* **2006,** *533*, 101-109.
20. Chen, R.; Liang, F.; Moriya, J.; Yamakawa, J.-i.; Takahashi, T.; Shen, L.; Kanda, T., Peroxisome proliferator-activated receptors (PPARs) and their agonists for hypertension and heart failure: Are the reagents beneficial or harmful? *International Journal of Cardiology* **2008,** *130*, 131-139.
21. Chetty, V. T.; Sharma, A. M., Can PPAR[gamma] agonists have a role in the management of obesity-related hypertension? *Vascular Pharmacology* **2006,** *45*, 46-53.
22. Dobrian, A. D.; Schriver, S. D.; Khraibi, A. A.; Prewitt, R. L., Pioglitazone Prevents Hypertension and Reduces Oxidative Stress

in Diet-Induced Obesity. *Hypertension* **2004,** *43*, 48-56.

23. Sarafidis, P. A.; Lasaridis, A. N., Actions of Peroxisome Proliferator-Activated Receptors-[gamma] Agonists Explaining a Possible Blood Pressure-Lowering Effect. *American Journal of Hypertension* **2006,** *19*, 646-653.
24. Allred, C. D.; Talbert, D. R.; Southard, R. C.; Wang, X.; Kilgore, M. W., PPAR{gamma}1 as a Molecular Target of Eicosapentaenoic Acid in Human Colon Cancer (HT-29) Cells. *J. Nutr.* **2008,** *138*, 250-256.
25. Han, S.; Roman, J., Peroxisome proliferator-activated receptor [gamma]: a novel target for cancer therapeutics? *Anti-Cancer Drugs* **2007,** *18*, 237-244 10.1097/CAD.0b013e328011e67d.
26. Michalik, L.; Desvergne, B.; Wahli, W., Peroxisome-proliferator-activated receptors and cancers: complex stories. *Nat Rev Cancer* **2004,** *4*, 61-70.
27. Ondrey, F., Peroxisome Proliferator-Activated Receptor {gamma} Pathway Targeting in Carcinogenesis: Implications for Chemoprevention. *Clin Cancer Res* **2009,** *15*, 2-8.
28. Elbrecht, A.; Chen, Y.; Cullinan, C. A.; Hayes, N.; Leibowitz, M. D.; Moller, D. E.; Berger, J., Molecular Cloning, Expression and Characterization of Human Peroxisome Proliferator Activated Receptors [gamma]1 and [gamma]2. *Biochemical and Biophysical Research Communications* **1996,** *224*, 431-437.
29. Fajas, L.; Auboeuf, D.; Raspé, E.; Schoonjans, K.; Lefebvre, A.-M.; Saladin, R.; Najib, J.; Laville, M.; Fruchart, J.-C.; Deeb, S.; Vidal-Puig, A.; Flier, J.; Briggs, M. R.; Staels, B.; Vidal, H.; Auwerx, J., The Organization, Promoter Analysis, and Expression of the Human PPARγ Gene. *Journal of Biological Chemistry* **1997,** *272*, 18779-18789.
30. Vidal-Puig, A.; Jimenez-Linan, M.; Lowell, B. B.; Hamann, A.; Hu, E.; Spiegelman, B.; Flier, J. S.; Moller, D. E., Regulation of PPAR gamma gene expression by nutrition and obesity in rodents. *The journal of Clinical Investigation* **1996,** *97*, 2553-61.
31. Vidal-Puig, A. J.; Considine, R. V.; Jimenez-Liñan, M.; Werman, A.; Pories, W. J.; Caro, J. F.; Flier, J. S., Peroxisome proliferator-activated receptor gene expression in human tissues. Effects of obesity, weight loss, and regulation by insulin and glucocorticoids. *The journal of Clinical Investigation* **1997,** *99*, 2416-2422.
32. Mueller, E.; Drori, S.; Aiyer, A.; Yie, J.; Sarraf, P.; Chen, H.; Hauser, S.; Rosen, E. D.; Ge, K.; Roeder, R. G.; Spiegelman, B. M., Genetic Analysis of Adipogenesis through Peroxisome Proliferator-activated Receptor γ Isoforms. *Journal of Biological Chemistry* **2002,** *277*, 41925-41930.
33. Ren, D.; Collingwood, T. N.; Rebar, E. J.; Wolffe, A. P.; Camp, H. S., PPARγ knockdown by engineered transcription factors: exogenous PPARγ2 but not PPARγ1 reactivates adipogenesis. *Genes & Development* **2002,** *16*, 27-32.
34. Medina-Gomez, G.; Virtue, S.; Lelliott, C.; Boiani, R.; Campbell, M.; Christodoulides, C.; Perrin, C.; Jimenez-Linan, M.; Blount, M.; Dixon, J.; Zahn, D.; Thresher, R. R.; Aparicio, S.; Carlton, M.; Colledge, W. H.; Kettunen, M. I.; Seppänen-Laakso, T.; Sethi, J. K.; O'Rahilly, S.; Brindle, K.; Cinti, S.; Orešič, M.; Burcelin, R.; Vidal-Puig, A., The Link Between Nutritional Status and Insulin Sensitivity Is Dependent on the Adipocyte-Specific Peroxisome Proliferator-Activated Receptor-γ2 Isoform. *Diabetes* **2005,** *54*, 1706-1716.
35. Jeninga, E. H.; van Beekum, O.; van Dijk, A. D. J.; Hamers, N.; Hendriks-Stegeman, B. I.; Bonvin, A. M. J. J.; Berger, R.; Kalkhoven, E., Impaired Peroxisome Proliferator-Activated Receptor {gamma} Function through Mutation of a Conserved Salt Bridge (R425C) in Familial Partial Lipodystrophy. *Mol Endocrinol* **2007,** *21*, 1049-1065.
36. Lucarelli, E.; Sangiorgi, L.; Maini, V.; Lattanzi, G.; Marmiroli, S.; Reggiani, M.; Mordenti, M.; Gobbi, G. A.; Scrimieri, F.; Bertoja, A. Z.; Picci, P., Troglitazione affects survival of human osteosarcoma cells. *International Journal of Cancer* **2002,** *98*, 344-351.
37. Sonneveld, E.; van den Brink, C.; van der Leede, B.; Schulkes, R.; Petkovich, M.; van der Burg, B.; van der Saag, P., Human retinoic acid (RA) 4-hydroxylase (CYP26) is highly specific for all-trans-RA and can be induced through RA receptors in human breast and colon carcinoma cells. *Cell Growth Differ* **1998,** *9*, 629-637.

38. Forman, B. M.; Tontonoz, P.; Chen, J.; Brun, R. P.; Spiegelman, B. M.; Evans, R. M., 15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. *Cell* **1995,** *83*, 803-12.

39. Sonneveld, E.; Jansen, H. J.; Riteco, J. A. C.; Brouwer, A.; van der Burg, B., Development of Androgen- and Estrogen-Responsive Bioassays, Members of a Panel of Human Cell Line-Based Highly Selective Steroid-Responsive Bioassays. *Toxicol. Sci.* **2005,** *83*, 136-148.

40. Quaedackers, M. E.; Van Den Brink, C. E.; Wissink, S.; Schreurs, R. H. M. M.; Gustafsson, J.-A.; Van Der Saag, P. T.; Van Der Burg, B., 4-Hydroxytamoxifen Trans-Represses Nuclear Factor-{{kappa}}B Activity in Human Osteoblastic U2-OS Cells through Estrogen Receptor (ER){{alpha}}, and Not through ER{{beta}}. *Endocrinology* **2001,** *142*, 1156-1166.

41. Reginato, M. J.; Bailey, S. T.; Krakow, S. L.; Minami, C.; Ishii, S.; Tanaka, H.; Lazar, M. A., A Potent Antidiabetic Thiazolidinedione with Unique Peroxisome Proliferator-activated Receptor Î³-activating Properties. *Journal of Biological Chemistry* **1998,** *273*, 32679-32684.

42. Sertznig, P.; Seifert, M.; Tilgen, W.; Reichrath, J., Present concepts and future outlook: Function of peroxisome proliferator-activated receptors (PPARs) for pathogenesis, progression, and therapy of cancer. *Journal of Cellular Physiology* **2007,** *212*, 1-12.

43. Willson, T. M.; Cobb, J. E.; Cowan, D. J.; Wiethe, R. W.; Correa, I. D.; Prakash, S. R.; Beck, K. D.; Moore, L. B.; Kliewer, S. A.; Lehmann, J. M., The Structure-Activity Relationship between Peroxisome Proliferator-Activated Receptor γ Agonism and the Antihyperglycemic Activity of Thiazolidinediones. *Journal of Medicinal Chemistry* **1996,** *39*, 665-668.

44. Leesnitzer, L. M.; Parks, D. J.; Bledsoe, R. K.; Cobb, J. E.; Collins, J. L.; Consler, T. G.; Davis, R. G.; Hull-Ryde, E. A.; Lenhard, J. M.; Patel, L.; Plunket, K. D.; Shenk, J. L.; Stimmel, J. B.; Therapontos, C.; Willson, T. M.; Blanchard, S. G., Functional Consequences of Cysteine Modification in the Ligand Binding Sites of Peroxisome Proliferator Activated Receptors by GW9662. *Biochemistry* **2002,** *41*, 6640-6650.

45. Lee, G.; Elwood, F.; McNally, J.; Weiszmann, J.; Lindstrom, M.; Amaral, K.; Nakamura, M.; Miao, S.; Cao, P.; Learned, R. M.; Chen, J.-L.; Li, Y., T0070907, a Selective Ligand for Peroxisome Proliferator-activated Receptor γ, Functions as an Antagonist of Biochemical and Cellular Activities. *Journal of Biological Chemistry* **2002,** *277*, 19649-19657.

46. Forman, B. M.; Chen, J.; Evans, R. M., Hypolipidemic Drugs, Polyunsaturated Fatty Acids, and Eicosanoids are Ligands for Peroxisome Proliferator-Activated Receptors α and δ. *Proceedings of the National Academy of Sciences of the United States of America* **1997,** *94*, 4312-4317.

47. Frederiksen, K. S.; Wulff, E. M.; Sauerberg, P.; Mogensen, J. P.; Jeppesen, L.; Fleckner, J., Prediction of PPAR-α ligand-mediated physiological changes using gene expression profiles. *Journal of Lipid Research* **2004,** *45*, 592-601.

48. Seimandi, M.; Lemaire, G.; Pillon, A.; Perrin, A.; Carlavan, I.; Voegel, J. J.; Vignon, F.; Nicolas, J.-C.; Balaguer, P., Differential responses of PPAR[alpha], PPAR[delta], and PPAR[gamma] reporter cell lines to selective PPAR synthetic ligands. *Analytical Biochemistry* **2005,** *344*, 8-15.

49. Henke, B. R.; Blanchard, S. G.; Brackeen, M. F.; Brown, K. K.; Cobb, J. E.; Collins, J. L.; Harrington, W. W.; Hashim, M. A.; Hull-Ryde, E. A.; Kaldor, I.; Kliewer, S. A.; Lake, D. H.; Leesnitzer, L. M.; Lehmann, J. M.; Lenhard, J. M.; Orband-Miller, L. A.; Miller, J. F.; Mook, R. A.; Noble, S. A.; Oliver, W.; Parks, D. J.; Plunket, K. D.; Szewczyk, J. R.; Willson, T. M., N-(2-Benzoylphenyl)-l-tyrosine PPARγ Agonists. 1. Discovery of a Novel Series of Potent Antihyperglycemic and Antihyperlipidemic Agents. *Journal of Medicinal Chemistry* **1998,** *41*, 5020-5036.

50. Mueller, M.; Jungbauer, A., Culinary plants, herbs and spices - A rich source of PPAR[gamma] ligands. *Food Chemistry* **2009,** *117*, 660-667.

51. Venkatraman, M. S.; Chittiboyina, A.; Meingassner, J.; Ho, C. I.; Varani, J.; Ellis, C. N.; Avery, M. A.; Pershadsingh, H. A.; Kurtz, T. W.; Benson, S. C., α-Lipoic acid-based PPARγ agonists for treating inflammatory skin diseases. *Archives of Dermatological Research* **2004,** *296*, 97-104.

52. Willson, T. M.; Brown, P. J.; Sternbach, D. D.; Henke, B. R., The PPARs: From Orphan Receptors to Drug Discovery. *Journal of Medicinal Chemistry* **2000,** *43*, 527-550.

53. Oliver, W. R.; Shenk, J. L.; Snaith, M. R.; Russell, C. S.; Plunket, K. D.; Bodkin, N. L.; Lewis, M. C.; Winegar, D. A.; Sznaidman, M. L.; Lambert, M. H.; Xu, H. E.; Sternbach, D. D.; Kliewer, S. A.; Hansen, B. C.; Willson, T. M., A selective peroxisome proliferator-activated receptor δ agonist promotes reverse cholesterol transport. *Proceedings of the National Academy of Sciences of the United States of America* **2001,** *98*, 5306-5311.

54. Pereira, R.; Gaudon, C.; Iglesias, B.; Germain, P.; Gronemeyer, H.; de Lera, A. R., Synthesis of the PPAR[beta]/[delta]-selective agonist GW501516 and C4-thiazole-substituted analogs. *Bioorganic & Medicinal Chemistry Letters* **2006,** *16*, 49-54.

55. Sznaidman, M. L.; Haffner, C. D.; Maloney, P. R.; Fivush, A.; Chao, E.; Goreham, D.; Sierra, M. L.; LeGrumelec, C.; Xu, H. E.; Montana, V. G.; Lambert, M. H.; Willson, T. M.; Oliver, W. R.; Sternbach, D. D., Novel selective small molecule agonists for peroxisome proliferator-activated receptor [delta] (PPAR[delta])--synthesis and biological activity. *Bioorganic & Medicinal Chemistry Letters* **2003,** *13*, 1517-1521.

56. Yamamoto, T.; de Crombrugghe, B.; Pastan, I., Identification of a functional promoter in the long terminal repeat of Rous sarcoma virus. *Cell* **1980,** *22*, 787-797.

57. pGL4 Luciferase Reporter Vectors Technical Manual. *Promega Corporation* **2009**.

58. Aranda, A.; Pascual, A., Nuclear Hormone Receptors and Gene Expression. *Physiol. Rev.* **2001,** *81*, 1269-1304.

59. Blanquart, C.; Barbier, O.; Fruchart, J. C.; Staels, B.; Glineur, C., Peroxisome proliferator-activated receptors: regulation of transcriptional activities and roles in inflammation. *The Journal of Steroid Biochemistry and Molecular Biology* **2003,** *85*, 267-273.

60. Leff, T.; Mathews, S. T.; Camp, H. S., Peroxisome Proliferator-Activated Receptor-γ and Its Role in the Development and Treatment of Diabetes. *Experimental Diabetes Research* **2004,** *5*, 11.

61. Werman, A.; Hollenberg, A.; Solanes, G.; Bjørbæk, C.; Vidal-Puig, A. J.; Flier, J. S., Ligand-independent Activation Domain in the N Terminus of Peroxisome Proliferator-activated Receptor γ (PPARγ). *Journal of Biological Chemistry* **1997,** *272*, 20230-20235.

62. Lehmann, J. r. M.; Moore, L. B.; Smith-Oliver, T. A.; Wilkison, W. O.; Willson, T. M.; Kliewer, S. A., An Antidiabetic Thiazolidinedione Is a High Affinity Ligand for Peroxisome Proliferator-activated Receptor (PPAR). *Journal of Biological Chemistry* **1995,** *270*, 12953-12956.

63. Giaginis, C.; Theocharis, S.; Tsantili-Kakoulidou, A., Investigation of the lipophilic behaviour of some thiazolidinediones: Relationships with PPAR-[gamma] activity. *Journal of Chromatography B* **2007,** *857*, 181-187.

64. Young, P. W.; Buckle, D. R.; Cantello, B. C. C.; Chapman, H.; Clapham, J. C.; Coyle, P. J.; Haigh, D.; Hindley, R. M.; Holder, J. C.; Kallender, H.; Latter, A. J.; Lawrie, K. W. M.; Mossakowska, D.; Murphy, G. J.; Cox, L. R.; Smith, S. A., Identification of High-Affinity Binding Sites for the Insulin Sensitizer Rosiglitazone (BRL-49653) in Rodent and Human Adipocytes Using a Radioiodinated Ligand for Peroxisomal Proliferator-Activated Receptor γ. *Journal of Pharmacology and Experimental Therapeutics* **1998,** *284*, 751-759.

65. Awais, M.; Sato, M.; Umezawa, Y., A fluorescent indicator to visualize ligand-induced receptor/coactivator interactions for screening of peroxisome proliferator-activated receptor [gamma] ligands in living cells. *Biosensors and Bioelectronics* **2007,** *22*, 2564-2569.

66. Burton, J. D.; Goldenberg, D. M.; Blumenthal, R. D., Potential of Peroxisome Proliferator-Activated Receptor Gamma Antagonist Compounds as Therapeutic Agents for a Wide Range of Cancer Types. *PPAR-research* **2008,** *2008*, 1-7.

67. Chen, Q.; Chen, J.; Sun, T.; Shen, J.; Shen, X.; Jiang, H., A yeast two-hybrid technology-based system for the discovery of PPAR[gamma] agonist and antagonist. *Analytical Biochemistry* **2004,** *335*, 253-259.

68. Kliewer, S. A.; Lenhard, J. M.; Willson, T. M.; Patel, I.; Morris, D. C.; Lehmann, J. M., A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor [gamma] and promotes adipocyte differentiation. *Cell* **1995,** *83*, 813-819.

69. Krey, G.; Braissant, O.; L'Horset, F.; Kalkhoven, E.; Perroud, M.; Parker, M. G.; Wahli, W., Fatty Acids, Eicosanoids, and Hypolipidemic Agents Identified as Ligands of Peroxisome Proliferator-Activated Receptors by Coactivator-Dependent Receptor Ligand Assay. *Mol Endocrinol* **1997,** *11*, 779-791.

4

INDUCTION OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR γ (PPARγ)-MEDIATED GENE EXPRESSION BY TOMATO (*SOLANUM LYCOPERSICUM* L.) EXTRACTS

Linda Gijsbers, Henriëtte D.L.M. van Eekelen, Laura H.J. de Haan, Jorik M. Swier, Nienke L. Heijink, Samantha K. Kloet, Hai-Yen Man, Arnaud G. Bovy, Jaap Keijer, Jac M.M.J.G. Aarts, Bart van der Burg, Ivonne M.C.M. Rietjens

Submitted for publication to Journal of Agricultural and Food chemistry

ABSTRACT

Since beneficial effects related to tomato consumption partially overlap with those related to PPARγ activation, our aim was to test tomato components (including polyphenols and isoprenoids) and extracts of tomato fruits and for their capacity to activate PPARγ. Thirty tomato compounds were tested, of which seven carotenoids and three polyphenols were able to induce PPARγ2-mediated gene expression. Two extracts of tomato fruit, one containing deglycosylated phenolic compounds and one containing isoprenoids, also induced PPARγ2-mediated expression at physiologically relevant concentrations. Furthermore, enzymatically hydrolyzed extracts of seven tomato varieties were all able to induce PPARγ-mediated expression, with a 1.6 fold difference between the least potent and the most potent variety. The two most potent varieties had high flavonoid content, while the twoleast potent varieties had low flavonoid content. These data indicate that tomato fruits are able to induce PPARγ-mediated gene expression, and that some tomato varieties are more potent than others.

KEYWORDS

Peroxisome proliferator-activated receptor gamma (PPARγ); tomato extracts; polyphenols; carotenoids; fatty acids; luciferase reporter gene assay; mixture effects.

INTRODUCTION

Peroxisome proliferator-activated receptor γ (PPARγ, NR1C3) belongs to the nuclear receptor superfamily. Upon activation by agonists, PPARγ forms a heterodimer with retinoid X receptor (RXR) and binds to a peroxisome proliferator-responsive element (PPRE) in the regulatory domain of target genes, thereby affecting their expression (*1, 2*). PPARγ is known to be an important stimulator of adipogenesis (*2, 3*). In addition, thiazolidinediones (TZDs) are a group of PPARγ agonists shown to improve insulin sensitivity (*4, 5*). Other beneficial health effects that have been related to PPARγ activation include, for example, anti-inflammatory activity (*6, 7*), effects on cholesterol levels (*8, 9*) and reduced risk of atherosclerosis (*10, 11*). Furthermore, PPARγ agonists have been related to inhibition of the development of prostate, breast and colon cancer (*12, 13*), although it is not yet clearly established to what extent the anticancer effects are PPARγ-mediated (*14-16*).

Tomato is one of the most consumed vegetables in the Western world and is a major component of the healthy Mediterranean diet (*17, 18*). The consumption of tomato has been related to reduced risk of prostate cancer and several other cancer types (*17, 19*). High intake of tomato is believed to modify lipid profiles towards a healthier pattern (*20, 21*) and to lower the risk of atherosclerosis and cardiovascular diseases (*22, 23*). In addition, some phytochemicals present in tomato have been related to improved glucose and insulin levels (*24, 25*). Tomato fruits contain many bioactive phytochemicals, for example flavonoids including quercetin, kaempferol and naringenin chalcone, and carotenoids including β-carotene and lycopene (*26-30*).

Given the overlap between the beneficial health effects associated with PPARγ-activation and those associated with tomato consumption, we hypothesized that bioactive components of tomato fruit may be able to activate PPARγ. Therefore, the aim of the present study was to investigate whether tomato components, including polyphenols, isoprenoids and fatty acids, as well as tomato extracts are able to activate PPARγ-mediated gene expression. To that end, the recently developed PPARγ2 CALUX reporter cell line (*31*) was used to investigate whether flavonoid-rich and isoprenoid-rich tomato extracts as well as individual compounds known to be present in tomato fruits are able to induce PPARγ-mediated gene expression.

MATERIALS AND METHODS

Chemicals

Rosiglitazone and palmitic acid were obtained from Cayman Chemical (Ann Arbor, USA). α-Linolenic acid (ALA), oleic acid (OA), linoleic acid (LA), stearic acid (SA), myristic acid (MA), quercetin dihydrate, ferulic acid, chlorogenic acid, β-carotene, α-tocopherol, γ-tocopherol, and δ-tocopherolwere purchased from Sigma Aldrich (St. Louis, USA). Kaempferol, kaempferol-3-O-rutinoside, cyanidin chloride, delphinidin chloride, lycopene and lutein were obtained from Extrasynthase (Genay, France). Caffeic acid and rutin were obtained from Acros (Geel, Belgium). Naringenin was obtained from ICN Biomedicals (Ohio, USA). Naringenin chalcone as a mixture with naringenin was purchased from Apin

Chemicals (Oxon, UK), since the pure compound was not commercially available. Phytoene, phytofluene, neoxanthin, violaxanthin, neurosporene, γ-carotene and α-carotene were obtained from Carotenature (Lupsingen, Switzerland).

Rosiglitazone and all phenolic compounds and tocopherols were dissolved in dimethylsulphoxide (DMSO, 99.9%, Acros, Geel, Belgium). The fatty acids were dissolved in ethanol (Merck KGaA, Darmstadt, Germany) and the carotenoids were dissolved in tetrahydrofuran (THF; Acros, Geel, Belgium).

Extraction of tomato samples

Tomato extracts containing semi-polar compounds

The model tomato sample "tomato mix" was previously described (*32*). In short, ripe beef, cherry and round tomatoes were pooled and snap frozen in liquid nitrogen. Then, the frozen tomatoes were ground to powder using an analytical mill (type A11 basic, IKA, Staufen, Germany). The powder was stored at -80°C until further use. A glycosidase-treated tomato extract which contains enzymatically hydrolyzed phenolic compounds was prepared from the tomato mix. To this end 300 µl of 0.1 M sodium acetate (pH 4.8) and 100 µl of Viscozyme L (Sigma Aldrich, St. Louis, USA) were added to 0.6 gram of tomato mixand incubated at 37°C for 1 hour. Then, 3 ml methanol was added and the Viscozyme-treatedtomato mix was put in an ultrasonic bath for 10 minutes and centrifuged at 1000x g for 10-15 minutes. The supernatant was filtered using 0.2 µm polytetrafluoroethylene filters (M-filter, Tiel, The Netherlands) and dried under a nitrogen stream and stored at -80°C. The Viscozyme-treated extract, referred to as enzymatically hydrolyzed tomato extract (containing hydrolyzed phenolic compounds) was dissolved in DMSO:assay medium (1:4 v/v) just before analysis in the PPARγ2 CALUX cells.

In addition to the tomato mix, nine different tomato varieties, which were kindly provided by Syngenta (Enkhuizen), were investigated. These nine tomato varieties vary in their polyphenolic content and included two high pigment tomato varieties (varieties G and H). Enzymatically hydrolyzed extracts of the nine tomato varieties were prepared using the method described above. These extracts were tested in the PPARγ2 CALUX cell line for their potency to induce PPARγ-mediated gene expression. In addition, chemical analyses of the seven extracts that appeared active in the PPARγ2 CALUX assay without showing cytotoxicity (varieties A-H) were performed using LC-MS.

Tomato extract containing isoprenoids

Roma tomatoes were purchased at a local supermarket and were pooled, snap frozen in liquid nitrogen and subsequently ground in an analytical mill. The powder, which will be further referred to as Roma tomato mix, was stored at -80°C until further use. A chloroform extract containing isoprenoids was prepared from the Roma tomato mix as described before (*26*). In short, 0.5 g Roma tomato mix was dissolved in 4.5 ml of methanol:chloroform (2.5:2.0 v/v) and put on ice for 10 minutes. Then, 2.5 ml of pre-cooled Tris-HCl (50 mM, pH 7.4) was added and the sample was mixed. After centrifugation for 10 minutes at 1000x g, the chloroform phase was transferred to a new tube. Extraction was repeated twice by adding fresh chloroform to the remaining methanol/Tris phase, mixing and centrifugation of the tubes. The three chloroform fractions were combined and dried under a stream of

nitrogen and stored at -80°C. Prior to analysis in the PPARγ2 CALUX cells, the isoprenoid-containing tomato extract was dissolved in THF: assay medium (1:1 v/v).

Cell culture

The construction and validation of the PPARγ2 CALUX cell line (BioDetection Systems, Amsterdam, The Netherlands) was described before (*31*). In short, human U2OS osteosarcoma cells were stably transfected with an expression vector for PPARγ2 and a pGL4–3xPPRE–tata–luc reporter construct. PPARγ2 CALUX cells were grown in culture medium: DMEM/F12 glutamax medium (Invitrogen, Breda, The Netherlands) supplemented with 7.5% fetal calf serum (Invitrogen), non-essential amino acids (Invitrogen) and penicillin/streptomycin (Invitrogen)(final concentrations 10 U/ml and 10 µg/ml, respectively). Once per week 200 µg/ml G418 (Duchefa Biochemie, Haarlem, The Netherlands) was added to the culture medium in order to maintain selection pressure. Cells were cultured at 37°C and 5% CO_2 in a humid atmosphere.

Reporter gene assays

The ability of tomato extracts or individual compounds to induce PPARγ2-mediated luciferase expression was tested by measuring luciferase activity in the PPARγ2 CALUX reporter cells. To this end, PPARγ2 CALUX cells were seeded in 96-well plates (Corning Incorporated, Cambridge, USA) at a density of 10,000 cells per well in 100 µL assay medium: DMEM/F12 without phenol red (Invitrogen) supplemented with 5% fetal calf serum treated with dextran-coated charcoal (Thermo Scientific, Waltham, USA), non-essential amino acids (Invitrogen) and penicillin/streptomycin (Invitrogen)(final concentrations 10 U/ml and 10 µg/ml, respectively). Before exposure to the fatty acids, the plated cells were treated with vitamin E by adding 20 µl of a 50 mM vitamin E solution (mixed isomers of (+)-α-tocopherol, CAS 59-02-9; Sigma Aldrich, St. Louis, USA) to 20 ml assay medium (final concentration 50 µM vitamin E). Vitamin E serves as an antioxidant to prevent oxidation of the unsaturated fatty acids. After 24 hours, when the cells formed a monolayer, 100 µL fresh assay medium supplemented with the test compounds was added to the wells. When testing the individual compounds, the percentage of solvent in the exposure medium was kept at or below 0.5%. Only for testing the highest concentrations of 100 µM, 1% of solvent had to be used. On each plate, 1 µM of rosiglitazone was included as a positive control. Although rosiglitazone is no longer used as an insulin-sensitizing drug in humans, it is still generally used as reference compound for PPARγ-activation (*33, 34*). During the exposure to fatty acids, the cells were co-exposed to 50 µM freshly added vitamin E (mixed isomers of (+)-α-tocopherol; Sigma Aldrich, St. Louis, USA) and 0.1% BSA (Sigma Aldrich, St. Louis, USA). BSA facilitates the solubility and cellular availability of the fatty acids. After 24 hours of exposure, the medium was removed and low salt buffer (30 µl per well) was added. The plates were subsequently frozen overnight at -80°C in order to lyse the cells. Luciferase activity was measured using a luminometer (Luminoscan Ascent, Thermo Scientific, Waltham, USA) by adding 100 µl flash mix (20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 2.0 mM dithiothreitol, 470 µM luciferine, 5.0 mM ATP) per well and measuring the light production as relative light units (RLU).

All individual tomato compounds were tested for cytotoxicity using the Cytotox CALUX cell line as described by Van der Linden *et al.* (*35*). These Cytotox CALUX cells are U2OS cells with an invariant luciferase expression and respond to cytotoxicity with a decreased luciferase activity compared to the solvent control. Only non-cytotoxic concentrations of the individual tomato compounds were used for testing in the PPARγ2 CALUX cell line.

Chemical analysis using LC-PDA-QTOF-MS

Using LC-PDA-QTOF-MS, semi-polar compounds in the sample can be separated using liquid chromatography (LC) and detected using photodiode array (PDA). In the next step, the compounds are ionized and masses are detected using quadrupole time of flight (QTOF) in combination with mass spectrometry (MS). This LC-PDA-QTOF-MS method provides insight in the semi-polar compounds present in the (tomato) sample since individual compounds can be identified based on their retention time and mass. LC-PDA-QTOF-MS analysis was performed as described before by De Vos and colleagues (*36*) in order to detect semi-polar compounds present in the enzymatically hydrolyzed extracts of the seven non-cytotoxic tomato varieties. In short, 5µl of the re-dissolved and filtered enzymatically hydrolyzed tomato extracts were injected onto a C18 column. Chromatographic separation was performed using ultrapure water (eluent A) and acetonitrile (eluent B), both acidified with 0.1% formic acid, using a linear gradient starting at 5% eluent B up to 35% eluent B in 45 minutes with a flow rate of 0.190 ml/min. Hereafter, the column was washed an equilibrated for 15 minutes, before the next injection. After separation and detection of the semi-polar compounds by LC-PDA, ionization was performed using an electrospray ionization source and masses were detected in positive mode (ESI+) using quadrupole time of flight high-resolution mass spectrometry QTOF-MS. Ion chromatograms obtained from LC-PDA-QTOF-MS were analyzed using MassLynx 4.1 (Waters) software.

Data analysis

Each test compound or extract was tested in at least two independent experiments, and one representative curve is presented (unless stated otherwise). In each of the independent experiments, all data points were performed in triplicate. The RLU data were converted into percentages of the positive control (1 µM rosiglitazone) and presented as mean values ± standard error (SE). Fold inductions were calculated by dividing the luciferase activity of the sample by the luciferase activity of the solvent control sample. Statistical significance was assessed using the one-sided Student's t-test and a cut off value of $p \leq 0.05$.

RESULTS AND DISCUSSION

PPARγ-activation by individual phytochemicals

In order to investigate whether compounds which are known to be present in tomato have the capacity to function as PPARγ agonists, PPARγ2 CALUX reporter cells were exposed for 24 hours to increasing concentrations of thirty individual compounds. These thirty compounds were selected based on their presence in tomato fruit (table 1) and include

eleven polyphenols, ten carotenoids, three tocopherols and six fatty acids. Of all thirty compounds tested, the phenolic compounds kaempferol, naringenin and naringenin chalcone and the carotenoids violaxanthin, phytofluene, neurosporene, lycopene,

Table 1. Overview of tomato compounds selected to be tested and summary of the results obtained in the PPARγ2 CALUX reporter cell line.

Compound	Reference(s) for presence in tomato	Fold induction at 10 µM
Polyphenolic compounds		
kaempferol	(*28, 30*)	3.8
kaempferol-3-O-rutinoside	(*28*)	inactive
quercetin	(*26, 30*)	inactive
rutin	(*26, 28*)	inactive
naringenin	(*28, 30, 37*)	5.3
naringenin chalcone	(*26, 28*)	2.6
delphinidin[a]	(*38*)	inactive
cyanidin[a]	(*39*)	inactive
chlorogenic acid	(*28, 37*)	inactive
ferulic acid	(*37, 40*)	inactive
caffeic acid	(*37, 40*)	inactive
Carotenoids		
lutein	(*26, 27*)	inactive
neoxanthin	(*26, 27*)	inactive
violaxanthin	(*26, 27*)	1.6 (fold at 100 µM: 2.6)
phytoene	(*27, 29*)	inactive
phytofluene	(*27, 29*)	1.5
neurosporene	(*27, 29*)	1.5
lycopene	(*27, 29*)	1.5 (fold at 100 µM: 2.5)
β-carotene	(*26, 27*)	2.3
γ-carotene	(*27*)	3.8
δ-carotene[a]	(*41, 42*)	2.3
Tocopherols		
α-tocopherol	(*26, 43*)	inactive
β-tocopherol	(*43, 44*)	inactive
δ-tocopherol	(*26, 43*)	inactive
Fatty acids		
α-linolenic acid (ALA; C18:3n3)	(*45, 46*)	2.7[b]
oleic acid (OA; C18:1n9)	(*45, 46*)	3.8[b]
linoleic acid (LA; C18:2n6)	(*45, 46*)	2.8[b]
palmitic acid (PA; C16:0)	(*45, 46*)	inactive
stearic acid (SA; C18:0)	(*45, 46*)	inactive
myristic acid (MA; C14:0)	(*45*)	inactive

[a]Although not found in conventional tomato, these compounds are found in special varieties like purple tomato (delphinidin and cyanidin) and Delta tomato (δ-carotene)(38, 41, 42).
[b]For fatty acids, the fold induction at 100 µM is presented.

β-carotene, γ-carotene and δ-carotene were able to induce PPARγ2-mediated expression of luciferase (table 1). The other phenolic compounds and carotenoids as well as the three tocopherols were not able to activate PPARγ2-mediated luciferase expression (table 1). Induction of PPARγ by β-carotene, lycopene, kaempferol, naringenin and naringenin chalcone is in line with findings in literature reporting that these compounds can bind to PPARγ when tested in receptor binding assays (*47-49*). These earlier findings, however, indicate binding to the PPARγ receptor, which does not automatically lead to activation of PPARγ and to subsequent changes in PPARγ-mediated gene expression patterns. For example, although quercetin was reported to bind to PPARγ (*47, 49*), it was not able to stimulate PPARγ-mediated gene expression in PPARγ2 CALUX cells. To our best knowledge, of the polyphenols and isoprenoids tested in the current research, only naringenin and naringenin chalcone have been shown to influence PPARγ-mediated gene expression before (*50, 51*). It is of interest to note that none of the flavonoid glycosides was able to induce PPARγ2-mediated expression, whereas some of the corresponding aglycones are active agonists (table 1). For example, the aglycone kaempferol was able to induce PPARγ2-mediated expression, while its glycoside kaempferol-3-O-rutinoside was not.

Of the six fatty acids tested for their ability to activate PPARγ2, α-linolenic acid (ALA), oleic acid (OA), and linoleic acid (LA) were found to act as PPARγ2 agonists, while palmitic acid, stearic acid and myristic acid were not functioning as PPARγ2 agonists (table 1). These findings are in line with data reported by Chou *et al.* showing that ALA, OA and LA function as PPARγ agonists and that palmitic acid and stearic acid do not activate PPARγ (*52*). Chou and colleagues, however, report that myristic acid also functions as PPARγ agonist, while our data did not show PPARγ agonism for this compound. Of all tested compounds, naringenin, kaempferol and γ-carotene were the most active inducers of PPARγ2-mediated luciferase gene expression: these compounds induce PPARγ2-mediated luciferase expression at concentrations of 1 μM or higher and showed the highest fold induction at a concentration of 10 μM (table 1).

PPARγ-activation by extracts of tomato fruit

To investigate the potential of tomato fruit to induce PPARγ2-mediated luciferase gene expression, two different types of tomato extracts were tested: one extract containing isoprenoids (which was obtained by chloroform extraction and is further referred to as isoprenoid-containing extract) and one extract containing semi-polar compounds, including flavonoids and other phenolic compounds. As the results with the individual compounds indicated that only the aglycones (and not the glycosides) were able to activate PPARγ, the tomato homogenate was treated with Viscozyme L before extraction with methanol in order to remove the glycosyl residues from the bioactive phenolic components and will be further referred to as enzymatically hydrolyzed tomato extract. After 24 hours of exposure, the isoprenoid-containing tomato extract was able to induce PPARγ2-mediated luciferase in a dose-dependent manner (figure 1). The PPARγ2-activating effect of the isoprenoid-containing tomato extract may be due to the presence of the carotenoids violaxanthin, phytofluene, neurosporene, lycopene, β-carotene, γ-carotene and δ-carotene, which were all found to function as PPARγ2-agonists (table 1) and are

known to be present in tomato fruits (*26, 27, 29, 42*). In addition, also the enzymatically hydrolyzed tomato extract was able to induce PPARγ2-mediated luciferase in a dose-dependent manner (figure 1). In the enzymatically hydrolyzed tomato extract, kaempferol, naringenin and naringenin chalcone may contribute to the activity since these compounds were able to activate PPARγ2 when tested as pure compounds. Previous studies have reported the presence of these compounds in tomato fruit (*28, 30*). In humans, enzymatic hydrolysis of flavonoid glycosides also occurs in the intestine before uptake (*53, 54*).

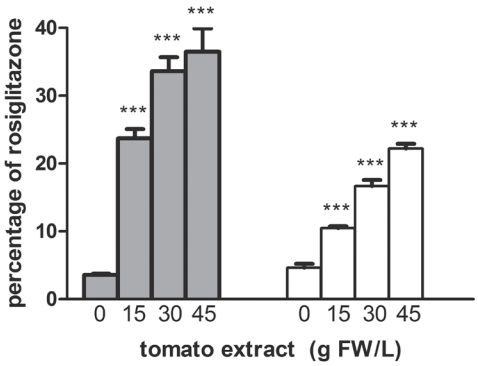

Figure 1. Enzymatically hydrolyzed tomato extract (grey bars) and isoprenoid-containing tomato extract (white bars) induce dose-dependent PPARγ2-mediated luciferase expression in the PPARγ2 CALUX cells. The amount of tomato extract is expressed as gram fresh weight per liter (g FW/L). Luciferase activity is expressed as percentage of the positive control (1 µM rosiglitazone). Data are corrected for background luciferase activity and are expressed as mean ± SEM (n=3). Asterisks indicate the level of significance compared to the solvent control: * p<0.05; ** p<0.01 and *** p<0.001.

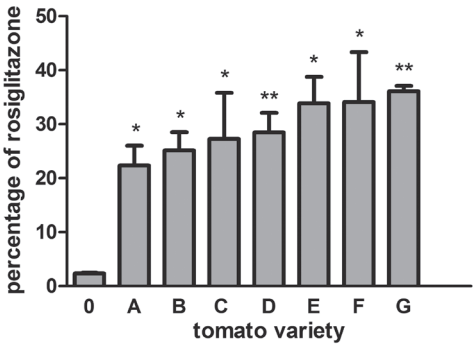

Figure 2. Enzymatically hydrolyzed extracts of seven tomato varieties induce PPARγ2-mediated luciferase expression in the PPARγ2 CALUX cells. The extracts were tested at 45 g FW/L. The solvent control (0) is included. Luciferase activity is expressed as percentage of the positive control (1 µM rosiglitazone). Data are corrected for background luciferase activity and are expressed as mean ± SEM of three independent experiments. Asterisks indicate the level of significance compared to the solvent control: * p<0.05; ** p<0.01; and *** p<0.001.

PPARγ-activation by seven different tomato varieties

In addition to the mixture of beef, cherry and round tomatoes, enzymatically hydrolyzed extracts of nine tomato varieties, designated A-I, were tested for their potency to induce PPARγ-mediated gene expression. These nine tomato varieties included two high pigment tomato varieties (G and H). Two tomato varieties, H and I, showed cytotoxicity at 45 g FW/L and were therefore not included in further analysis. The other seven varieties were all able to significantly induce PPARγ-mediated gene expression at 45 g FW/L (figure 2). There was a 1.6 fold difference in the induction found with the least potent variety (variety A) and the most potent variety (variety G)(figure 2).

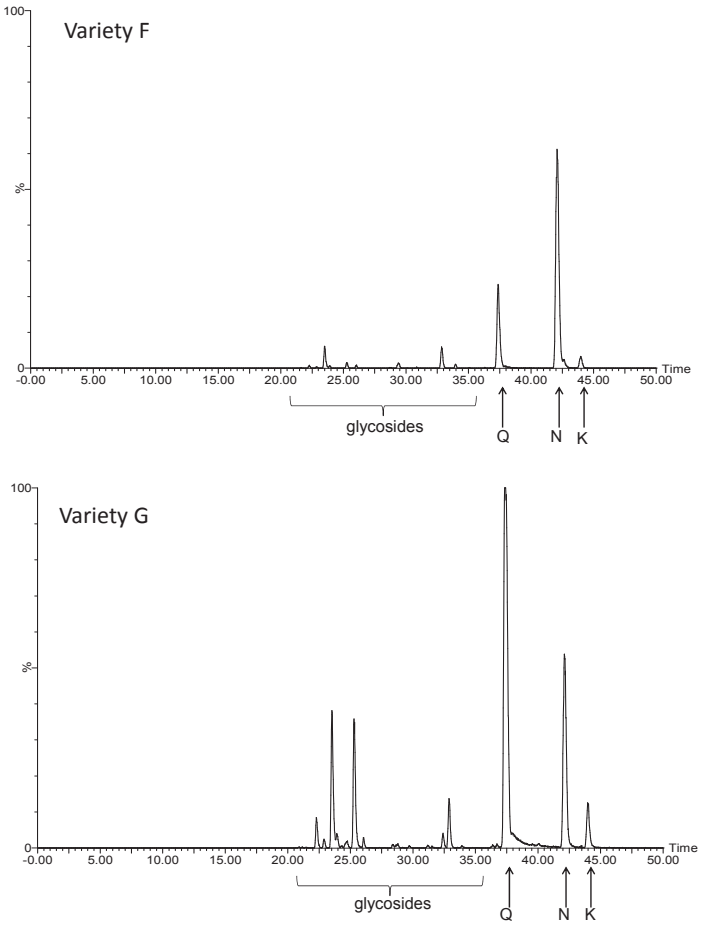

Figure 3. Chromatograms of enzymatically hydrolyzed extracts of the most potent tomato varieties (F and G), representing the total ion signal of selected masses: 273.075 (naringenin); 287.055 (kaempferol); 303.050 (quercetin). Maximal intensity (100%) of 15000. Indicated with arrows are: N = naringenin; K = kaempferol; Q = Quercetin; glycosides = flavonoid glycosides. For complete chromatograms, see supplemental data.

The extracts of the seven tomato varieties not showing cytotoxicity, were chemically analyzed using LC-MS, and chromatograms representing the total ion signal of each variety are presented in the supplemental data. Chromatograms showing selected masses of flavonoid aglycones indicated that considerable amounts of kaempferol, naringenin and quercetin were present in the most potent tomato varieties F and G (figure 3). In addition, levels of kaempferol and naringenin, both found to function as PPARγ2-agonists when tested individually, were quantified in the enzymatically hydrolyzed extracts of the seven tomato varieties (table 2). It is interesting to note that the least potent varieties, A and B, had the lowest content of kaempferol and naringenin, while the two most potent varieties, F and G, had the highest content of kaempferol and naringenin. These results suggest that the differences in potency to induce PPARγ-mediated gene expression may be partly explained by the differences in levels of kaempferol and naringenin and/or other flavonoids.

Table 2. Levels of kaempferol and naringenin found in enzymatically hydrolyzed extracts of seven tomato varieties.

Tomato variety	Kaempferol (μM)[a]	Naringenin (μM)[a]
A	0.07	0.59
B	0.04	0.04
C	1.13	7.76
D	0.03	1.30
E	0.02	1.59
F	0.31	13.19
G[b]	1.33	10.56

[a]Kaempferol and naringenin are presented as μM present in extract dissolved and diluted to 45 g FW/L, which is the concentration at which the extracts were tested in the PPARγ2 CALUX cell line.
[b]High pigment variety.

PPARγ-activation by phytochemical mixtures

Most of the individual compounds induced PPARγ2-mediated luciferase expression at concentrations of 1 μM and higher. These concentrations seem relatively high, since the concentrations of these compounds in human plasma normally do not exceed 1 μM (*55, 56*). After tomato consumption, plasma concentrations of several phytochemicals, including β-carotene, lycopene and naringenin increase, but still stay below 1 μM (*21, 37, 55*). The total concentration of phytochemicals, however, is higher than 1 μM (*57, 58*) and may be high enough to activate PPARγ and lead to PPARγ-mediated changes in gene expression. To investigate whether an additive effect of different tomato compounds on PPARγ2 activation can be expected, induction of PPARγ2-mediated gene expression by combinations of kaempferol, naringenin and β-carotene was investigated. This reveals that upon combining different compounds at concentrations of 1 μM, which individually do not induce PPARγ2-mediated gene expression, induction of PPARγ2-mediated expression

can be obtained (figure 4). The effect was most striking for the combination of β-carotene with either kaempferol or naringenin: the individual compounds were not able to induce PPARγ2-mediated expression at 1 µM, but the combination of 1 µM β-carotene with either 1 µM kaempferol or 1 µM naringenin resulted in a significant induction. Our results suggest an additive effect when combining individual phytochemicals. Additive effects between phytochemicals have been reported before (*59*). This additive effect might be achieved by the fact that these phytochemicals all act by binding to the same receptor and therefore their concentrations can be added up. Another explanation could be that the mixtures of phytochemicals are also able to activate RXR, which is the dimerization partner for PPARγ, leading to an additive effect in activation of the PPARγ-RXR complex.

Furthermore, phytochemicals are not only present in plasma, but also in tissues. In tissues the total carotenoid level has been reported to reach concentrations of 5.1, 9.4 and 7.6 nmol/gram wet tissue (equal to 5.1, 9.4 and 7.6 µM, assuming that 1 kg of wet tissue corresponds to 1 liter) in liver, adrenal glands and testes, respectively (*60*). Based on the results of the present paper it can be concluded that these levels are high enough to induce PPARγ-mediated gene expression. To substantiate functional effects of the food compounds and extracts *in vivo*, however, additional data are needed, in particular for polyphenols that do not occur as aglycones in plasma.

The beneficial effects of tomato consumption have often been linked to lycopene (*61*). Our data indicate that many other phytochemicals present in tomato may be involved in possible PPARγ-mediated beneficial health effects of tomato fruits.

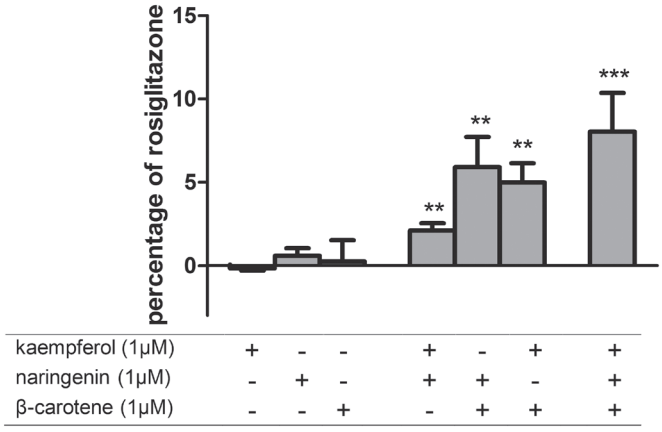

Figure 4. PPARγ2-mediated luciferase expression in the PPARγ2 CALUX cells induced by individual phytochemicals and combinations of phytochemicals at 1 µM each. Luciferase activity is expressed as percentage of the positive control (1 µM rosiglitazone). Data are corrected for background luciferase activity and for luciferase activity observed with the solvent control. Data are expressed as mean ± SEM of five independent experiments. Asterisks indicate the level of significance compared to the solvent control: * $p<0.05$; and ** $p<0.01$.

Tomato, PPARγ and health

Induction of PPARγ-mediated gene expression has frequently been suggested to play a role in insulin-sensitization (*62*), protection against prostate, breast and colon cancer (*12, 13*) and protection against atherosclerosis (*10, 11*). Although our data indicate that tomato extracts are able to induce PPARγ-mediated changes in gene expression, additional data are needed to confirm that tomato consumption *in vivo* leads to PPARγ-mediated gene expression and to PPARγ-related beneficial health effects. Several *in vivo* studies provide a link between tomato consumption and beneficial effects on lipid peroxidation rate (*63*), lipid profile (*19*) and blood pressure (*64, 65*). A role for PPARγ-mediated gene expression in endpoints like serum levels of free fatty acids and HDL cholesterol, blood pressure and glucose tolerance has been reported (*4, 8, 66*) and activation of PPARγ may thus provide a potential mode of action of several of the beneficial health effects of tomato consumption.

Using reporter gene assays, it was previously demonstrated that PPARγ1 and PPARγ2 are activated by PPARγ agonists in a similar way (*31, 67*). This suggests that tomato compounds and tomato extracts which are able to activate PPARγ2, may be able to also activate PPARγ1.

In conclusion, our data show that isolated tomato compounds as well as the enzymatically hydrolyzed tomato extract containing phenolic compounds and the isoprenoid-containing tomato extract were able to induce PPARγ2-mediated gene expression. Taking into account concentrations at which PPARγ activation was detected and reported physiological levels of PPARγ-activating compounds in plasma and various tissues, our results indicate that beneficial health effects associated with tomato consumption may be (partly) mediated by PPARγ2-mediated induction of gene transcription.

ACKNOWLEDGMENTS

This work was supported by the Food and Nutrition Delta (grant number FND07007). The authors want to thank Syngenta for providing fruits of the nine tomato varieties.

REFERENCES

1. Berger, J.; Moller, D. E., The mechanisms of action of PPARs. *Annual Review of Medicine* **2002,** *53*, 409-435.
2. Tontonoz, P.; Hu, E.; Spiegelman, B. M., Stimulation of adipogenesis in fibroblasts by PPARγ2, a lipid-activated transcription factor. *Cell* **1994,** *79*, 1147-1156.
3. Barak, Y.; Nelson, M. C.; Ong, E. S.; Jones, Y. Z.; Ruiz-Lozano, P.; Chien, K. R.; Koder, A.; Evans, R. M., PPARγ Is Required for Placental, Cardiac, and Adipose Tissue Development. *Molecular Cell* **1999,** *4*, 585-595.
4. Bennett, S. M. A.; Agrawal, A.; Elasha, H.; Heise, M.; Jones, N. P.; Walker, M.; Wilding, J. P. H., Rosiglitazone improves insulin sensitivity, glucose tolerance and ambulatory blood pressure in subjects with impaired glucose tolerance. *Diabetic Medicine* **2004,** *21*, 415-422.
5. Raji, A.; Seely, E. W.; Bekins, S. A.; Williams, G. H.; Simonson, D. C., Rosiglitazone Improves Insulin Sensitivity and Lowers Blood Pressure in Hypertensive Patients. *Diabetes Care* **2003,** *26*, 172-178.
6. Martin, H., Role of PPAR-gamma in inflammation. Prospects for therapeutic intervention by food components. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **2010,** *690*, 57-63.
7. Széles, L.; Töröcsik, D.; Nagy, L., PPARγ in immunity and inflammation: cell types and diseases. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **2007,** *1771*, 1014-1030.
8. Davidson, M.; Meyer, P. M.; Haffner, S.; Feinstein, S.; D'Agostino, R.; Kondos, G. T.; Perez, A.; Chen, Z.; Mazzone, T., Increased High-Density Lipoprotein Cholesterol Predicts the Pioglitazone-Mediated Reduction of Carotid Intima-Media Thickness Progression in Patients With Type 2 Diabetes Mellitus. *Circulation* **2008,** *117*, 2123-2130.
9. Goldberg, R. B.; Kendall, D. M.; Deeg, M. A.; Buse, J. B.; Zagar, A. J.; Pinaire, J. A.; Tan, M. H.; Khan, M. A.; Perez, A. T.; Jacober, S. J., A Comparison of Lipid and Glycemic Effects of Pioglitazone and Rosiglitazone in Patients With Type 2 Diabetes and Dyslipidemia. *Diabetes Care* **2005,** *28*, 1547-1554.
10. Hamblin, M.; Chang, L.; Fan, Y.; Zhang, J.; Chen, Y. E., PPARs and the Cardiovascular System. *Antioxidants & Redox Signaling* **2009,** *11*, 1415-1452.
11. Walczak, R.; Tontonoz, P., PPARadigms and PPARadoxes: expanding roles for PPARγ in the control of lipid metabolism. *Journal of Lipid Research* **2002,** *43*, 177-186.
12. Grommes, C.; Landreth, G. E.; Heneka, M. T., Antineoplastic effects of peroxisome proliferatoractivated receptor γ agonists. *The Lancet Oncology* **2004,** *5*, 419-429.
13. Michalik, L.; Desvergne, B.; Wahli, W., Peroxisome-proliferator-activated receptors and cancers: complex stories. *Nat Rev Cancer* **2004,** *4*, 61-70.
14. Chen, G. G.; Xu, H.; Lee, J. F. Y.; Subramaniam, M.; Leung, K. L.; Wang, S. H.; Chan, U. P. F.; Spelsberg, T. C., 15-hydroxy-eicosatetraenoic acid arrests growth of colorectal cancer cells via a peroxisome proliferator-activated receptor gamma-dependent pathway. *Int. J. Cancer* **2003,** *107*, 837-843.
15. Girnun, G. D.; Naseri, E.; Vafai, S. B.; Qu, L.; Szwaya, J. D.; Bronson, R.; Alberta, J. A.; Spiegelman, B. M., Synergy between PPARγ Ligands and Platinum-Based Drugs in Cancer. *Cancer Cell* **2007,** *11*, 395-406.
16. Yang, C.-C.; Ku, C.-Y.; Wei, S.; Shiau, C.-W.; Chen, C.-S.; Pinzone, J. J.; Ringel, M. D.; Chen, C.-S., Peroxisome Proliferator-Activated Receptor γ-Independent Repression of Prostate-Specific Antigen Expression by Thiazolidinediones in Prostate Cancer Cells. *Molecular Pharmacology* **2006,** *69*, 1564-1570.
17. Canene-Adams, K.; Campbell, J. K.; Zaripheh, S.; Jeffery, E. H.; Erdman, J. W., The Tomato As a Functional Food. *The Journal of Nutrition* **2005,** *135*, 1226-1230.
18. Gómez-Romero, M.; Arráez-Román, D.; Segura-Carretero, A.; Fernández-Gutiérrez, A., Analytical determination of antioxidants in tomato: Typical components of the Mediterranean diet. *J. Sep. Science* **2007,** *30*, 452-461.
19. Blum, A.; Monir, M.; Wirsansky, I.; Ben-Arzi, S., The beneficial effects of tomatoes. *European Journal of Internal Medicine* **2005,** *16*, 402-404.

20. Ibrahim, H. S.; Ahmed, L. A.; El-din, M. M., The functional role of some tomato products on lipid profile and liver function in adult rats. *Journal of medicinal food* **2008,** *11*, 551-559.

21. Shen, Y.-C.; Chen, S.-L.; Wang, C.-K., Contribution of Tomato Phenolics to Antioxidation and Down-regulation of Blood Lipids. *Journal of Agricultural and Food Chemistry* **2007,** *55*, 6475-6481.

22. Sesso, H. D.; Liu, S.; Gaziano, J. M.; Buring, J. E., Dietary Lycopene, Tomato-Based Food Products and Cardiovascular Disease in Women. *The Journal of Nutrition* **2003,** *133*, 2336-2341.

23. Visioli, F.; Riso, P.; Grande, S.; Galli, C.; Porrini, M., Protective activity of tomato products on in vivo markers of lipid oxidation. *European Journal of Nutrition* **2003,** *42*, 201-206.

24. Ylönen, K.; Alfthan, G.; Groop, L.; Saloranta, C.; Aro, A.; Virtanen, S. M.; Group, t. B. R., Dietary intakes and plasma concentrations of carotenoids and tocopherols in relation to glucose metabolism in subjects at high risk of type 2 diabetes: the Botnia Dietary Study. *The American Journal of Clinical Nutrition* **2003,** *77*, 1434-1441.

25. Mulvihill, E. E.; Allister, E. M.; Sutherland, B. G.; Telford, D. E.; Sawyez, C. G.; Edwards, J. Y.; Markle, J. M.; Hegele, R. A.; Huff, M. W., Naringenin Prevents Dyslipidemia, Apolipoprotein B Overproduction, and Hyperinsulinemia in LDL Receptor–Null Mice With Diet-Induced Insulin Resistance. *Diabetes* **2009,** *58*, 2198-2210.

26. Bino, R. J.; De Vos, C. H. R.; Lieberman, M.; Hall, R. D.; Bovy, A.; Jonker, H. H.; Tikunov, Y.; Lommen, A.; Moco, S.; Levin, I., The light-hyperresponsive high pigment-2dg mutation of tomato: alterations in the fruit metabolome. *New Phytologist* **2005,** *166*, 427-438.

27. Fraser, P. D.; Truesdale, M. R.; Bird, C. R.; Schuch, W.; Bramley, P. M., Carotenoid Biosynthesis during Tomato Fruit Development (Evidence for Tissue-Specific Gene Expression). *Plant Physiology* **1994,** *105*, 405-413.

28. Slimestad, R.; Verheul, M. J., Seasonal Variations in the Level of Plant Constituents in Greenhouse Production of Cherry Tomatoes. *Journal of Agricultural and Food Chemistry* **2005,** *53*, 3114-3119.

29. Tonucci, L. H.; Holden, J. M.; Beecher, G. R.; Khachik, F.; Davis, C. S.; Mulokozi, G., Carotenoid Content of Thermally Processed Tomato-Based Food Products. *Journal of Agricultural and Food Chemistry* **1995,** *43*, 579-586.

30. Torres, C. A.; Davies, N. M.; Yanez, J. A.; Andrews, P. K., Disposition of Selected Flavonoids in Fruit Tissues of Various Tomato (Lycopersicon esculentum Mill.) Genotypes. *Journal of Agricultural and Food Chemistry* **2005,** *53*, 9536-9543.

31. Gijsbers, L.; Man, H.-Y.; Kloet, S. K.; de Haan, L. H. J.; Keijer, J.; Rietjens, I. M. C. M.; van der Burg, B.; Aarts, J. M. M. J. G., Stable reporter cell lines for peroxisome proliferator-activated receptor γ (PPARγ)-mediated modulation of gene expression. *Analytical Biochemistry* **2011,** *414*, 77-83.

32. Tikunov, Y.; Lommen, A.; de Vos, C. H. R.; Verhoeven, H. A.; Bino, R. J.; Hall, R. D.; Bovy, A. G., A Novel Approach for Nontargeted Data Analysis for Metabolomics. Large-Scale Profiling of Tomato Fruit Volatiles. *Plant Physiology* **2005,** *139*, 1125-1137.

33. Barros, C. D.; Amato, A. A.; Oliveira, T. B. d.; Iannini, K. B. R.; Silva, A. L. d.; Silva, T. G. d.; Leite, E. S.; Hernandes, M. Z.; Lima, M. d. C. A. d.; Galdino, S. L.; Neves, F. d. A. R.; Pitta, I. d. R., Synthesis and anti-inflammatory activity of new arylidene-thiazolidine-2, 4-diones as PPARγ ligands. *Bioorganic & Medicinal Chemistry* **2010,** *18*, 3805-3811.

34. Seto, S.; Okada, K.; Kiyota, K.; Isogai, S.; Iwago, M.; Shinozaki, T.; Kitamura, Y.; Kohno, Y.; Murakami, K., Design, Synthesis, and Structure−Activity Relationship Studies of Novel 2, 4, 6-Trisubstituted-5-pyrimidinecarboxylic Acids as Peroxisome Proliferator-Activated Receptor γ (PPARγ) Partial Agonists with Comparable Antidiabetic Efficacy to Rosiglitazone. *Journal of Medicinal Chemistry* **2010,** *53*, 5012-5024.

35. Van der Linden, S. C.; von Bergh, A. R. M.; Van Vugt-Lussenburg, B.; Jonker, L.; Brouwer, A.; Teunis, M.; Krul, C. A. M.; Van der Burg, B., Development of a panel of high throughput reporter gene assays to detect genotoxicity and oxidative stress. **Submitted for publication**.

36. De Vos, R. C. H.; Moco, S.; Lommen, A.; Keurentjes, J. J. B.; Bino, R. J.; Hall, R. D., Untargeted large-scale plant metabolomics using liquid chromatography coupled to mass spectrometry. **2007,** *2*, 778-791.

37. Tulipani, S.; Martinez Huelamo, M.; Rotches Ribalta, M.; Estruch, R.; Ferrer, E. E.; Andres-Lacueva, C.; Illan, M.; Lamuela-Raventós, R. M., Oil matrix effects on plasma exposure and urinary excretion of phenolic compounds from tomato sauces: Evidence from a human pilot study. *Food Chemistry* **2012,** *130*, 581-590.

38. Butelli, E.; Titta, L.; Giorgio, M.; Mock, H.-P.; Matros, A.; Peterek, S.; Schijlen, E. G. W. M.; Hall, R. D.; Bovy, A. G.; Luo, J.; Martin, C., Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. **2008,** *26*, 1301-1308.

39. Li, H.; Deng, Z.; Liu, R.; Young, J. C.; Zhu, H.; Loewen, S.; Tsao, R., Characterization of Phytochemicals and Antioxidant Activities of a Purple Tomato (Solanum lycopersicum L.). *Journal of Agricultural and Food Chemistry* **2011,** *59*, 11803-11811.

40. Luthria, D. L.; Mukhopadhyay, S.; Krizek, D. T., Content of total phenolics and phenolic acids in tomato (Lycopersicon esculentum Mill.) fruits as influenced by cultivar and solar UV radiation. *Journal of Food Composition and Analysis* **2006,** *19*, 771-777.

41. Lewinsohn, E.; Sitrit, Y.; Bar, E.; Azulay, Y.; Meir, A.; Zamir, D.; Tadmor, Y., Carotenoid Pigmentation Affects the Volatile Composition of Tomato and Watermelon Fruits, As Revealed by Comparative Genetic Analyses. *Journal of Agricultural and Food Chemistry* **2005,** *53*, 3142-3148.

42. Rubio-Diaz, D. E.; Santos, A.; Francis, D. M.; Rodriguez-Saona, L. E., Carotenoid Stability during Production and Storage of Tomato Juice Made from Tomatoes with Diverse Pigment Profiles Measured by Infrared Spectroscopy. *Journal of Agricultural and Food Chemistry* **2010,** *58*, 8692-8698.

43. Capanoglu, E.; Beekwilder, J.; Boyacioglu, D.; Hall, R.; de Vos, R., Changes in Antioxidant and Metabolite Profiles during Production of Tomato Paste. *Journal of Agricultural and Food Chemistry* **2008,** *56*, 964-973.

44. Abushita, A. A.; Daood, H. G.; Biacs, P. A., Change in Carotenoids and Antioxidant Vitamins in Tomato as a Function of Varietal and Technological Factors. *Journal of Agricultural and Food Chemistry* **2000,** *48*, 2075-2081.

45. Cook, D.; Grierson, D.; Jones, C.; Wallace, A.; West, G.; Tucker, G., Modification of fatty acid composition in tomato (Lycopersicon esculentum) by expression of a borage Δ^6-desaturase. *Molecular Biotechnology* **2002,** *21*, 123-128.

46. Czapski, J.; Horbowicz, M.; Saniewski, M., The effect of methyl jasmonate on free fatty acids content in ripening tomato fruits. *Biologia Plantarum* **1992,** *34*, 71-76.

47. Fang, X.-K.; Gao, J.; Zhu, D.-N., Kaempferol and quercetin isolated from Euonymus alatus improve glucose uptake of 3T3-L1 cells without adipogenesis activity. *Life Sciences* **2008,** *82*, 615-622.

48. Goto, T.; Takahashi, N.; Hirai, S.; Kawada, T., Various Terpenoids Derived from Herbal and Dietary Plants Function as PPAR Modulators and Regulate Carbohydrate and Lipid Metabolism. *PPAR Research* **2010,** *2010*.

49. Zoechling, A.; Liebner, F.; Jungbauer, A., Red wine: A source of potent ligands for peroxisome proliferator-activated receptor [gamma]. *Food & Function* **2011,** *2*, 28-38.

50. Horiba, T.; Nishimura, I.; Nakai, Y.; Abe, K.; Sato, R., Naringenin chalcone improves adipocyte functions by enhancing adiponectin production. *Molecular and Cellular Endocrinology* **2010,** *323*, 208-214.

51. Liu, L.; Shan, S.; Zhang, K.; Ning, Z.-Q.; Lu, X.-P.; Cheng, Y.-Y., Naringenin and hesperetin, two flavonoids derived from Citrus aurantium up-regulate transcription of adiponectin. *Phytother. Res.* **2008,** *22*, 1400-1403.

52. Chou, Y.-C.; Prakash, E.; Huang, C.-F.; Lien, T.-W.; Chen, X.; Su, I.-J.; Chao, Y.-S.; Hsieh, H.-P.; Hsu, J. T.-A., Bioassay-guided purification and identification of PPARα/γ agonists from Chlorella sorokiniana. *Phytother. Res.* **2008,** *22*, 605-613.

53. Day, A. J.; DuPont, M. S.; Ridley, S.; Rhodes, M.; Rhodes, M. J. C.; Morgan, M. R. A.; Williamson, G., Deglycosylation of flavonoid and isoflavonoid glycosides by human small

intestine and liver β-glucosidase activity. *FEBS Letters* **1998,** *436*, 71-75.

54. Rechner, A. R.; Smith, M. A.; Kuhnle, G.; Gibson, G. R.; Debnam, E. S.; Srai, S. K. S.; Moore, K. P.; Rice-Evans, C. A., Colonic metabolism of dietary polyphenols: influence of structure on microbial fermentation products. *Free Radical Biology and Medicine* **2004,** *36*, 212-225.
55. Paetau, I.; Khachik, F.; Brown, E.; Beecher, G.; Kramer, T.; Chittams, J.; Clevidence, B., Chronic ingestion of lycopene-rich tomato juice or lycopene supplements significantly increases plasma concentrations of lycopene and related tomato carotenoids in humans. *The American Journal of Clinical Nutrition* **1998,** *68*, 1187-1195.
56. Scalbert, A.; Williamson, G., Dietary Intake and Bioavailability of Polyphenols. *The Journal of Nutrition* **2000,** *130*, 2073S-2085S.
57. Scott, K. J.; Thurnham, D. I.; Hart, D. J.; Bingham, S. A.; Day, K., The correlation between the intake of lutein, lycopene and β-carotene from vegetables and fruits, and blood plasma concentrations in a group of women aged 50-65 years in the UK. *British Journal of Nutrition* **1996,** *75*, 409-418.
58. Thornquist, M. D.; Kristal, A. R.; Patterson, R. E.; Neuhouser, M. L.; Rock, C. L.; Neumark-Sztainer, D.; Cheskin, L. J., Olestra Consumption Does Not Predict Serum Concentrations of Carotenoids and Fat-Soluble Vitamins in Free-Living Humans: Early Results from the Sentinel Site of the Olestra Post-Marketing Surveillance Study. *The Journal of Nutrition* **2000,** *130*, 1711-1718.
59. Liu, R. H., Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. *The American Journal of Clinical Nutrition* **2003,** *78*, 517S-520S.
60. Stahl, W.; Schwarz, W.; Sundquist, A. R.; Sies, H., cis-trans isomers of lycopene and β-carotene in human serum and tissues. *Archives of Biochemistry and Biophysics* **1992,** *294*, 173-177.
61. Giovannucci, E., Tomatoes, Tomato-Based Products, Lycopene, and Cancer: Review of the Epidemiologic Literature. *Journal of the National Cancer Institute* **1999,** *91*, 317-331.
62. Moller, D. E.; Berger, J. P., Role of PPARs in the regulation of obesity-related insulin sensitivity and inflammation. **2003,** *27*, S17-S21.
63. Subhash, K.; Bose, C.; Agrawal, B., Effect of short term supplementation of tomatoes on antioxidant enzymes and lipid peroxidation in type-II diabetes. *Indian Journal of Clinical Biochemistry* **2007,** *22*, 95-98.
64. Engelhard, Y. N.; Gazer, B.; Paran, E., Natural antioxidants from tomato extract reduce blood pressure in patients with grade-1 hypertension: A double-blind, placebo-controlled pilot study. *American Heart Journal* **2006,** *151*, 100.e6-100.e1.
65. Paran, E.; Novack, V.; Engelhard, Y.; Hazan-Halevy, I., The Effects of Natural Antioxidants from Tomato Extract in Treated but Uncontrolled Hypertensive Patients. *Cardiovascular Drugs and Therapy* **2009,** *23*, 145-151.
66. Yamauchi, T.; Kamon, J.; Waki, H.; Murakami, K.; Motojima, K.; Komeda, K.; Ide, T.; Kubota, N.; Terauchi, Y.; Tobe, K.; Miki, H.; Tsuchida, A.; Akanuma, Y.; Nagai, R.; Kimura, S.; Kadowaki, T., The Mechanisms by Which Both Heterozygous Peroxisome Proliferator-activated Receptor γ (PPARγ) Deficiency and PPARγ Agonist Improve Insulin Resistance. *Journal of Biological Chemistry* **2001,** *276*, 41245-41254.
67. Elbrecht, A.; Chen, Y.; Cullinan, C. A.; Hayes, N.; Leibowitz, M. D.; Moller, D. E.; Berger, J., Molecular Cloning, Expression and Characterization of Human Peroxisome Proliferator Activated Receptors [gamma]1 and [gamma]2. *Biochemical and Biophysical Research Communications* **1996,** *224*, 431-437.

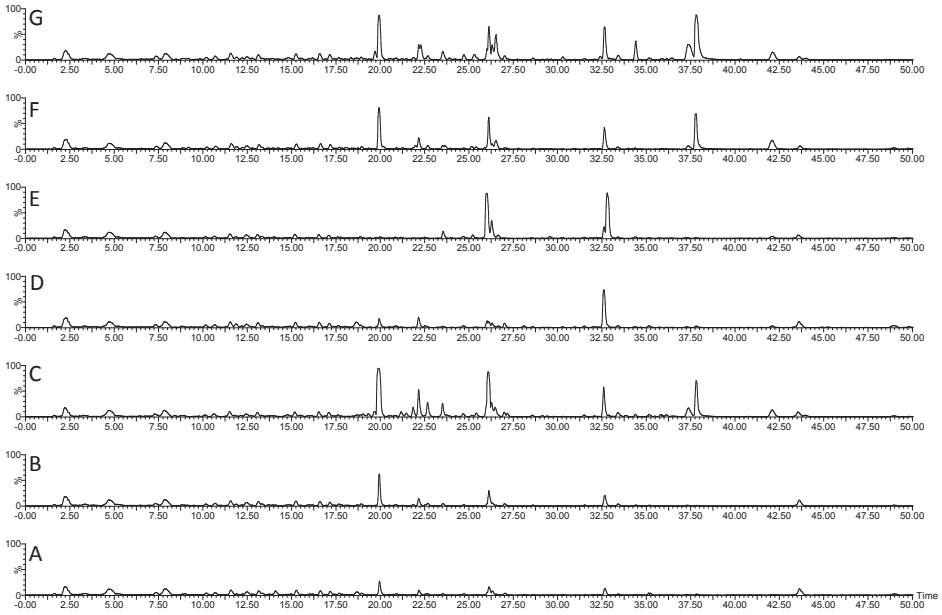

Figure S1. Chromatograms of enzymatically hydrolyzed extracts of tomato varieties, representing the total ion signal obtained from QTOF-MS analysis (with max intensity = 100% of 35000).

5

INDUCTION OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR α (PPARα)-MEDIATED GENE EXPRESSION BY EXTRACTS OF TOMATO

Linda Gijsbers, Hai-Yen Man, Henriëtte D.L.M. van Eekelen, Jaap Keijer, Jac M.M.J.G. Aarts, Ivonne M.C.M. Rietjens, Bart van der Burg

In preparation

ABSTRACT

In the present study, a stable reporter gene assay for detection of PPARα activation was developed and characterized. This assay, designated PPARα CALUX assay, showed a concentration-dependent increase of PPARα-mediated luciferase expression upon exposure to the PPARα agonists GW7647, GW9578, WY14643 and bezafibrate. The PPARα CALUX assay was further used to test extracts of tomato fruit and phytochemicals present in tomato for their ability to induce PPARα-mediated gene expression. An isoprenoid-containing extract of tomato was able to induce PPARα-mediated gene expression, which is consistent with our findings that β-carotene, γ-carotene and lycopene also induce PPARα-mediated gene expression. In addition, it was shown that an extract of tomato containing semi-polar compounds is only a weak inducer of PPARα-mediated gene expression, but enzymatic hydrolysis of the tomato homogenate before extraction greatly increases this capacity. This is in line with our findings that all tested flavonoid glycosides failed to induce PPARα-mediated gene expression, but that the aglycones kaempferol and naringenin were able to induce PPARα-mediated gene expression. Furthermore, nine tomato varieties were tested for their capacity to induce PPARα-mediated gene expression, and the varieties showing the highest capacity were the ones containing the highest amounts of kaempferol and naringenin. Although this suggests that naringenin and kaempferol contribute to the induction of PPARα-mediated gene expression found with the enzymatically hydrolyzed tomato extracts, kaempferol and naringenin cannot fully explain the high (up to 12-fold) induction found with these tomato varieties. Presumably there are more, yet unidentified PPARα-activating compounds present in tomato. Taken together the data presented indicate that the beneficial effects associated with tomato consumption may partly be explained by induction of PPARα-mediated transcription by tomato compounds.

KEYWORDS

Peroxisome proliferator-activated receptor alpha (PPARα); polyphenols; isoprenoids; tomato; stable reporter gene assay.

INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear receptor family (*1, 2*). Three PPAR isoforms are known: PPARα (NR1C1), PPARβ/δ (NR1C2) and PPARγ (NR1C3)(*3*). Once activated by ligands, PPARs influence the expression of their target genes, thereby influencing important physiological processes. For example, PPARγ is well-known for its role in adipogenesis (*4, 5*) while PPARβ/δ has more pleiotropic effects and is involved in for example lipid metabolism, inflammation and wound healing (*6-10*). PPARα is known for its role in lipid metabolism (*11, 12*): it modulates the expression of genes involved in cellular uptake and β-oxidation of fatty acids (*11, 13, 14*). PPARα is mainly expressed in liver, skeletal muscle and brown adipose tissue (*2*) and is activated by both endogenous and exogenous ligands. Endogenous ligands include several fatty acids and their derivatives, like for example arachidonic acid, docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), linolenic acid, linoleic acid and eicosanoids (*15, 16*). Exogenous ligands for PPARα are for example fibrates, a class of lipid-lowering drugs including gemfibrozil, clofibrate, fenofibrate, bezafibrate, and ciprofibrate (*16*).

Activation of PPARα by its ligands has been related to several beneficial health effects. Fibrates, which are potent PPARα agonists, are known to improve risk factors for cardiovascular disease (CVD): fibrates decrease plasma levels of triglycerides and LDL cholesterol, and increase HDL cholesterol (*17, 18*). In addition, PPARα activation has been related to a decreased risk on development of atherosclerosis by improving risk factors (*17-20*). Furthermore, activation of PPARα has been related to slower progression of atherosclerosis by inhibition of leukocyte infiltration, reduction of foam cells formation and enhanced plaque stability (*21, 22*). PPARα activation has also been related to improved insulin sensitivity (*18, 23, 24*).

Beneficial health effects related to PPARα activation partially overlap with those related to the consumption of tomato; epidemiological studies indicate that consumption of tomato or tomato products is related to a reduced risk of several chronic diseases, including CVD, prostate cancer and several other cancer types (*25-28*). It is of interest to know whether PPARα activation by tomato extracts and tomato constituents could have a role in explaining these beneficial effects. Therefore, the aim of the present study was to investigate whether tomato extracts and tomato constituents, including flavonoids and carotenoids, are able to induce PPARα-mediated gene expression. In order to test this, a luciferase reporter gene assay to measure PPARα agonism was developed and validated. This cell-based assay was then used to test tomato constituents and extracts of tomato fruit for their ability to activate PPARα-mediated gene expression.

MATERIALS AND METHODS

Materials

GW7647 (CAS no: 265129-71-3) was obtained from Sigma Aldrich (St. Louis, USA), bezafibrate (CAS no: 41859-67-0), WY14643 (CAS no: 50892-23-4), GW9578 (CAS no: 247923-29-1), rosiglitazone (CAS no: 122320-73-4), pioglitazone (CAS no: 111025-46-8), L165041 (CAS no:

79558-091) and GW501516 (CAS no: 317318-70-0) were purchased from Cayman Chemical (Ann Arbor, USA). Lycopene, lutein, kaempferol, kaempferol-3-O-rutinoside, cyanidin chloride and delphinidin chloride were purchased from Extrasynthase (Genay, France). Phytoene, phytofluene, neoxanthin, violaxanthin, neurosporene, γ-carotene and α-carotene were obtained from Carotenature (Lupsingen, Switzerland). Quercetin, chlorogenic acid, ferulic acid, β-carotene, α-tocopherol, γ-tocopherol, and δ-tocopherol were obtained from Sigma Aldrich (St. Louis, USA). Caffeic acid and rutin were purchased from Acros (Geel, Belgium) and naringenin from ICN Biomedicals (Irvin, USA). The carotenoids were dissolved in tetrahydrofuran (THF; VWR Prolabo, Radnor, USA), all other compounds were dissolved in dimethylsulphoxide (DMSO, 99.9%, Acros, Geel, Belgium).

Development of the PPARα CALUX cell line

Human U2OS osteosarcoma cells (obtained from the American Type Culture Collection (ATCC), Manassas, USA) were stably transfected as described before (*29*), using reporter construct 3xPPRE-tata-luc (*30*) and expression vector pSG5-neo-PPARα expressing human PPARα. The PPARα gene was excised from pCMV6-XL4-PPARα (purchased from Origene, Rockville, USA) and inserted into the unique NOTI site in the multiple cloning site of pSG5-neo, which contains the neomycin resistance gene (*29*). Stable transfection was carried out using a calcium phosphate co-precipitation method, as described before (*31*). In short, after transfection G418-disulfate (CAS no: 108321-42-2, DuchefaBiochemie, Haarlem, The Netherlands) was added to the culture medium (final concentration 150 µg/ml) to maintain selection pressure and all G418-resistant clones were tested for their responsiveness to the well-known PPARα agonist GW7674 (*32*). The responsive clones were tested to generate full dose-response curves and the clone showing the best response was selected and used for further validation.

Cell culture

Stably transfected PPARα CALUX cells were cultured in culture medium: DMEM/F12 glutamax medium (Invitrogen, Breda, The Netherlands) supplemented with 7.5% fetal calf serum (FCS, Invitrogen), non-essential amino acids (NEAA, Invitrogen) and penicillin/streptomycin (Invitrogen)(final concentrations 10 U/ml and 10 µg/ml, respectively). Once per week 200 µg/ml G418 was added to the culture medium, in order to maintain the selection pressure. Cells were cultured at 37°C and 5% CO_2 in a humid atmosphere.

Tomato Extracts

Roma tomatoes, cherry tomatoes and cocktail tomatoes were purchased at a local supermarket in Wageningen (The Netherlands). For each variety, several tomato fruits were snap-frozen in liquid nitrogen. Next, the frozen tomato was ground to powder using an analytical mill (IKA, Staufen, Germany) and stored at -80°C until further use. Prior to extract preparation, the frozen powders were pooled in order to get a mix of roma tomatoes, cherry tomatoes and cocktail tomatoes, further referred to as tomato mix. Three different extracts were generated from this tomato mix: one extract containing isoprenoids and two extracts containing semi-polar compounds.

Isoprenoids, including carotenoids and tocopherols, were extracted from the tomato mix by a chloroform extraction method described before (*33*). This resulted in an extract further referred to as isoprenoid-containing tomato extract.

The two extracts containing semi-polar compounds were generated. The first extract containing semi-polar compounds was prepared by methanol extraction using a method described before (*33*). This extract contains for example flavonoid glycosides and other polyphenols, and is further referred to as nonhydrolyzed tomato extract. For the second extract containing semi-polar compounds, tomato mix was treated with Viscozyme L for two hours prior to the methanol extraction. The Viscozyme L treatment was performed in order to remove glycosyl groups from glycosylated ingredients. Viscozyme L treatment and subsequent methanol extraction were performed as previously described (*33*). The resulting extract contains for example flavonoid aglycones and is further referred to as enzymatically hydrolyzed tomato extract.

In addition to the tomato mix, nine different tomato varieties were tested for their capacity to induce PPARα-mediated gene expression. These varieties include two high pigment tomato varieties (varieties 7 and 8) and were kindly provided by Syngenta (Enkhuizen, The Netherlands). Enzymatically hydrolyzed extracts of these nine tomato varieties were prepared using the method described above. Furthermore, chemical analysis of these extracts was performed using LC-MS as described below.

Chemical analysis using LC-PDA-QTOF-MS

The LC-PDA-QTOF-MS method combines four techniques: liquid chromatography (LC), photodiode array (PDA), quadrupole time of flight (QTOF) and mass spectrometry (MS). Semi-polar compounds in the sample can be separated with LC and detected with PDA. Then, the compounds are ionized and masses are detected using QTOF and MS. As individual compounds can be identified based on their retention time and mass, the LC-PDA-QTOF-MS method provides insight in the semi-polar compounds present in the sample. LC-PDA-QTOF-MS analysis was performed as described before by De Vos and colleagues (*34*). In short, 5 µl of enzymatically hydrolyzed tomato extracts were injected onto a C18 column. Separation was performed using ultrapure water (eluent A) and acetonitrile (eluent B), both acidified with 0.1 % formic acid, and a flow rate of 190 µl/min. A linear gradient was applied increasing eluent B from 5% to 35% in 45 minutes. Before the next injection, the column was washed and equilibrated for 15 minutes. After separation and detection of the semipolar compounds by LC-PDA, positive electrospray ionization was applied and masses were detected using QTOF-MS. Chromatograms obtained in this way were analyzed using MassLynx 4.1 (Waters) software.

Exposure experiments

PPARα CALUX cells were plated in 96-well plates at a density of 10,000 cells per well using 100 µl assay medium: phenol red-free DMEM/F12 medium (Invitrogen) supplemented with 5% dextran-coated charcoal-stripped FCS, NEAA and penicillin/streptomycin (final concentrations 10 U/ml and 10 µg/ml, respectively). The next day, the medium was replaced by 200 µl fresh assay medium supplemented with the test compounds or

tomato extracts. The percentage of solvent in the exposure medium was kept at 0.5% for experiments with individual compounds and at or below 1.0% for experiments with tomato extracts. After 24 hours, the exposure medium was removed and the cells were lysed. Luciferase activity in the lysate was measured using a luminometer (Berthold LB941, Bad Widbad, Germany), as described before (*29*). Experiments with isoprenoids and the isoprenoid-containing tomato extract were performed in the dark.

All extracts of tomato and all individual compounds were tested for cytotoxicity using the Cytotox CALUX cell line as described before (*35*). The Cytotox CALUX cells show an invariant luciferase expression and a decrease in luciferase activity therefore indicates a cytotoxic effect. Moreover, an increase in luciferase activity in the Cytotox CALUX cells may indicate stabilization of the luciferase enzyme and possible false positives for gene expression in the PPARα CALUX assay (*33*). All individual tomato compounds and tomato extracts were tested for cytotoxicity using the Cytotox CALUX cells and only non-cytotoxic concentrations were used for testing in the PPARα CALUX cell line. Furthermore, the Cytotox CALUX cells were used to investigate whether stabilization of the luciferase enzyme was occurring during the exposure to tomato compounds and tomato extracts.

Data analysis

Luciferase activity per well was measured as relative light units (RLUs). Induction factors were calculated using the following formula: RLU for exposed well / average RLU of the solvent control. For each test compound, at least two independent experiments were performed. Data are presented as mean values ± standard error of the mean (SEM). Statistical significance was assessed using the one-sided Student's t-test and a cut-off value of $p \leq 0.05$.

RESULTS

Characterization and validation of the PPARα CALUX cell line

PPARα CALUX cells were exposed to four known PPARα agonists: GW7647, GW9578, WY14643, and bezafibrate. Upon exposure to these compounds, PPARα-mediated luciferase expression was induced (figure 1A). GW7647 had an EC_{50} value of 4.3 nM and GW9578 had an EC_{50} of 0.23 µM. For WY14643 and bezafibrate, no complete dose-response curves were generated in the concentration range that could be tested without causing cytotoxicity, and therefore EC_{50} values could not be determined. The concentrations at which the tested agonists start to induce PPARα-mediated luciferase expression and EC_{50} values found for GW7647 and GW9578, are in line with data from other reporter gene assays reported in literature (*32, 36, 37*). GW7647 was the most potent of the tested agonists asreflected by the lowest EC_{50} value, followed by GW9578, and then by WY14643 and bezafibrate which have comparable potencies. This potency ranking is consistent with rankings reported in literature (*32, 38*).

In order to verify that the PPARα CALUX cells specifically respond to PPARα activation and not to activation via PPARβ/δ or PPARγ, the PPARα CALUX cells were

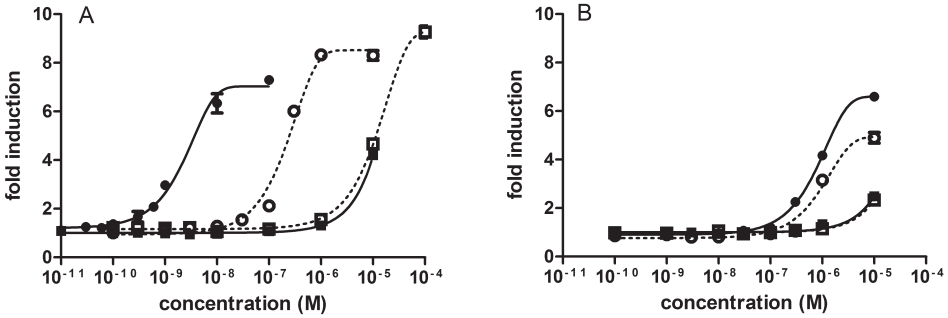

Figure 1. Luciferase activity is induced in the PPARα CALUX assay upon exposure to A) PPARα agonists GW7647 (●), GW9578 (○), WY14643 (■) and bezafibrate (□); and B) PPARβ/δ agonists GW501516 (●) and L165041 (○), and PPARγ agonists rosiglitazone (■) and pioglitazone (□).

exposed to two agonists selective for PPARγ (rosiglitazone and pioglitazone (*39*)) and to two agonists selective for PPARβ/δ (L165041 and GW501516 (*40*)). PPARα CALUX cells showed increased luciferase expression upon exposure to the PPARγ agonists rosiglitazone and pioglitazone, but only in concentrations higher than 1 µM (figure 1B). This is higher than their concentrations needed for PPARγ agonist activity (two orders of magnitude for rosiglitazone and one order of magnitude for pioglitazone), which underlines that these responses cannot be mediated by PPARγ. It has been shown before that rosiglitazone and pioglitazone activate PPARα at relatively high concentrations (>1µM)(*16, 40, 41*). Luciferase induction in the PPARα CALUX cells was also found upon exposure to the PPARβ/δ agonists L165041 and GW501516 (figure 1B). This is consistent with data in literature showing that L165041 and GW501516 activate PPARα at concentrations of 1 µM and higher (*40, 42*), which is higher than their concentrations needed for PPARβ/δ agonist activity (two orders of magnitude for L165041 and three orders of magnitude for GW501516), which underlines that these responses cannot be mediated by PPARβ/δ.

Induction of PPARα-mediated gene expression by tomato extracts

Next, extracts of tomato fruit were tested for their ability to induce PPARα-mediated gene expression. To this end, PPARα CALUX cells were exposed for 24 hours to the isoprenoid-containing tomato extract, the nonhydrolyzed tomato extract and the enzymatically hydrolyzed tomato extract. The isoprenoid-containing tomato extract showed an induction in PPARα-mediated luciferase expression up to 2.1 fold at 45 g FW/L (figure 2). The nonhydrolyzed tomato extract, containing semi-polar compounds including flavonoid glycosides, only showed significant induction (1.4 fold) of PPARα-mediated luciferase at the highest concentration tested (figure 2). The enzymatically hydrolyzed extract, in which flavonoid glycosides were hydrolyzed into flavonoid aglycones, showed significant induction of PPARα-mediated luciferase even at 5 g FW/L (2.4 fold) and reached an induction of 9.3 fold at 45 g FW/L (figure 2).

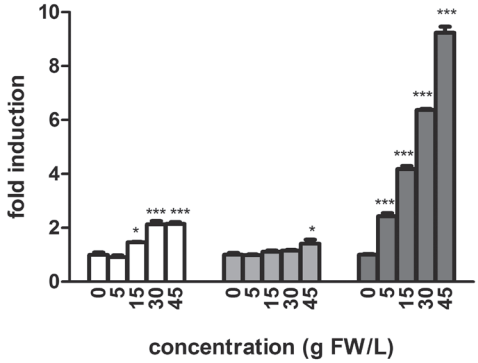

Figure 2. Luciferase activity induced in the PPARα CALUX assay upon exposure to tomato extracts: isoprenoid-containing extract (white bars), nonhydrolyzed tomato extract (light grey bars) and enzymatically hydrolyzed extract (dark grey bars). The concentration of tomato extract is given as gram fresh weight (FW) per liter. Asterisks indicate the level of significance compared to the solvent control: * $p<0.05$; ** $p<0.01$; and *** $p<0.001$.

The PPARα-mediated luciferase induction found with the enzymatically hydrolyzed tomato extract at 45 g FW/L (9.3-fold) is much higher than with the isoprenoid-containing tomato extract (2.1-fold). This may suggest that in tomato, semi-polar compounds including polyphenols are more important than isoprenoids in activating PPARα.

Induction of PPARα-mediated gene expression by tomato compounds

To assess which tomato constituents may contribute to the induction of PPARα-mediated gene expression by tomato extracts, 23 compounds known to be present in tomato (table 1) were tested for their capacity to induce PPARα-mediated gene expression. Of the 10 polyphenols tested, only naringenin and kaempferol were able to induce PPARα-mediated gene expression (table 1), but only at concentrations higher than 1 µM (data not shown). Our results are in line with data reported in literature showing that naringenin is able to activate PPARα at concentrations in the micromolar range (*43, 44*).

Of the 10 carotenoids investigated, lycopene, β-carotene and γ-carotene were able to induce PPARα-mediated gene expression, while the three tested tocopherols were not able to do so (table 1). These data indicate that lycopene, β-carotene and γ-carotene might contribute to the induction of PPARα-mediated gene expression by the isoprenoid-containing tomato extract.

The induction of PPARα-mediated luciferase expression with kaempferol, naringenin, lycopene, β-carotene and γ-carotene at a concentration of 17 µM is comparable with the induction found with 1 µM bezafibrate.

Induction of PPARα-mediated gene expression by various tomato varieties

Besides extracts of a mix of roma, cherry and cocktail tomatoes, enzymatically hydrolyzed extracts of nine tomato varieties, designated 1-9, were tested for their potency to induce PPARα-mediated gene expression using the PPARα CALUX cells. These nine varieties include

Table 1. Luciferase expression induced by tomato compounds in the PPARα CALUX assay.

Compound	Reference(s) for presence in tomato	Fold induction at 17 µM
Polyphenolic compounds		
naringenin	(*45-47*)	1.3
kaempferol	(*45, 46*)	1.5
kaempferol-3-O-rutinoside	(*45*)	inactive
quercetin	(*46, 48*)	inactive
rutin	(*45, 48*)	inactive
delphinidin[a]	(*49*)	inactive
cyanidin[a]	(*50*)	inactive
chlorogenic acid	(*45, 47*)	inactive
ferulic acid	(*47, 51*)	inactive
caffeic acid	(*47, 51*)	inactive
Carotenoids		
lutein	(*48, 52*)	inactive
neoxanthin	(*48, 52*)	inactive
violaxanthin	(*48, 52*)	inactive
phytoene	(*52, 53*)	inactive
phytofluene	(*52, 53*)	inactive
neurosporene	(*52, 53*)	inactive
lycopene	(*52, 53*)	1.6
β-carotene	(*48, 52*)	1.7
γ-carotene	(*52*)	1.8
δ-carotene*	(*54, 55*)	Inactive
Tocopherols		
α-tocopherol	(*48, 56*)	inactive
β-tocopherol	(*56, 57*)	inactive
δ-tocopherol	(*48, 56*)	inactive

[a]Although these compounds are not found in conventional tomato, they are found in special varieties like purple tomato (delphinidin and cyanidin) and Delta tomato (δ-carotene)(49, 50, 54, 55).

two high pigment varieties (7 and 8). Enzymatically hydrolyzed extracts of these nine tomato varieties were prepared and tested for their potency to induce PPARα-mediated gene expression using the PPARα CALUX cells. Varieties 8 and 9 showed cytotoxicity at 45 g FW/L and these varieties were therefore not included in further analysis. The seven varieties not showing cytotoxicity at this concentration were all able to significantly induce PPARα-mediated gene expression at 45 g FW/L and fold induction ranged from 7.5-fold (variety 1) to 12.8-fold (variety 7)(figure 3A). This means that there was a 1.7-fold difference in induction between the least potent variety and the most potent variety. Using the Cytotox CALUX cell line, it was shown that the tomato varieties had hardly any effect on the luciferase activity measured in those cells (figure 3B). This indicates that the induction of luciferase activity upon exposure of the PPARα CALUX cells to tomato extracts is not due to stabilization of the luciferase enzyme.

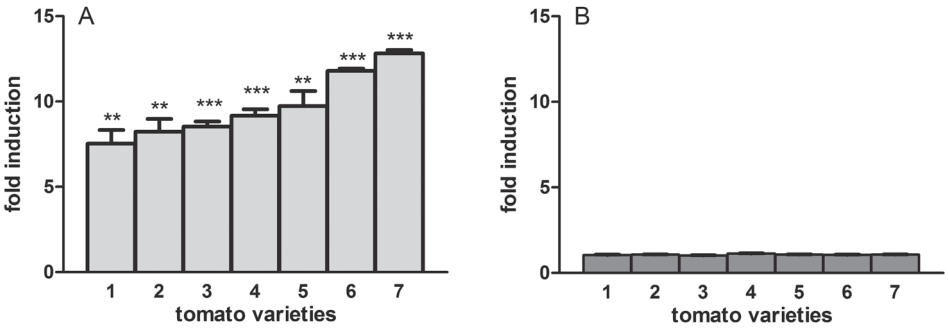

Figure 3. Luciferase activity is induced in the PPARα CALUX assay (A) but not in the Cytotox CALUX assay (B) upon exposure to enzymatically hydrolyzed extracts of seven tomato varieties at 45 g FW/L. Variety 7 is a high-pigment tomato variety. Asterisks indicate the level of significance compared to the solvent control: * $p<0.05$; ** $p<0.01$; and *** $p<0.001$.

The extracts of tomato varieties 1-7 were analyzed using LC-PDA-QTOF-MS and the levels of kaempferol and naringenin in these extracts were quantified (table 2). It is interesting to note that the most potent varieties (5, 6 and 7) had the highest content of kaempferol and naringenin. Altogether, these data suggest that kaempferol and naringenin present in tomato may contribute to potency differences between the tomato varieties.

Table 2. Levels of kaempferol and naringenin found in enzymatically hydrolyzed extracts of seven tomato varieties.

Tomato variety	Kaempferol (μM)[a]	Naringenin (μM)[a]
1	0.02	1.59
2	0.07	0.59
3	0.03	1.30
4	0.04	0.004
5	0.31	13.19
6	1.13	7.76
7[b]	1.33	10.56

[a]Kaempferol and naringenin present in tomato extract diluted to 45 g FW/L, which is the concentration at which the extracts were tested in the PPARα CALUX assay.
[b]High pigment variety.

DISCUSSION

In the present study, a stable reporter gene assay for activation of PPARα was developed and characterized. This PPARα CALUX assay enables fast and low-cost screening of synthetic and natural compounds for their potency to induce PPARα-mediated gene expression. This reporter gene assay was based on the U2OS cell line, a cell line of human

origin with low endogenous expression of the three PPAR subtypes (*58, 59*). By stably transfecting the U2OS cells with an expression vector for human PPARα, a cell line was generated which is specific for PPARα-mediated gene expression. This was supported by the results obtained with GW7647, GW9578, WY14643 and bezafibrate: induction of PPARα-mediated luciferase expression was seen with all four PPARα agonists. In order to verify that the PPARα CALUX cells specifically respond to PPARα agonism, the PPARα CALUX cells were exposed to rosiglitazone and pioglitazone (PPARγ agonists) and to L165041 and GW501516 (PPARβ/δ agonists). The PPARα CALUX cells showed increased luciferase expression upon exposure to the PPARγ agonists as well, but only at concentrations much higher than needed for PPARγ activation. This underlines that these responses cannot be mediated by PPARγ. The same holds for the response to the PPARβ/δ agonists: they induce luciferase expression at concentrations much higher than their concentrations needed for PPARβ/δ agonist activity, which underlines that the responses observed in the PPARα CALUX cells cannot be mediated by PPARβ/δ.

The present results indicate that tomato is able to induce PPARα-mediated gene expression. This is in line with an earlier report in which a tomato extract was fractionated using HPLC, with several of the generated fractions being able to induce PPARα-mediated gene expression (*60*). Recent studies towards PPARα-activating effects of tomato and of food compounds mainly used chimera systems combining the ligand binding domain of PPARα with the DNA binding domain of GAL4 (*43, 60, 61*). These chimera systems therefore mainly reflect binding to the ligand binding domain of PPARα, which does not necessarily lead to activation of PPARα and subsequent PPARα-mediated effects on gene expression. The PPARα CALUX cells express the complete human PPARα receptor and detect PARα-mediated gene expression, and may therefore be closer to physiological effects on gene expression.

The newly developed PPARα CALUX cell line was applied to test extracts of tomato fruit and phytochemicals present in tomato for their ability to induce PPARα. It was shown that both tomato extracts and phytochemicals present in tomato are able to induce PPARα-mediated changes in gene expression. Our data indicate that an isoprenoid-containing extract of tomato is able to induce PPARα-mediated gene expression, which is consistent with our findings that β-carotene, γ-carotene and lycopene are able to induce PPARα-mediated gene expression as well. To our knowledge, this is the first time that these carotenoids are reported to activate PPARα, but it is consistent with the recently reported finding that another carotenoid, astaxanthin, which is present in for example sea food, is able to activate PPARα (*62*). Besides activating PPARα, β-carotene, γ-carotene and lycopene were also able to activate PPARγ-mediated gene expression (*63*). It would be of interest to see whether these compounds also activate PPARβ/δ and/or the dimerization partner of PPARs, RXR.

In addition, it was shown that an extract of tomato containing semi-polar compounds is only a weak inducer of PPARα-mediated gene expression, but that enzymatic hydrolysis of the tomato sample before extraction greatly increases this capacity. This is in line with our findings that none of the flavonoid glycosides was able to induce PPARα-mediated gene expression, but that the aglycones kaempferol and naringenin were. Furthermore,

of the seven tomato varieties tested for their capacity to induce PPARα-mediated gene expression without showing cytotoxicity, the varieties showing the highest potency are also the ones containing the highest amounts of kaempferol and naringenin. These results together suggest that kaempferol and naringenin contribute to the induction of PPARα-mediated gene expression found with the enzymatically hydrolyzed tomato extracts, and that differences in the levels of kaempferol and naringenin may contribute to the differences in PPARα-activating capacity of the tomato varieties. The amounts of kaempferol and naringenin, however, cannot fully explain the high induction found with the enzymatically hydrolyzed tomato extracts: variety 7 contains approximately 1.5 µM kaempferol and 10 µM naringenin (table 2). When these phytochemicals were tested individually, these concentrations gave about 1.1-fold induction each, while the enzymatically hydrolyzed extract of tomato variety 7 gave an induction of 12.8 fold (figure 3). This might point at an additive or even synergistic effect. Additive effects in mixtures of phytochemicals have been reported before (*64*). It also might point to other phytochemicals present in enzymatically hydrolyzed extracts of tomato fruit which may be able to induce PPARα-mediated gene expression. For example, it has been shown before that fatty acids and fatty acid derivatives like eicosanoids, which are also present in tomato fruits, are able to induce PPARα-mediated gene expression (*15, 16, 60, 61*). Furthermore, it was recently shown that 9-oxo-10(E),12(E)-octadecadienoic acid (9-oxo-ODA) from tomato and 13-oxo-9,11-octadecadienoic acid (13-oxo-ODA) from tomato juice are potent PPARα activators (*60, 61*). In addition, the tomato extracts may also contain agonists for RXR – the dimerization partner for PPARγ – which may lead to an additive or even synergistic effect in activation of the PPARα-RXR complex.

It was shown here that PPARα CALUX cells can be used to investigate differences in PPARα-activating capacity between tomato varieties. This implies that the PPARα CALUX cells can be used to select tomato varieties showing the highest capacity to induce PPARα-mediated gene expression. Although the present results indicate that tomato and phytochemicals present in tomato are able to induce PPARα-mediated gene expression, *in vivo* experiments are needed to confirm that PPARα-activation by tomato and its phytochemicals also leads to beneficial health effects *in vivo*. *In vivo* data from literature indicate that tomato consumption leads to decreased blood pressure, decreased total cholesterol and LDL cholesterol levels and decreased myocardial lesions after myocardial infarctions (*65-68*), but it remains to be elucidated to what extent these effects induced by tomato are PPARα-dependent.

ACKNOWLEDGEMENTS

This work was financially supported by the Food and Nutrition Delta (grant number FND07007). The PPARα CALUX assay was developed with financial support of the EU FP7 project PlantLibra (grant number 245199). The authors want to cordially thank Syngenta for providing the nine tomato varieties.

REFERENCES

1. Berger, J.; Moller, D. E., The mechanisms of action of PPARs. *Annual Review of Medicine* **2002,** *53*, 409-435.
2. Issemann, I.; Green, S., Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature* **1990,** *347*, 645-650.
3. Michalik, L.; Wahli, W., Peroxisome proliferator-activated receptors: three isotypes for a multitude of functions. *Current Opinion in Biotechnology* **1999,** *10*, 564-570.
4. Barak, Y.; Nelson, M. C.; Ong, E. S.; Jones, Y. Z.; Ruiz-Lozano, P.; Chien, K. R.; Koder, A.; Evans, R. M., PPAR[gamma] Is Required for Placental, Cardiac, and Adipose Tissue Development. *Molecular Cell* **1999,** *4*, 585-595.
5. Tontonoz, P.; Hu, E.; Spiegelman, B. M., Stimulation of adipogenesis in fibroblasts by PPARγ2, a lipid-activated transcription factor. *Cell* **1994,** *79*, 1147-1156.
6. Bishop-Bailey, D.; Bystrom, J., Emerging roles of peroxisome proliferator-activated receptor-β/δ in inflammation. *Pharmacology & Therapeutics* **2009,** *124*, 141-150.
7. Grimaldi, P. A., Regulatory functions of PPARβ in metabolism: implications for the treatment of metabolic syndrome. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **2007,** *1771*, 983-990.
8. Michalik, L.; Desvergne, B.; Tan, N. S.; Basu-Modak, S.; Escher, P.; Rieusset, J.; Peters, J. M.; Kaya, G.; Gonzalez, F. J.; Zakany, J.; Metzger, D.; Chambon, P.; Duboule, D.; Wahli, W., Impaired skin wound healing in peroxisome proliferator–activated receptor (PPAR)α and PPARβ mutant mice. *The Journal of Cell Biology* **2001,** *154*, 799-814.
9. Michalik, L.; Desvergne, B.; Wahli, W., Peroxisome proliferator-activated receptors [beta]/[delta]: emerging roles for a previously neglected third family member. *Current Opinion in Lipidology* **2003,** *14*, 129-135.
10. Tan, N. S.; Michalik, L.; Di-Poi, N.; Desvergne, B.; Wahli, W., Critical roles of the nuclear receptor PPARbeta (peroxisome-proliferator-activated receptor beta) in skin wound healing. *Biochemical Society transactions* **2004,** *32*, 97-102.
11. Kersten, S.; Seydoux, J.; Peters, J. M.; Gonzalez, F. J.; Desvergne, B.; Wahli, W., Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting. *The Journal of clinical investigation* **1999,** *103*, 1489-1498.
12. Reddy, J. K.; Hashimoto, T., Peroxisomal beta-oxidation and peroxisome proliferator-activated receptor alpha: an adaptive metabolic system. *Annual review of nutrition* **2001,** *21*, 193-230.
13. Aoyama, T.; Peters, J. M.; Iritani, N.; Nakajima, T.; Furihata, K.; Hashimoto, T.; Gonzalez, F. J., Altered Constitutive Expression of Fatty Acid-metabolizing Enzymes in Mice Lacking the Peroxisome Proliferator-activated Receptor α (PPARα). *Journal of Biological Chemistry* **1998,** *273*, 5678-5684.
14. Mandard, S.; Müller, M.; Kersten, S., Peroxisome proliferator-activated receptor a target genes. *Cellular and Molecular Life Sciences* **2004,** *61*, 393-416.
15. Kliewer, S. A.; Sundseth, S. S.; Jones, S. A.; Brown, P. J.; Wisely, G. B.; Koble, C. S.; Devchand, P.; Wahli, W.; Willson, T. M.; Lenhard, J. M.; Lehmann, J. r. M., Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors α and γ. *Proceedings of the National Academy of Sciences of the United States of America* **1997,** *94*, 4318-4323.
16. Krey, G.; Braissant, O.; L'Horset, F.; Kalkhoven, E.; Perroud, M.; Parker, M. G.; Wahli, W., Fatty Acids, Eicosanoids, and Hypolipidemic Agents Identified as Ligands of Peroxisome Proliferator-Activated Receptors by Coactivator-Dependent Receptor Ligand Assay. *Mol Endocrinol* **1997,** *11*, 779-791.
17. Idzior-Walus, B.; Sieradzki, J.; Rostworowski, W.; Zdzienicka, A.; Kawalec, E.; Wójcik, J.; Żarnecki, A.; Blane, G., Effects of comicronised fenofibrate on lipid and insulin sensitivity in patients with polymetabolic syndrome X. *European Journal of Clinical Investigation* **2000,** *30*, 871-878.
18. Koh, K. K.; Han, S. H.; Quon, M. J.; Yeal Ahn, J.; Shin, E. K., Beneficial Effects of Fenofibrate to Improve Endothelial Dysfunction and

Raise Adiponectin Levels in Patients With Primary Hypertriglyceridemia. *Diabetes Care* **2005,** *28*, 1419-1424.

19. Remick, J.; Weintraub, H.; Setton, R.; Offenbacher, J.; Fisher, E.; Schwartzbard, A., Fibrate Therapy: An Update. *Cardiology in Review* **2008,** *16*, 129-141.
20. Ericsson, C. G.; de Faire, U.; Grip, L.; Svane, B.; Hamsten, A.; Nilsson, J., Angiographic assessment of effects of bezafibrate on progression of coronary artery disease in young male postinfarction patients. *The Lancet* **1996,** *347*, 849-853.
21. Fruchart, J.-C., Peroxisome proliferator-activated receptor-alpha (PPARα): At the crossroads of obesity, diabetes and cardiovascular disease. *Atherosclerosis* **2009,** *205*, 1-8.
22. Zandbergen, F.; Plutzky, J., PPARα in atherosclerosis and inflammation. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **2007,** *1771*, 972-982.
23. Guerre-Millo, M.; Gervois, P.; Raspé, E.; Madsen, L.; Poulain, P.; Derudas, B.; Herbert, J.-M.; Winegar, D. A.; Willson, T. M.; Fruchart, J.-C.; Berge, R. K.; Staels, B., Peroxisome Proliferator-activated Receptor α Activators Improve Insulin Sensitivity and Reduce Adiposity. *Journal of Biological Chemistry* **2000,** *275*, 16638-16642.
24. Wägner, A. M.; Sánchez-Quesada, J. L.; Benítez, S.; Bancells, C.; Ordóñez-Llanos, J.; Pérez, A., Effect of statin and fibrate treatment on inflammation in type 2 diabetes. A randomized, cross-over study. *Diabetes Research and Clinical Practice* **2011,** *93*, e25-e28.
25. Blum, A.; Monir, M.; Wirsansky, I.; Ben-Arzi, S., The beneficial effects of tomatoes. *European Journal of Internal Medicine* **2005,** *16*, 402-404.
26. Canene-Adams, K.; Campbell, J. K.; Zaripheh, S.; Jeffery, E. H.; Erdman, J. W., The Tomato As a Functional Food. *The Journal of Nutrition* **2005,** *135*, 1226-1230.
27. Sesso, H. D.; Liu, S.; Gaziano, J. M.; Buring, J. E., Dietary Lycopene, Tomato-Based Food Products and Cardiovascular Disease in Women. *The Journal of Nutrition* **2003,** *133*, 2336-2341.
28. Visioli, F.; Riso, P.; Grande, S.; Galli, C.; Porrini, M., Protective activity of tomato products on in vivo markers of lipid oxidation. *European Journal of Nutrition* **2003,** *42*, 201-206.
29. Sonneveld, E.; Jansen, H. J.; Riteco, J. A. C.; Brouwer, A.; van der Burg, B., Development of Androgen- and Estrogen-Responsive Bioassays, Members of a Panel of Human Cell Line-Based Highly Selective Steroid-Responsive Bioassays. *Toxicol. Sci.* **2005,** *83*, 136-148.
30. Gijsbers, L.; Man, H.-Y.; Kloet, S. K.; de Haan, L. H. J.; Keijer, J.; Rietjens, I. M. C. M.; van der Burg, B.; Aarts, J. M. M. J. G., Stable reporter cell lines for peroxisome proliferator-activated receptor γ (PPARγ)-mediated modulation of gene expression. *Analytical Biochemistry* **2011,** *414*, 77-83.
31. Quaedackers, M. E.; Van Den Brink, C. E.; Wissink, S.; Schreurs, R. H. M. M.; Gustafsson, J.-A.; Van Der Saag, P. T.; Van Der Burg, B., 4-Hydroxytamoxifen Trans-Represses Nuclear Factor-{{kappa}}B Activity in Human Osteoblastic U2-OS Cells through Estrogen Receptor (ER){{alpha}}, and Not through ER{{beta}}. *Endocrinology* **2001,** *142*, 1156-1166.
32. Brown, P. J.; Stuart, L. W.; Hurley, K. P.; Lewis, M. C.; Winegar, D. A.; Wilson, J. G.; Wilkison, W. O.; Ittoop, O. R.; Willson, T. M., Identification of a subtype selective human PPARα agonist through parallel-array synthesis. *Bioorganic & Medicinal Chemistry Letters* **2001,** *11*, 1225-1227.
33. Gijsbers, L.; van Eekelen, H. D. L. M.; Nguyen, T. H.; de Haan, L. H. J.; van der Burg, B.; Aarts, J. M. M. J. G.; Rietjens, I. M. C. M.; Bovy, A. G., Induction of electrophile-responsive element (EpRE)-mediated gene expression by tomato extracts in vitro. *Food Chemistry* **2012,** *135*, 1166-1172.
34. De Vos, R. C. H.; Moco, S.; Lommen, A.; Keurentjes, J. J. B.; Bino, R. J.; Hall, R. D., Untargeted large-scale plant metabolomics using liquid chromatography coupled to mass spectrometry. **2007,** *2*, 778-791.
35. Van der Linden, S. C.; von Bergh, A. R. M.; Van Vugt-Lussenburg, B.; Jonker, L.; Brouwer, A.; Teunis, M.; Krul, C. A. M.; Van der Burg, B., Development of a panel of high throughput reporter gene assays to detect genotoxicity and oxidative stress. **Submitted for publication**.
36. Brown, P. J.; Winegar, D. A.; Plunket, K. D.; Moore, L. B.; Lewis, M. C.; Wilson, J. G.; Sundseth, S. S.; Koble, C. S.; Wu, Z.; Chapman,

J. M.; Lehmann, J. M.; Kliewer, S. A.; Willson, T. M., A ureido-thioisobutyric acid (GW9578) is a subtype-selective PPARalpha agonist with potent lipid-lowering activity. *Journal of medicinal chemistry* **1999,** *42*, 3785-3788.

37. Liu, G.; Moon, T. W.; Metcalfe, C. D.; Lee, L. E. J.; Trudeau, V. L., A teleost in vitro reporter gene assay to screen for agonists of the peroxisome proliferator-activated receptors. *Environmental Toxicology and Chemistry* **2005,** *24*, 2260-2266.
38. Zhou, Z.; Sun, W.; Liang, Y.; Gao, Y.; Kong, W.; Guan, Y.; Feng, J.; Wang, X., Fenofibrate Inhibited the Differentiation of T Helper 17 Cells In Vitro. *PPAR Research* **2012,** *2012*, 10.
39. Willson, T. M.; Brown, P. J.; Sternbach, D. D.; Henke, B. R., The PPARs: from orphan receptors to drug discovery. *Journal of medicinal chemistry* **2000,** *43*, 527-550.
40. Seimandi, M.; Lemaire, G.; Pillon, A.; Perrin, A.; Carlavan, I.; Voegel, J. J.; Vignon, F.; Nicolas, J.-C.; Balaguer, P., Differential responses of PPARα, PPARδ, and PPARγ reporter cell lines to selective PPAR synthetic ligands. *Analytical Biochemistry* **2005,** *344*, 8-15.
41. Sakamoto, J.; Kimura, H.; Moriyama, S.; Odaka, H.; Momose, Y.; Sugiyama, Y.; Sawada, H., Activation of Human Peroxisome Proliferator-Activated Receptor (PPAR) Subtypes by Pioglitazone. *Biochemical and Biophysical Research Communications* **2000,** *278*, 704-711.
42. Oliver, W. R.; Shenk, J. L.; Snaith, M. R.; Russell, C. S.; Plunket, K. D.; Bodkin, N. L.; Lewis, M. C.; Winegar, D. A.; Sznaidman, M. L.; Lambert, M. H.; Xu, H. E.; Sternbach, D. D.; Kliewer, S. A.; Hansen, B. C.; Willson, T. M., A selective peroxisome proliferator-activated receptor δ agonist promotes reverse cholesterol transport. *Proceedings of the National Academy of Sciences* **2001,** *98*, 5306-5311.
43. Goldwasser, J.; Cohen, P. Y.; Yang, E.; Balaguer, P.; Yarmush, M. L.; Nahmias, Y., Transcriptional Regulation of Human and Rat Hepatic Lipid Metabolism by the Grapefruit Flavonoid Naringenin: Role of PPARα, PPARγ and LXRα. *PLoS ONE* **2010,** *5*, e12399.
44. Liu, L.; Shan, S.; Zhang, K.; Ning, Z.-Q.; Lu, X.-P.; Cheng, Y.-Y., Naringenin and hesperetin, two flavonoids derived from Citrus aurantium up-regulate transcription of adiponectin. *Phytother. Res.* **2008,** *22*, 1400-1403.
45. Slimestad, R.; Verheul, M. J., Seasonal Variations in the Level of Plant Constituents in Greenhouse Production of Cherry Tomatoes. *Journal of Agricultural and Food Chemistry* **2005,** *53*, 3114-3119.
46. Torres, C. A.; Davies, N. M.; Yanez, J. A.; Andrews, P. K., Disposition of Selected Flavonoids in Fruit Tissues of Various Tomato (Lycopersicon esculentum Mill.) Genotypes. *Journal of Agricultural and Food Chemistry* **2005,** *53*, 9536-9543.
47. Tulipani, S.; Martinez Huelamo, M.; Rotches Ribalta, M.; Estruch, R.; Ferrer, E. E.; Andres-Lacueva, C.; Illan, M.; Lamuela-Raventós, R. M., Oil matrix effects on plasma exposure and urinary excretion of phenolic compounds from tomato sauces: Evidence from a human pilot study. *Food Chemistry* **2012,** *130*, 581-590.
48. Bino, R. J.; De Vos, C. H. R.; Lieberman, M.; Hall, R. D.; Bovy, A.; Jonker, H. H.; Tikunov, Y.; Lommen, A.; Moco, S.; Levin, I., The light-hyperresponsive high pigment-2dg mutation of tomato: alterations in the fruit metabolome. *New Phytologist* **2005,** *166*, 427-438.
49. Butelli, E.; Titta, L.; Giorgio, M.; Mock, H.-P.; Matros, A.; Peterek, S.; Schijlen, E. G. W. M.; Hall, R. D.; Bovy, A. G.; Luo, J.; Martin, C., Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. **2008,** *26*, 1301-1308.
50. Li, H.; Deng, Z.; Liu, R.; Young, J. C.; Zhu, H.; Loewen, S.; Tsao, R., Characterization of Phytochemicals and Antioxidant Activities of a Purple Tomato (Solanum lycopersicum L.). *Journal of Agricultural and Food Chemistry* **2011,** *59*, 11803-11811.
51. Luthria, D. L.; Mukhopadhyay, S.; Krizek, D. T., Content of total phenolics and phenolic acids in tomato (Lycopersicon esculentum Mill.) fruits as influenced by cultivar and solar UV radiation. *Journal of Food Composition and Analysis* **2006,** *19*, 771-777.
52. Fraser, P. D.; Truesdale, M. R.; Bird, C. R.; Schuch, W.; Bramley, P. M., Carotenoid Biosynthesis during Tomato Fruit Development (Evidence for Tissue-Specific Gene Expression). *Plant Physiology* **1994,** *105*, 405-413.

53. Tonucci, L. H.; Holden, J. M.; Beecher, G. R.; Khachik, F.; Davis, C. S.; Mulokozi, G., Carotenoid Content of Thermally Processed Tomato-Based Food Products. *Journal of Agricultural and Food Chemistry* **1995,** *43*, 579-586.

54. Lewinsohn, E.; Sitrit, Y.; Bar, E.; Azulay, Y.; Meir, A.; Zamir, D.; Tadmor, Y., Carotenoid Pigmentation Affects the Volatile Composition of Tomato and Watermelon Fruits, As Revealed by Comparative Genetic Analyses. *Journal of Agricultural and Food Chemistry* **2005,** *53*, 3142-3148.

55. Rubio-Diaz, D. E.; Santos, A.; Francis, D. M.; Rodriguez-Saona, L. E., Carotenoid Stability during Production and Storage of Tomato Juice Made from Tomatoes with Diverse Pigment Profiles Measured by Infrared Spectroscopy. *Journal of Agricultural and Food Chemistry* **2010,** *58*, 8692-8698.

56. Capanoglu, E.; Beekwilder, J.; Boyacioglu, D.; Hall, R.; de Vos, R., Changes in Antioxidant and Metabolite Profiles during Production of Tomato Paste. *Journal of Agricultural and Food Chemistry* **2008,** *56*, 964-973.

57. Abushita, A. A.; Daood, H. G.; Biacs, P. A., Change in Carotenoids and Antioxidant Vitamins in Tomato as a Function of Varietal and Technological Factors. *Journal of Agricultural and Food Chemistry* **2000,** *48*, 2075-2081.

58. Jeninga, E. H.; van Beekum, O.; van Dijk, A. D. J.; Hamers, N.; Hendriks-Stegeman, B. I.; Bonvin, A. M. J. J.; Berger, R.; Kalkhoven, E., Impaired Peroxisome Proliferator-Activated Receptor {gamma} Function through Mutation of a Conserved Salt Bridge (R425C) in Familial Partial Lipodystrophy. *Mol Endocrinol* **2007,** *21*, 1049-1065.

59. Lucarelli, E.; Sangiorgi, L.; Maini, V.; Lattanzi, G.; Marmiroli, S.; Reggiani, M.; Mordenti, M.; Gobbi, G. A.; Scrimieri, F.; Bertoja, A. Z.; Picci, P., Troglitazione affects survival of human osteosarcoma cells. *International Journal of Cancer* **2002,** *98*, 344-351.

60. Kim, Y.-I.; Hirai, S.; Takahashi, H.; Goto, T.; Ohyane, C.; Tsugane, T.; Konishi, C.; Fujii, T.; Inai, S.; Iijima, Y.; Aoki, K.; Shibata, D.; Takahashi, N.; Kawada, T., 9-oxo-10(E), 12(E)-octadecadienoic acid derived from tomato is a potent PPAR α agonist to decrease triglyceride accumulation in mouse primary hepatocytes. *Mol. Nutr. Food Res.* **2011,** *55*, 585-593.

61. Kim, Y.-i.; Hirai, S.; Goto, T.; Ohyane, C.; Takahashi, H.; Tsugane, T.; Konishi, C.; Fujii, T.; Inai, S.; Iijima, Y.; Aoki, K.; Shibata, D.; Takahashi, N.; Kawada, T., Potent PPARα Activator Derived from Tomato Juice, 13-oxo-9, 11-Octadecadienoic Acid, Decreases Plasma and Hepatic Triglyceride in Obese Diabetic Mice. *PLoS ONE* **2012,** *7*, e31317.

62. Jia, Y.; Kim, J.-Y.; Jun, H.-J.; Kim, S.-J.; Lee, J.-H.; Hoang, M. H.; Hwang, K.-Y.; Um, S.-J.; Chang, H. I.; Lee, S.-J., The natural carotenoid astaxanthin, a PPAR-α agonist and PPAR-γ antagonist, reduces hepatic lipid accumulation by rewiring the transcriptome in lipid-loaded hepatocytes. *Mol. Nutr. Food Res.* **2012,** *56*, 878-888.

63. Gijsbers, L.; van Eekelen, H. D. L. M.; de Haan, L. H. J.; Swier, J. M.; Heijink, N. L.; Man, H.-Y.; Bovy, A. G.; Keijer, J.; Aarts, J. M. M. J. G.; van der Burg, B.; Rietjens, I. M. C. M., Induction of peroxisome proliferator-activated receptor γ (PPARγ)-mediated gene expression by tomato (Solanum lycopersicum L.) extracts. **Submitted to Journal of Agricultural and Food Chemistry**.

64. Liu, R. H., Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. *The American Journal of Clinical Nutrition* **2003,** *78*, 517S-520S.

65. Engelhard, Y. N.; Gazer, B.; Paran, E., Natural antioxidants from tomato extract reduce blood pressure in patients with grade-1 hypertension: A double-blind, placebo-controlled pilot study. *American Heart Journal* **2006,** *151*, 100.e6-100.e1.

66. Paran, E.; Novack, V.; Engelhard, Y.; Hazan-Halevy, I., The Effects of Natural Antioxidants from Tomato Extract in Treated but Uncontrolled Hypertensive Patients. *Cardiovascular Drugs and Therapy* **2009,** *23*, 145-151.

67. Parvin, R.; Akhter, N., *Protective effect of tomato against adrenaline-induced myocardial infarction in rats*. 2009; Vol. 34.

68. Silaste, M. L.; Alfthan, G.; Aro, A.; Kesäniemi, Y. A.; Hörkkö, S., Tomato juice decreases LDL cholesterol levels and increases LDL resistance to oxidation. *The British journal of nutrition* **2007,** *98*, 1251-1258.

6

IDENTIFICATION VIA METABOLOMICS SCREENING OF TOMATO COMPOUNDS RESPONSIBLE FOR INDUCING ELECTROPHILE-RESPONSIVE ELEMENT (EpRE)-MEDIATED GENE TRANSCRIPTION

Henriëtte D.L.M. van Eekelen, Linda Gijsbers, Chris Maliepaard, Robert A.M. Vreeburg, Richard Finkers, Yuri M. Tikunov, Victoria M. Gomez Roldan, Laura H.J. de Haan, Ric C.H. de Vos, Jac M.M.J.G. Aarts, Ivonne M.C.M. Rietjens, Arnaud G. Bovy

Submitted for publication to Food Chemistry

ABSTRACT

Reporter gene assays may be fast and useful tools for breeders and food industry to select for health-beneficial traits of crops or food processing methods that lead to an improved level and range of health-beneficial compounds. In this study, the ability of extracts of tomato fruit from different accessions to induce EpRE-mediated luciferase expression was measured using the EpRE-LUX reporter cells. By combining data from reporter gene assays obtained with tomato extracts with metabolic profiles from the same extracts, using multivariate analysis, it was possible to identify the flavonoid aglycone quercetin as one of the main compounds responsible for the ability of tomato extracts to induce EpRE-mediated gene expression. It seems likely that also other, yet unidentified compounds contribute to EpRE-mediated gene expression induced by tomato extracts. The results indicate that combining reporter gene assays with metabolomics of tomatoes provides a powerful tool towards the identification of health beneficial constituents of foods.

KEY WORDS

Tomato, *Solanum lycopersicum*, electrophile-responsive element (EpRE), phenolic compounds, LC–MS, metabolomics, reporter gene assay, multivariate analysis.

INTRODUCTION

Tomato fruit (*Solanum lycopersicum*) is one of the most important vegetables consumed worldwide. It is used both as fresh and as processed food. The annual production of tomatoes worldwide was >145 million metric tons in 2010 according to FAOSTAT (http://faostat.fao.org). Tomato breeding programmes have mainly focussed on producer traits such as disease resistance, yield and shelf life. However, consumer traits relating to taste, nutritional value and health have become more importantduring the last decade (*1*). A number of epidemiological and intervention studies show that a diet rich in tomato results in a decreased incidence of certain cancers and coronary heart diseases (*2-4*). In these studies, the major carotenoid in tomato, lycopene, has gained much attention as health beneficial phytochemical. However, in addition to carotenoids, tomatoes contain many other potentially health beneficial phytochemicals such as flavonoids, chlorogenic acid and vitamin C (*5*). Flavonoids are able to induce the transcription of enzymes such as the quinone-reducing enzyme NAD(P)H: quinone oxidoreductase (NQO1) and glutathione S-transferases (GSTs)(*6-8*). Induction of these enzymes has been linked to reduced toxicity of chemical carcinogens due to efficient detoxification and an enhanced redox status to sustain optimal physiologic conditions in the body (*7, 9, 10*). Transcription of many of these detoxification enzymes, or phase II enzymes, is under control of the Antioxidant-Responsive Element (ARE) also called Electrophile-Responsive Element (EpRE, which will be used here). The EpRE mainly controls expression through the Kelch-like ECH associated protein 1 / nuclear factor-E2-related factor 2 (Keap1/Nrf2) system; oxidation of cysteines in Keap1 releases the transcription factor Nrf2, which will translocate to the nucleus and activate EpRE-mediated gene expression (*11*). In our previous study, a method was developed to prepare tomato extracts in order to test them for potential health effects in cell-based reporter gene assays. Furthermore, induction of EpRE-controlled gene expression by tomato extracts was monitored in such a cell system using the EpRE-LUX reporter cell line (*6*). It was shown that neither chloroform extracts nor methanol extracts of the tomato fruits induced EpRE-controlled luciferase expression. It is known that many plant polyphenols are present as sugar conjugates in plants and have to be hydrolyzed to their aglycones before uptake (*12, 13*). Deglycosylation of polyphenols normally occurs in the small intestine by the membrane-bound lactase-phlorizin hydrolase (LPH) and putatively by the cytosolic β-glucosidase (CBG)(*14, 15*). When deglycosylation was mimicked using fungal glycosidases, methanol extracts did activate EpRE-mediated luciferase expression.

The present study describes the use of the previously developed method, to screen a collection of 97 different tomato accessions for their ability to induce EpRE-mediated gene expression. The metabolic variation between the different accessions appeared to influence their potency to induce EpRE-mediated gene expression. In order to identify metabolites putatively involved in the induction of EpRE-mediated gene expression, the reporter gene-assay data were linked to metabolomic profiles of the same extracts and a regression analysis was performed using various methods. The regression analysis yielded candidate metabolites (mostly flavonoids) for the EpRE-LUX induction. These results were validated using a transgenic tomato line accumulating high levels of flavonoids.

MATERIALS AND METHODS

Chemicals and Reagents

Quercetin dihydrate, rutin, quercetin 3-glucoside, kaempferol, tert-butylhydroquinone (tBHQ), acetic acid, sodium acetate and Viscozyme L were purchased from Sigma-Aldrich (St. Louis, USA). Dimethyl sulfoxide (DMSO) and naringenin were obtained from Acros Organics (Geel, Belgium). Methanol and acetonitrile were purchased from Biosolve (Valkenswaard, The Netherlands). Minimum Essential Medium alpha (further referred to as α-MEM), Minimum Essential Medium alpha without phenol red, Dulbecco's modified fetal calf serum (FCS), Hank's balanced salt solution (HBSS), trypsin, gentamicin and G418 were purchased from Invitrogen Corporation (Breda, The Netherlands). HLB solid phase extraction (SPE) cartridges (3cc) were obtained from OASIS, Waters, (Etten-Leur, The Netherlands). Formic acid was obtained from Merck Millipore (Amsterdam, The Netherlands). Kaempferol-3-rutinoside was purchased from Apin Chemicals (Abingdon, UK).

Tomato Material

A diverse set of 97 different tomato (*Solanum lycopersicon*) accessions was selected and provided by Syngenta (Enkhuizen, The Netherlands). Six plants per accession were grown in Morocco in the summer of 2009. Preferably at least one fruit per plant was harvested by picking the fruit next to the ripest on the truss. In this way, at least six fruits were harvested for each accession. The picked fruits were stored in bags overnight to facilitate further ripening. The next day fruits were pooled and blended using a Whring blender for 30 sec at maximal speed. Juice obtained this way was aliquoted into cryovials, snap-frozen in liquid nitrogen and stored at -20°C until transportation on dry ice to The Netherlands. Samples were finally stored at -80°C until further use. Additionally, a reference sample was prepared by blending frozen fruit juice of one cryovial of each of the 97 different accessions.

A commercial tomato accession was purchased at a local supermarket. These tomatoes were peeled. Peel and flesh samples were separated, snap frozen in liquid nitrogen and subsequently ground in an analytical mill. The powder, was stored at -80°C until further use. Furthermore, tomato peel samples were prepared from wild type (wt) and flavonoid enriched tomato, which is overexpressing the *petunia* chalcone isomerase1 (CHI1) gene and the *Gerbera* flavone synthase II (FNSII) gene as described previously (*16, 17*).

Enzymatic hydrolysis and extraction of semi-polar metabolites

Tomato samples were enzymatically hydrolyzed using a method that has previously been described by (*6*). In short, enzymatically deglycosylated tomato methanolextracts were prepared by adding 500 µl of 0.1M sodium acetate buffer (pH 4.8) and 167 µl of Viscozyme L per gram tomato juice, followed by incubation in a waterbath at 37°C. After two hours of incubation, samples were put on ice and extracts were prepared by adding 3 ml methanol per ml of deglycosylated tomato sample, followed by 10 minutes sonication and 15 minutes centrifugation at 1000 g. The supernatant was aliquoted in portions of 1.5 ml in eppendorf tubes. Methanol was evaporated under a nitrogen atmosphere and the remaining residue was dried using a centrifugal vacuum concentrator. The dried

extracts were stored at -80°C until further analysis. Prior to analysis, the dried extracts were re-dissolved in 375 µL DMSO:α-MEM without phenol red (1:4) and filtered through a Captiva 96-well 0.45µm polypropylene filter plate, (Agilent Technologies Netherlands B.V., Amstelveen). Samples were split to be used for either EpRE-mediated gene expression measurements or metabolomic analysis using LC-MS.

The freeze dried wt and flavonoid enriched tomato peel samples were first re-dissolved in water to give a final concentration of 60 mg dried sample/ml. Immediately thereafter, enzymatic hydrolysis and extraction were performed as described above.

Cell culture

Hepa-1c1c7 mouse liver hepatoma cells containing a stably transfected luciferase reporter gene under transcriptional control of an EpRE, as described by Boerboom *et al.* (*18*) and in this article further referred to as: EpRE-LUX cells, were cultivated in α-MEM, supplemented with FCS and 0.5 mg/ml G418 and 50 µg/ml gentamicin, under a humidified atmosphere with 5% CO_2 at 37°C.

EpRE-LUX assay

The EpRE-LUX cells were used to test the ability of deglycosylated tomato extracts to induce EpRE-mediated gene expression. Cells were plated onto 96-wells view plates by adding 100 µl EpRE-LUX cell suspension ($2*10^5$ cells/ml) to the inner 60 wells of the plate. In the outer wells 100 µl HBSS was added to maintain physical homogeneity throughout the plate. To allow the cells to attach to the bottom and to form a confluent monolayer, the 96-wells view plate was incubated for 24 hours.

Exposure medium was prepared by adding 60 µl of the re-dissolved and filtered deglycosylated tomato extracts to 740 µl of α-MEM without addition of FCS and antibiotics, resulting in a final concentration of the tomato extracts tested in the EpRE-LUX assay of 45 g FW/L. The final concentration of DMSO was kept at 1.5% (a concentration tested not to be toxic to the cells). As a positive control, each plate contained six wells with tBHQ, a well-known inducer of EpRE-mediated gene expression, at a final concentration of 15 µM. Furthermore, on each plate an additional six wells were used for the solvent control (1.5 % DMSO) and six wells for an extract of enzymatically hydrolyzed reference tomato sample.

After exposure of the EpRE-LUX cells to the tomato extracts for 24 hours under a humidified atmosphere with 5% CO_2 at 37°C, absence of cytotoxicity was checked visually using a microscope. Exposure medium was removed, cells were lysed using a low salt lysis buffer and at least overnight incubated at -80°C. Upon addition of flash mix, the luciferase activity (in relative light units (RLUs)) of the lysate was measured using a luminometer (Luminoscan Ascent, Termo Scientific, Waltham USA)(*18*).

LC-MS machinery and methods

Semi-polar compounds present in the enzymatically deglycosylated tomato extracts were analysed using C18-reversed-phase liquid chromatography coupled to a photodiode array detector and a quadrupole time of flight high-resolution mass spectrometer (LC-PDA-QTOF-MS) as described by de Vos *et al.* 2007 (*19*). In short, 5 µl of the re-dissolved

and filtered deglycosylated tomato extracts were injected in the LC-system. Separation was performed using degassed solutions of ultrapure water (eluent A) and acetonitrile (eluent B), both acidified with 0.1 % formic acid, which were pumped into the LC system with a flow rate of 190 µl/min. A linear gradient was applied increasing eluent B from 5% to 35% in 45 minutes. Hereafter, the column was washed and equilibrated at the initial conditions for 15 minutes before the next injection. After separation and detection of the semi-polar compounds by LC-PDA, positive electrospray ionization was applied and masses were detected using QTOF-MS.

LC-PDA-QTOF-MS data processing and putative identification

The compound profiles obtained from LC-PDA-QTOF-MS were analysed using MassLynx (4.1)(Waters) after prepossessing using the MetAlign™ software package (http://www.metalign.nl) for baseline correction, noise estimation, and ion-wise mass spectral alignment (*19*). Noise was subtracted and signals that were present in at least six tomato accessions were selected for further processing using MS-CLUST software (http://www.metalign.nl) as described by Tikunov *et al.* (*20*). In short, MS-CLUST clusters signals that are derived from the same compound, such as isotopes, adducts and in-source fragments based on their signal intensity patterns and retention time over all samples, in order to remove data redundancy. This resulted in 313 reconstructed metabolite clusters (centrotypes) of which for each cluster the signal of the most unique mass was chosen as a representative for the respective cluster and was used for further (statistical) analysis.

For putative identification of the semi-polar compound clusters UV spectra, molecular weight and (in source) fragmentation patterns were used in combination with different metabolite databases such as the MotoDB (http://appliedbioinformatics.wur.nl/moto), Dictionary of Natural Products (http://dnp.chemnetbase.com).

Downstream data processing and statistical procedures

For each experiment, at least three independent repetitions were performed. Within each experiment at least six technical replicates (six wells with cells were exposed to the same sample) were measured. Using Microsoft Excel 2010, EpRE-mediated luciferase induction of the different tomato accessions were expressed as the percentage of the luciferase induction by the reference sample (mix of all 97 tomato accessions) and the least significant difference (lsd) over all tomato accessions at a confidence level of 95% was calculated by one way ANOVA using IBM SPSS statistics 19 software package.

EpRE-mediated luciferase induction of the flesh and peel samples of the commercial tomato accession as well as the peel samples of the wt tomato and its transgenic high flavonoid line were expressed as the percentage of the luciferase induction by the reference sample (mix of all 97 tomato accessions) ± standard error of the mean (SEM). Statistical significance was assessed using the one-sided Student's t-test with a significance threshold of α=0.01.

For further statistical analysis, intensities of the metabolomic dataset were log-transformed and mean-centred. The log-normalised metabolomics data for the 7 highest and 7 lowest inducing tomato accessions were selected to perform principal components

analysis (PCA) using GeneMaths XT version 2.12. Furthermore, statistical significance was assessed using a two sided Student's t-test with a significance threshold of α=0.05.

The log-transformed metabolomics data set was also used for a non-targeted correlative approach to find metabolites that show a correlation with the response in the EpRE-LUX assay of different tomato accessions. To do so, different statistical algorithms were applied using the web-based tool OmicsFusion (http://www.plantbreeding. wur.nl/omicsFusion/). This tool combines nine different statistical algorithms, including univariate regression, a machine learning algorithm (random forest), and a number of regularized regression techniques (principal components regression (PCR), partial least squares (PLS), and ridge regression) without variable selection and regularized regression analysis with variable selection (lasso regression, elastic net, and sparse PLS), to regress the EpRE-mediated luciferase induction on the metabolics dataset.

In order to identify which metabolites show significant correlation with EpRE-mediated luciferase induction, the Random Forest (RF) regression approach (*21*) was combined with a permutation test using the Random Forest package in the R statistical software package. First, RF was applied 100 times to correlate the metabolomics data with the EpRE-LUX response (based on increase in node purity values). Second, the EpRE-LUX luciferase levels of the 93 tomato samples were randomised 500 times and for each of these 500 permutated sets RF was applied. The significance of each individual metabolite, was determined by the increase in mean square error after permutation. Significance levels were set at a permutation threshold of α=0.05.

RESULTS AND DISCUSSION

EpRE-mediated luciferase induction by tomato lines

Deglycosylated extracts of 97 tomato accessions were tested for their ability to induce EpRE-mediated gene expression using the EpRE-LUX assay (figure 1). Four of the 97 accessions caused cytotoxic effects and for this reason were not used for further analysis and are not shown in figure 1. Significant differences (one-way ANOVA, α=0.001) in EpRE-mediated luciferase induction between the 93 accessions were found. Induction of EpRE-mediated luciferase expression ranged from 52.5% to 151.9% relative to the reference tomato sample (a mix of all accessions used)(figure 1). The tomato collection thus showed almost three-fold variation in EpRE-controlled luciferase induction. This variation was used to link the potency of the tomato accessions to induce EpRE-mediated gene expression with their metabolic profiles in order to identify metabolites responsible for this potentially health beneficial effect.

Metabolic contrasts

Deglycosylated tomato extracts that had been characterised with respect to EpRE-mediated luciferase induction potential, were subjected to an untargeted metabolic profiling using Liquid Chomatography – Mass Spectrometry (LC-MS). MsClust was used to reduce redundancy in the LC-MS data, as described by Tikunov *et al.* (*20*). This resulted in 313 unique molecular fragment clusters which each represent a putative compound mass

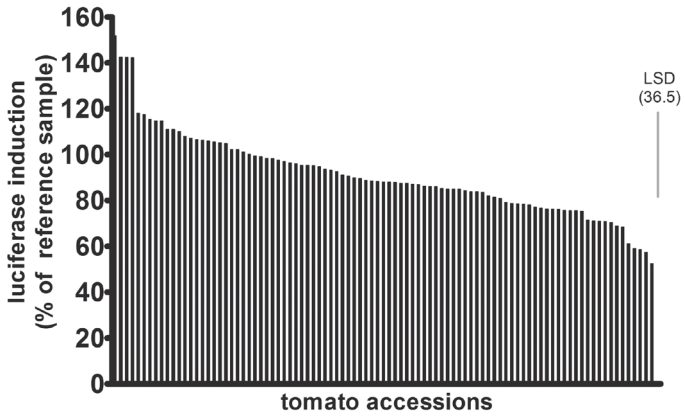

Figure 1. Average (of n=3) luciferase induction in EpRE-LUX cells by enzymatically hydrolyzed tomato extracts of 93 different tomato accessions (45 g fresh weight/L) after 24 hours of exposure. Induction is given relative to the luciferase induction of a reference tomato sample (a mix of all individual samples used in this study). Samples are ordered in descending induction activity. The error bar represents the Least Significant Difference (LSD).

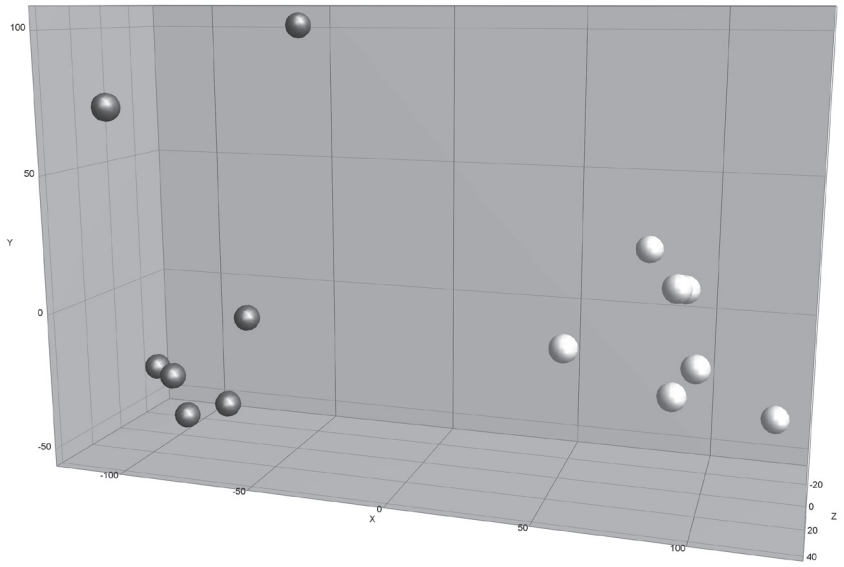

Figure 2. Principal components analysis of the LC-MS data obtained from the 14 tomato accessions that showed either highest (dark dots) or lowest (light dots) EpRE-mediated luciferase induction. Clustering of these tomato accessions along the first three principal components (PC): PC1, PC2, and PC3 describe 42.1%, 19.4%, and 8.3% of the total metabolic variation between the 14 different accessions.

spectrum. The metabolic clusters of the 7 accessions that showed at least 30% increased and the 7 accessions that showed at least 15% lower luciferase inductions compared to the reference sample were subjected to principal components analysis. A clear separation of the accession groups was observed (figure 2). This suggests that the difference in EpRE-mediated gene expression levels observed in the two groups can be explained based on contrasts in metabolic profiles. 64 out of the 313 metabolites were selected to be most different between the two groups based on 2 criteria: (1) $P<0.01$ between the two accession groups in a two sided Students t-test and (2) the average intensity within the two accession groups needed to be at least 10-fold different. These 64 metabolites included most of the putatively annotated flavonoids, among which the aglycones quercetin and kaempferol ($p = 0.0001$ and $p = 0.0003$ respectively) which have previously been shown to be able to induce EpRE-mediated gene expression as pure compounds (*6, 8*). Interestingly, five out of the seven accessions that gave the highest EpRE-mediated luciferase induction in the EpRE-LUX assay, are high pigment (HP) ripening mutants. Such mutants are known to have elevated levels of carotenoids, flavonoids and vitamin C (*5, 22, 23*). Moreover, two of the seven lowest inducing accessions are white heirloom varieties which are generally low in flavonoid and carotenoid levels (*23*). Together these findings suggest that compounds such as flavonoids might play a role in the potential of tomato extracts to induce EpRE-mediated gene expression.

Regression analysis

By using PCA it was possible to visualise clear metabolic contrast between the accessions that showed either highest or lowest expression of EpRE-mediated luciferase. In addition to this PCA, a non-targeted regression approach was used on all genotypes tested to find metabolites that are associated with induction of EpRE-mediated gene expression. A number of different regression approaches, each suitable for prediction of a trait from a high-dimensional ~omics data set were used. Metabolites were ranked per method based on the correlation and these ranks were combined to come to an overall ranking. These analyses were performed using the web-based tool OmicsFusion. Compounds with highest combined ranking over all the methods have been (partly) annotated as flavonoid glycosides and flavonoid aglycones (Table 1). In a previous study, flavonoid glycosides could not induce EpRE-mediated gene expression (*6*) and their presence indicates that the enzymatic treatment was not sufficient to fully deglycosylate all flavonoid glycosides. Not surprisingly, the levels of glycosides show high correlation with the flavonoid aglycones, such asquercetin and kaempferol, which were able to induce EpRE-mediated gene expression (*6, 8*). High ranking in the regression analysis in combination with shown activity make flavonoid aglycones the prime candidate metabolites present in the tomato extracts responsible for EpRE-mediated gene expression. The top ranked flavonoid aglycones were quercetin (ranking 12) and kaempferol (ranking 35). The flavonoid aglycones naringenin and eriodictyol show a weaker relationship and are ranked at position 75 and 85 respectively. Moreover, an isomer of chlorogenic acid, tri-caffeoylquinic acid, also showed a strong association (rank 8) with EpRE-mediated

Table 1. Top 35 metabolites that showed best association with EpRE-mediated gene expression by the 93 tomato accession according to OmicsFusion and their putative identification.

OF	RF	incMSE	rt	id
1	9	*0.002*	23.54	Quercetin hexose pentose
2	10	*0.006*	32.41	Quercetin 3-O-beta-(6''-O-E-p-coumaroylglucoside)-7-O-beta-blucoside
3	1	*0.002*	22.31	Quercetin-hexose-deoxyhexose, -pentose
4	3	*0.002*	31.52	Quercetin hexose
5	4	*0.006*	44.04	Unknown 1
6	15	*0.006*	25.32	Quercetin 3-O-glucoside
7	2	*0.002*	41.15	Unknown 2
8	8	*0.012*	39.22	Tri caffeoylquinic acid
9	7	*0.002*	44.50	Unknown 3
10	31	*0.040*	27.32	Unknown 4
11	12	*0.050*	36.90	Unknown 5
12	5	*0.002*	37.39	Quercetin
13	23	*0.014*	25.00	Unknown 6
14	11	*0.004*	24.57	Kaempferol hexose deoxyhexose pentose
15	26	*0.004*	30.99	Unknown 7
16	25	0.074	26.80	Unknown 8
17	18	*0.002*	20.62	Unknown 9
18	27	0.090	30.82	Unknown 10
19	30	0.234	42.84	Unknown 11
20	20	*0.048*	41.78	Unknown 12
21	37	*0.018*	24.33	Quercetin 3-O-rutinoside
22	28	0.226	36.44	Unknown 13
23	16	*0.004*	39.73	Unknown 14
24	22	0.126	30.32	Unknown 15
25	44	*0.034*	15.94	Unknown 16
26	29	*0.022*	14.82	Unknown 17
27	96	0.259	25.75	Unknown 18
28	68	0.331	41.99	Unknown 19
29	71	0.581	2.26	Unknown 20
30	36	0.515	14.44	Unknown 21
31	32	0.216	11.84	Unknown 22
32	72	0.353	26.63	Unknown 23
33	13	*0.030*	33.12	Unknown 24
34	14	*0.044*	32.90	Naringeninchalcone hexose
35	34	*0.040*	44.00	Kaempferol

Abbreviations:

OF	Rank OmicsFusion
RF	Rank Random Forest
incMSE	Increase in mean square error after permutation (p-value) *[Italic = p<0.05]*
rt	Retention time (minutes)
id	Putative identification of the metabolite

mol form	m/z theory	m/z found	ppm error	fragments
C26H28O16	597.1450	597.1472	3.6	465.10 – 303.05
C36H36O19	773.1924	773.1957	4.3	465.10 – 303.05
C32H38O20	743.2029	743.2046	2.2	611.16 – 465.10 – 303.05
C21H20O12	465.1028	465.1042	3.1	303.05
		659.2748		
C21H20O12	465.1028	465.1042	3.1	303.05
		823.3423		
C34H30O15	679.1657	679.1696	5.7	499.12 – 517.13
		275.2018		
		209.1554		
		725.3073		
C15H10O7	303.0499	303.0498	0.5	
		525.2372		
C32H38O19	727.2080	727.2113	4.5	595.40 – 449.10 – 287.05
		431.0632		
		400.1406		
		433.2109		
		526.2215		
		275.2000		
		823.3432		
C27H30O16	611.1607	611.1602	0.8	465.10 – 303.05
		693.2795		
		481.0793		
		693.2784		
		335.1351		
		378.1679		
		443.1900		
		425.2536		
		381.0792		
		381.1175		
		288.1913		
		319.0523		
		677.2855		
C21H22O10	435.1311	435.1286	5.8	273.08
C15H10O6	287.0550	287.0555	1.7	

mol form Molecular formula of the metabolite
m/z theory Theoretical monoisotopic mass calculated for the ion (M+H)+
m/z found Found m/z- mass detected in the experiment
ppm error Difference between theoretical and found m/z values in ppm
fragments Insource fragments

luciferase induction. Some studies show that chlorogenic acid is able to induce EpRE-mediated gene expression (*24, 25*). However, when chlorogenic acid was tested as a model compound in our cell assay, it was not shown to induce EpRE-mediated luciferase expression (*6*). Several unidentified metabolites are highly ranked for their correlation with the response in the EpRE-LUX (Table 1). It is not unlikely that these compounds might also (partially) play a role in the induction of EpRE-mediated gene expression by the tomato extracts. Interestingly, most of the high ranked unidentified metabolites have a retention time >30 minutes on C18 column, which suggests a more a-polar nature. Further identification of these compounds will be a major target of a follow-up study. Nevertheless, finding these unidentified metabolites shows that the use of reporter gene assays in combination with metabolomics could potentially lead to the discovery of "new" metabolites with potential health beneficial properties.

Random Forest and permutation test for statistical significance

By combining several statistical algorithms, it was possible to relate the luciferase induction level as measured in the EpRE-LUX assay to metabolic profiles and to rank the importance of each individual metabolite. However, this approach does not give a significance level to individual metabolites. Therefore a Random Forest regression approach was performed in combination with a permutation test. This showed that 24 metabolites have a significant correlation with EpRE-controlled reporter gene induction. Nine of these metabolites have been putatively identified and include flavonoid glycosides, tri-caffeoylquinic acid and quercetin, which shows to be most significant (permutation p-value< 0.002)(Table 1).

Moreover, this approach also showed that the relationship between metabolites and EpRE-induced activity of the tomato accessions is statistically significant, since the non-permutated (true) data showed that the variance explained in RF (prediction R^2) is significantly higher ($R^2 = 0.46 \pm 0.01$) than any of the prediction R^2 of the permutated data sets (R^2= -0.19 to 0.13 with an average of -0.05).

Flesh vs. peel and wild type vs. genetically engineered high flavonoid tomato

To verify that flavonoid aglycones are a major factor responsible for the EpRE-mediated gene expression, extracts of enzymatically deglycosylated peel sample from a transgenic high flavonoid tomato and its corresponding wild type tomato (*17*) were tested in the EpRE-LUX assay. This flavonoid-enriched tomato contains significantly increased levels of kaempferol-rutinoside (37 fold), quercetin-rutinoside (19 fold), luteolin-glucoside, luteolin and quercetin (not detected in wt)(*16, 17*). These tomatoes were chosen since they contain the same genetic background, in contrast to the high and low EpRE-inducing accessions, which all have different genetic background and thus a different food matrix besides differences in flavonoid levels. Induction of EpRE-mediated luciferase appeared to be significantly higher in transgenic (80.1% relative to the reference tomato sample) compared to the wt tomato (55.6% relative to the reference tomato sample)(figure 3). Therefore it can be concluded that the difference in the potency to induce EpRE-mediated gene expression is most likely due to the increased flavonoid levels present in

the transgenic peel extracts. This suggests that flavonoids present in tomato play a major role in the induction of EpRE-mediated expression. In the same experiment also peel and flesh sample from a commercial tomato accession have been tested for their ability to induce EpRE-mediated-expression. The peel sample showed significantly higher EpRE-mediated luciferase induction (112.8 percent relative to the reference tomato sample) compared to flesh (53.9 percent relative to the reference tomato sample)(figure 3). Again, this indicates that flavonoids present in tomato extracts are good candidates for the induction of the Keap1/Nrf2/EpRE pathway, since flavonoids are known to be mainly present in peel and are hardly detectable in flesh (*26*). Despite the quantitative difference, both peel and flesh sample were able to significantly induce EpRE-mediated gene expression, although the levels of quercetin and kaempferol in tomato flesh were shown to be low (*16, 27, 28*). This indicates that in addition to flavonoids, also other metabolites may play a role in the induction of EpRE-controlled gene expression by tomato extracts. This was also suggested by a quantitative analysis of the levels of quercetin and kaempferolin the 93 tomato accessions. Quercetin levels ranged from 0 to almost 1.1 µmol/gram fresh weight and kaempferol levels ranged from 0 to 0.35 µmol/gram fresh weight. For some of the 93 accessions, these levels of quercetin and kaempferol were sufficient to fully explain the EpRE-mediated luciferase induction based on the inducing activity of pure compounds (*6*), whereas for several other accessions these levels are too low to explain induction. This again indicates that there may be other metabolites present in the tomato extracts which are also able to induce EpRE-mediated gene expression.

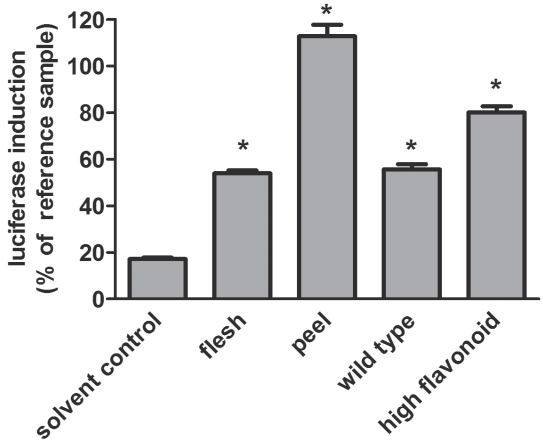

Figure 3. Luciferase activity is induced in the EpRE-LUX reporter cells by 24 hour exposure to enzymatically hydrolyzed extracts (27.5 g fresh weight/L) of flesh and peel of a commercial tomato accession and of peel samples of a transgenic, high flavonoid tomato and its wild-type. Luciferase induction is expressed as induction factor compared to solvent control. Error bars represent the ± SEM of 12 replicates. Asterisks (*) indicate a significant difference from the solvent control: $p < 0.01$.

CONCLUSION

In this study, 97 different tomato accessions were screened for their ability to induce EpRE-mediated gene expression. The 93 accessions that did not show cytotoxicity were all able to induce EpRE-mediated gene expression, with a three-fold difference between the most potent and the least potent accession. The variation in EpRE-mediated gene expression between the accessions was combined with the metabolic profiles of the tomatoes and showed a significant correlation between the metabolites present and EpRE-inducing ability of the tomato accessions. It was shown that the flavonoid aglycone quercetin can partly explain the EpRE-inducing ability of the semi-polar extracts of 93 enzymatically deglycosylated tomato accessions. Yet, it seems that also other metabolites present in the extracts play a role in EpRE-mediated gene induction and that activity of the complex tomato extracts in the EpRE-LUX assay cannot simply be explained by flavonoid aglycones only. Even the semi-polar tomato extracts, that represent only a part of the complete tomato metabolome, are complex mixtures of many metabolites that are likely to interact with each other and/or cellular components present in the hepatoma cell line. Additive, synergistic or even antagonistic effects are likely to play a role (*29*). Although the extraction and detection methods show limitations by only representing the semi-polair metabolites present in the tomato metabolome, this study shows a clear example on how cell-based reporter gene assays, such as the EpRE-LUX assay, might provide useful tools for breeders and/or food (processing) industry to make a fast selection of varieties/ processing methods, that result in products with a composition of active ingredients optimal for inducing specific health beneficial gene expression pathways. Furthermore, it was shown that combining the output of these cell based reporter gene assays with metabolome analysis enables putative identification compounds that might be responsible for the health beneficial effects. This might also be of interest for the discovery of “new” potentially health-beneficial metabolites.

ACKNOWLEDGEMENTS

This work was financially supported by the Food and Nutrition Delta grant number FND07007. We thank Syngenta for providing us the tomato accessions.

REFERENCES

1. Foolad, M. R., Genome Mapping and Molecular Breeding of Tomato. *International Journal of Plant Genomics* **2007,** *2007*.
2. Agarwal, S.; Rao, A. V., Tomato lycopene and its role in human health and chronic diseases. *Canadian Medical Association Journal* **2000,** *163*, 739-744.
3. Campbell, J. K.; Canene-Adams, K.; Lindshield, B. L.; Boileau, T. W. M.; Clinton, S. K.; Erdman Jr, J. W., Tomato phytochemicals and prostate cancer risk. *Journal of Nutrition* **2004,** *134*.
4. Giovannucci, E., Tomatoes, tomato-based products, lycopene, and cancer: Review of the epidemiologic literature. *Journal of the National Cancer Institute* **1999,** *91*, 317-331.
5. Levin, I.; Ric De Vos, C. H.; Tadmor, Y.; Bovy, A.; Lieberman, M.; Oren-Shamir, M.; Segev, O.; Kolotilin, I.; Keller, M.; Ovadia, R.; Meir, A.; Bino, R. J., High pigment tomato mutants - More than just lycopene (a review). *Israel Journal of Plant Sciences* **2006,** *54*, 179-190.
6. Gijsbers, L.; van Eekelen, H. D. L. M.; Nguyen, T. H.; de Haan, L. H. J.; van der Burg, B.; Aarts, J. M. M. J. G.; Rietjens, I. M. C. M.; Bovy, A. G., Induction of electrophile-responsive element (EpRE)-mediated gene expression by tomato extracts in vitro. *Food Chemistry* **2012,** *135*, 1166-1172.
7. Hayes, J. D.; McMahon, M.; Chowdhry, S.; Dinkova-Kostova, A. T., Cancer chemoprevention mechanisms mediated through the keap1-Nrf2 pathway. *Antioxidants and Redox Signaling* **2010,** *13*, 1713-1748.
8. Lee-Hilz, Y. Y.; Boerboom, A. M. J. F.; Westphal, A. H.; Van Berkel, W. J. H.; Aarts, J. M. M. J. G.; Rietjens, I. M. C. M., Pro-oxidant activity of flavonoids induces EpRE-mediated gene expression. *Chemical Research in Toxicology* **2006,** *19*, 1499-1505.
9. Chen, C.; Kong, A. N. T., Dietary chemopreventive compounds and ARE/EpRE signaling. *Free Radical Biology and Medicine* **2004,** *36*, 1505-1516.
10. Talalay, P., Chemoprotection against cancer by induction of Phase 2 enzymes. *BioFactors* **2000,** *12*, 5-11.
11. Baird, L.; Dinkova-Kostova, A. T., The cytoprotective role of the Keap1-Nrf2 pathway. *Archives of Toxicology* **2011,** *85*, 241-272.
12. Arts, I. C. W.; Sesink, A. L. A.; Faassen-Peters, M.; Hollman, P. C. H., The type of sugar moiety is a major determinant of the small intestinal uptake and subsequent biliary excretion of dietary quercetin glycosides. *British Journal of Nutrition* **2004,** *91*, 841-847.
13. Walgren, R. A.; Walle, U. K.; Walle, T., Transport of quercetin and its glucosides across human intestinal epithelial Caco-2 cells. *Biochemical Pharmacology* **1998,** *55*, 1721-1727.
14. Day, A. J.; DuPont, M. S.; Ridley, S.; Rhodes, M.; Rhodes, M. J. C.; Morgan, M. R. A.; Williamson, G., Deglycosylation of flavonoid and isoflavonoid glycosides by human small intestine and liver [beta]-glucosidase activity. *FEBS Letters* **1998,** *436*, 71-75.
15. Németh, K.; Plumb, G. W.; Berrin, J. G.; Juge, N.; Jacob, R.; Naim, H. Y.; Williamson, G.; Swallow, D. M.; Kroon, P. A., Deglycosylation by small intestinal epithelial cell Î²-glucosidases is a critical step in the absorption and metabolism of dietary flavonoid glycosides in humans. *European Journal of Nutrition* **2003,** *42*, 29-42.
16. Rein, D.; Schijlen, E.; Kooistra, T.; Herbers, K.; Verschuren, L.; Hall, R.; Sonnewald, U.; Bovy, A.; Kleemann, R., Transgenic Flavonoid Tomato Intake Reduces C-Reactive Protein in Human C-Reactive Protein Transgenic Mice More Than Wild-Type Tomato. *J. Nutr.* **2006,** *136*, 2331-2337.
17. Schijlen, E.; Ric De Vos, C. H.; Jonker, H.; Van Den Broeck, H.; Molthoff, J.; Van Tunen, A.; Martens, S.; Bovy, A., Pathway engineering for healthy phytochemicals leading to the production of novel flavonoids in tomato fruit. *Plant Biotechnology Journal* **2006,** *4*, 433-444.
18. Boerboom, A. M. J. F.; Vermeulen, M.; van der Woude, H.; Bremer, B. I.; Lee-Hilz, Y. Y.; Kampman, E.; van Bladeren, P. J.; Rietjens, I. M. C. M.; Aarts, J. M. M. J. G., Newly constructed stable reporter cell lines for mechanistic studies on electrophile-responsive element-mediated gene expression reveal a role for flavonoid planarity. *Biochemical Pharmacology* **2006,** *72*, 217-226.

19. De Vos, R. C. H.; Moco, S.; Lommen, A.; Keurentjes, J. J. B.; Bino, R. J.; Hall, R. D., Untargeted large-scale plant metabolomics using liquid chromatography coupled to mass spectrometry. *Nature Protocols* **2007,** *2*, 778-791.
20. Tikunov, Y. M.; Laptenok, S.; Hall, R. D.; Bovy, A.; de Vos, R. C. H., MSClust: a tool for unsupervised mass spectra extraction of chromatography-mass spectrometry ion-wise aligned data. *Metabolomics* **2011**, 1-5.
21. Breiman, L., Random forests. *Machine Learning* **2001,** *45*, 5-32.
22. Bino, R. J.; De Vos, C. H. R.; Lieberman, M.; Hall, R. D.; Bovy, A.; Jonker, H. H.; Tikunov, Y.; Lommen, A.; Moco, S.; Levin, I., The light-hyperresponsive high pigment-2dg mutation of tomato: Alterations in the fruit metabolome. *New Phytologist* **2005,** *166*, 427-438.
23. Paran, I.; van der Knaap, E., Genetic and molecular regulation of fruit and plant domestication traits in tomato and pepper. *Journal of Experimental Botany* **2007,** *58*, 3841-3852.
24. Feng, R.; Lu, Y.; Bowman, L. L.; Qian, Y.; Castranova, V.; Ding, M., Inhibition of activator protein-1, NF-κB, and MAPKs and induction of phase 2 detoxifying enzyme activity by chlorogenic acid. *Journal of Biological Chemistry* **2005,** *280*, 27888-27895.
25. Higgins, L. G.; Cavin, C.; Itoh, K.; Yamamoto, M.; Hayes, J. D., Induction of cancer chemopreventive enzymes by coffee is mediated by transcription factor Nrf2. Evidence that the coffee-specific diterpenes cafestol and kahweol confer protection against acrolein. *Toxicology and Applied Pharmacology* **2008,** *226*, 328-337.
26. Bovy, A.; Schijlen, E.; Hall, R., Metabolic engineering of flavonoids in tomato (Solanum lycopersicum): the potential for metabolomics. *Metabolomics* **2007,** *3*, 399-412.
27. Bovy, A.; de Vos, R.; Kemper, M.; Schijlen, E.; Almenar Pertejo, M.; Muir, S.; Collins, G.; Robinson, S.; Verhoeyen, M.; Hughes, S.; Santos-Buelga, C.; van Tunen, A., High-Flavonol Tomatoes Resulting from the Heterologous Expression of the Maize Transcription Factor Genes LC and C1. *Plant Cell* **2002,** *14*, 2509-2526.
28. Moco, S.; Capanoglu, E.; Tikunov, Y.; Bino, R. J.; Boyacioglu, D.; Hall, R. D.; Vervoort, J.; De Vos, R. C. H., Tissue specialization at the metabolite level is perceived during the development of tomato fruit. *Journal of Experimental Botany* **2007,** *58*, 4131-4146.
29. Liu, R. H., Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. *American Journal of Clinical Nutrition* **2003,** *78*, 517S-520S.

Web references

FAOSTAT:	http://faostat.fao.org
MetAlign™ & MS-CLUST:	http://www.metalign.nl
MotoDB	http://appliedbioinformatics.wur.nl/moto
Dictionary of Natural Products	http://dnp.chemnetbase.com
OmicsFusion	http://www.plantbreeding.wur.nl/omicsFusion/

7

DISCUSSION

DISCUSSION

The use of functional foods, such as margarines with plant sterols, fruit juice enriched with calcium and cereals with (soluble) fibre, has increased rapidly during the last years (*1-3*). The world health organisation (WHO) defines a functional food as "any food claiming to have a health-promoting or disease-preventing property beyond the basic function of supplying nutrients". The health-promoting effects of functional foods may be (partly) due to the presence of bioactive compounds (*4, 5*). In order to predict health-promoting effects of functional foods and to identify bioactive compounds accounting for these effects, tools for identification of bio-functional characteristics of food items are essential. Reporter gene assays may be fast and useful tools to facilitate detection of effects of food products and bioactive compounds present in these foods, on gene expression pathways. Therefore, the aim of this thesis was to develop and validate stable reporter cell lines for several health-related endpoints and to demonstrate their usefulness by assessing tomato extracts and bioactive compounds known to be present in tomato fruits.

Three health-related endpoints were chosen for this approach; electrophile response element (EpRE)-mediated gene expression, peroxisome proliferator-activated receptor γ (PPARγ)-mediated gene expression and peroxisome proliferator-activated receptor α (PPARα)-mediated gene expression. Genes regulated by EpRE include phase II detoxifying enzymes, which stimulate the conversion of reactive electrophiles into less toxic metabolites which can be easily excreted from the body (*6, 7*). In this way, phase II enzymes may be able to detoxify carcinogenic metabolites and help in the protection against carcinogenesis (*6, 8, 9*). The second selected endpoint was activation of PPARγ and PPARγ-mediated gene expression. PPARγ is an important stimulator of lipogenesis and adipogenesis (*10, 11*) and activation of PPARγ has been related to improved insulin sensitivity in patients with type 2 diabetes mellitus (*12*). The third and last selected endpoint was activation of PPARα and PPARα-mediated gene expression. PPARα is an important factor in lipid metabolism (*13, 14*) and activation of PPARα has been related to improved lipid levels in plasmaincluding an increase in HDL cholesterol and a decrease in LDL cholesterol and triglyceride plasma levels (*15-17*).

In order to test fruits and vegetables for the selected functions in reporter gene assays, extraction methods for vegetables and fruits are needed which result in extracts suitable for testing in reporter gene assays. In **chapter 2** of this thesis, methods are described to prepare three tomato extracts: an isoprenoid-containing tomato extract, a nonhydrolyzed tomato extract containing semi-polar compounds, and an enzymatically hydrolyzed tomato extract containing deglycosylated semi-polar compounds. EpRE-LUX reporter cells were exposed to these tomato extracts and all three tomato extracts were compatible with the EpRE-LUX reporter cells. The isoprenoid-containing chloroform extract of tomato fruit was unable to induce EpRE-regulated gene expression, which was in line with the finding that most individual isoprenoids also were unable to do so. The nonhydrolyzed tomato extract also failed to induce EpRE-regulated gene expression, just as the flavonoid glycosides failed to induce this response. The enzymatically hydrolyzed tomato extract was able to induce EpRE-mediated luciferase expression, both at mRNA level and at

protein level. Furthermore, the flavonoid aglycones quercetin, kaempferol, naringenin and naringenin chalcone were also able to induce EpRE-mediated gene expression and may therefore contribute to the induction seen with the enzymatically hydrolyzed tomato extract. So, tomato extracts and tomato compounds were able to induce EpRE-mediated gene expression *in vitro*. Whether tomato consumption indeed leads to EpRE-mediated up-regulation of phase II enzymes and to protection against cancer, should be endorsed by results from *in vivo* studies. From literature it is known that in mice, a combination of tomato and garlic leads to increased activity of the phase II enzyme GST and to reduced frequencies of chemically-induced genotoxicity (*18*). This indicates that tomato may indeed induce EpRE-mediated changes in gene expression and may thereby increase the levels of phase II detoxifying enzymes, which in turn may play a role in protection against cancer. Altogether, results presented in chapter 2 suggest that induction of EpRE-regulated genes, such as detoxifying phase II and antioxidant enzymes, may contribute to the beneficial health effects of tomato (*19*).

In **chapter 3**, two reporter gene assays, PPARγ1 CALUX and PPARγ2 CALUX, were developed in order to enable fast and low-cost measurement of PPARγ agonist and antagonist activity. These cell lines were constructed by stable transfection of U2OS cells with an expression vector for PPARγ1 or PPARγ2, and a pGL3-3xPPRE-tata-luc or pGL4-3xPPRE-tata-luc reporter construct, respectively. PPARγ1 CALUX and PPARγ2 CALUX cells showed similar concentration-dependent luciferase induction upon exposure to several well-known PPARγ agonists. The potency to induce PPARγ-mediated gene expression of the investigated PPARγ agonists decreased in the following order: rosiglitazone > troglitazone = pioglitazone > netoglitazone > ciglitazone. A concentration-dependent decrease in the response to 50 nM rosiglitazone was observed upon addition of the PPARγ antagonists GW9662 or T0070907, both in PPARγ1 CALUX and PPARγ2 CALUX cells, suggesting that the response was indeed mediated by PPARγ activation. The PPARα agonists WY14643 and fenofibrate failed to induce luciferase activity, confirming the specificity of these cell lines for PPARγ agonists. It was concluded that PPARγ1 CALUX and PPARγ2 CALUX cells presented in this chapter provide a reliable and useful tool to screen (bio-)chemicals for PPARγ agonist or antagonist activity (*20*).

Since beneficial effects related to tomato consumption partially overlap with those related to PPARγ activation, the aim of **chapter 4** was to test extracts of tomato fruits and tomato components, including polyphenols, isoprenoids and fatty acids, for their potency to activate PPARγ. Thirty tomato compounds were investigated, of which seven carotenoids and three polyphenols were able to induce PPARγ2-mediated gene expression. This is in line with the findings that two extracts of tomato fruit, one isoprenoid-containing tomato extract and one enzymatically hydrolyzed tomato extract containing deglycosylated phenolic compounds, also induced PPARγ2-regulated expression at physiologically relevant concentrations. Furthermore, enzymatically hydrolyzed extracts of seven tomato varieties were all able to induce PPARγ-mediated expression, with a 1.6 fold difference between the least potent and the most potent variety. The two most potent varieties had high flavonoid content, while the two least potent varieties had low flavonoid content,

suggesting that the differences in potency to induce PPARγ-mediated gene expression may be partly explained by the differences in levels of kaempferol and naringenin and/or other flavonoids. Mixtures of the tomato components kaempferol, naringenin and β-carotene were found to produce an additive effect, showing that various classes of bioactive compounds might be involved in induction of the PPARγ-mediated response observed with the tomato extracts. Activation of PPARγ has been related to improved insulin sensitivity in patients with type 2 diabetes mellitus and here it was shown that tomato and phytochemicals present in tomato are able to induce PPARγ-mediated gene expression. Literature data confirming that consumption of tomato also leads to PPARγ-mediated effects on insulin sensitivity and glucose tolerance *in vivo* are currently lacking. In summary, the data in chapter 4 indicate that tomato fruits and tomato compounds are able to induce PPARγ-mediated gene expression, and that some tomato varieties are more potent than others. Moreover, these data indicate that induction of PPARγ2-mediated transcription by tomato compounds may contribute to the beneficial effects associated with tomato consumption.

The development and validation of a stable reporter gene assay for PPARα-mediated gene expression, called the PPARα CALUX assay, was described in **chapter 5**. The PPARα CALUX cells showed a concentration-dependent increase of PPARα-mediated luciferase activity upon exposure to the PPARα agonists GW7647, GW9578, WY14643 and bezafibrate. The newly developed PPARα CALUX assay was then used to test tomato extracts as well as individual tomato components, including flavonoids and carotenoids, for their potency to activate PPARα-mediated gene expression. An isoprenoid-containing extract of tomato was able to induce PPARα-mediated gene expression, which is in line with our findings that β-carotene, γ-carotene and lycopene induce PPARα-mediated gene expression as well. In addition, it was shown that an extract of tomato containing semi-polar compounds is only a weak inducer of PPARα-mediated gene expression, but that enzymatic hydrolysis of the tomato homogenate before extraction greatly increases its potency. This is in line with our findings that none of the flavonoid glycosides is able to induce PPARα-mediated gene expression, but that the aglycones kaempferol and naringenin are able to do so. Furthermore, of the seven tomato varieties tested for their capacity to induce PPARα-mediated gene expression, the varieties showing the highest potency are also the ones containing the highest amounts of kaempferol and naringenin. Although this suggests that naringenin and kaempferol would contribute to the induction of PPARα-mediated gene expression found with the enzymatically hydrolyzed tomato extracts, the actual levels of kaempferol and naringenin detected in the extracts cannot fully explain the high (up to 12-fold) induction found with these tomato varieties. Presumably there are more, yet unidentified PPARα-activating compounds present in tomato. Tomato and its phytochemicals were shown to induce PPARα-mediated gene expression *in vitro*, and *in vivo* data are needed to confirm that PPARα activation by tomato and tomato compounds leads to beneficial health effects *in vivo*. Data from literature indicate that tomato consumption leads to decreased blood pressure, decreased total cholesterol and LDL cholesterol levels, and decreased myocardial lesions after myocardial infarctions *in vivo*

(*21-24*), although it is not shown that these effects are PPARα-dependent. Altogether, the data presented in chapter 5 indicate that the beneficial effects associated with tomato consumption may be partly explained by induction of PPARα-mediated gene transcription by tomato compounds.

In order to corroborate that reporter gene assays are capable to reveal potency differences between different tomato varieties, extracts of 97 different tomato accessions were screened for their capacity to induce EpRE-mediated reporter gene expression in the EpRE-LUX assay in **chapter 6**. There was almost three-fold difference in the observed luciferase activity between the least potent tomato variety and the most potent tomato variety. Metabolomic profiles of the 97 extracts were generated in order to identify phytochemicals responsible for the differences in potency between these 97 tomato varieties to induce EpRE-mediated gene expression. By combining the reporter gene assay data with the metabolic profile of each tomato extract, and performing multivariate analysis, it was possible to identify the flavonoid aglycone quercetin as one of the main compounds responsible for the ability of tomato extracts to induce EpRE-mediated gene expression. However, spiking the low-quercetin tomato extracts with extra quercetin did not always result in proportional activation. This indicates that matrix effects may play a role. It also seems likely that some yet unidentified compounds contribute to EpRE-mediated gene expression induced by tomato extracts. The results presented in chapter 6 indicate that combining data from reporter gene assays with metabolomics data of tomatoes presents a powerful approach towards the identification of health beneficial constituents of foods.

It was shown in chapters 2, 4 and 5 that reporter gene assays can be implemented in screening bioactive food compounds as well as whole fruit and vegetable extracts for their capacity to modulate transcription factor-mediated gene expression. Reporter gene assays are rapid and low-cost assays which are easy to perform. Using reporter gene assays, many compounds can be screened for their effects on gene expression mediated by a specific receptor, in a short period. Reporter cell lines, however, contain only one cell type, and therefore miss the interaction with other cell types which is occurring in tissues, organs and organ systems in *in vivo* studies. This means that results from reporter gene assays cannot be directly translated into *in vivo* effects (*25*). So, although our data presented in chapters 2, 4 and 5 indicate that tomato extracts and tomato compounds are able to induce EpRE-, PPARγ- and/or PPARα-mediated gene expression, *in vivo* experiments are needed to confirm that activation of these pathways by tomato and its phytochemicals also leads to EpRE-, PPARγ- and/or PPARα-mediated gene expression and possible related health effects *in vivo*. On the other hand, reporter gene assays are useful in a tiered screening approach enabling testing of many food items and/or food compounds and making a selection of the most interesting ones for further *in vivo* testing. In this way, the use of reporter gene assays may lead to a reduction in animal experiments. This is relevant in view of both economic and animal welfare perspectives.

In the three reporter gene assays used in this thesis, the induction found with tomato extracts is higher than expected based on the data of individual phytochemicals investigated so far. Additive and/or synergistic effects may occur in mixtures of phytochemicals, as reported in chapter 5 and in literature (*26, 27*). Furthermore, a matrix effect may enhance the effects of phytochemicals in whole tomato extracts but may be absent when testing individual compounds. For example, the cellular uptake, bioactivation and biodegradation of a compound might be influenced by the surrounding matrix (*28*). Furthermore, for PPARγ and PPARα, it is known that they bind to the peroxisome proliferator-response element (PPRE) as a heterodimer with RXR (*29, 30*). If tomato contains phytochemicals which are able to activate RXR, this may (partly) explain the high induction found with tomato extracts. Last but not least, other, yet unidentified compounds in tomato may be contributing to the effects on EpRE-, PPARγ- and PPARα-mediated gene expression.

FUTURE PERSPECTIVES

In chapter 2 of this thesis, a robust method was developed to prepare crude extracts of ripe tomato fruits that are compatible with cell-physiological and cell culture conditions and thus enable testing of these extracts in the cell-based EpRE-LUX reporter assay. It was shown in chapters 4 and 5 that these extraction methods also enabled the testing of whole tomato extracts in other reporter cell lines. Furthermore, in chapters 4, 5 and 6 it was shown that it is possible to detect differences between extracts of different tomato varieties in inducing transcription factor-mediated gene expression in reporter gene assays. This means that reporter gene assays provide a fast and low-cost method for breeders to select tomato varieties with an optimal composition of bioactive ingredients for inducing specific health-beneficial gene expression pathways. A next step could be to verify that this method is also applicable to other fruits and vegetables.

In a pilot study, nonhydrolyzed extracts and enzymatically hydrolyzed extracts of Brussels sprouts, Braeburn apple, broccoli and onion were prepared using the methods described in chapter 2 of this thesis. All extracts were compatible with cell-physiological and cell culture conditions and induced EpRE-mediated and PPARγ-mediated gene expression. Interestingly, nonhydrolyzed extracts of all four vegetables and fruits showed an induction in both the EpRE-LUX assay (figure 1A) and the PPARγ2 CALUX assay (figure 1B), while tomato only showed induction after enzymatic hydrolysis. The enzymatically hydrolyzed extracts of braeburn apple, broccoli and onion and Brussels sprouts gave an induction up to 25-fold in the EpRE-LUX assay (figure 1C) and an induction of 10- to 20-fold in the PPARγ CALUX assay (figure 1D). Especially broccoli and Brussels sprouts, both belonging to the cruciferous vegetables, showed a high capacity to induce EpRE-mediated gene expression. These data suggest that besides polyphenols, other compounds present in braeburn apple, onion, broccoli and Brussels sprouts are able to induce EpRE-mediated and PPARγ-mediated gene expression. Glucosinolates may be a good candidate, as they are present in cruciferous vegetables (like broccoli and Brussels sprouts) and as metabolites of glucosinolates, such as isothiocyanates have been shown before to induce EpRE-mediated gene expression (*31, 32*).

As a next step, it would be interesting to verify that the extraction methods described in chapter 2 can also be applied to processed foods, such as tomato sauce, tomato juice, tomato ketchup and tomato soup. Results thus obtained will reveal whether food processing has an effect on the capacity of tomato and its phytochemicals to induce health-beneficial gene expression pathways. Furthermore, it may provide a useful tool for food industry in selecting food items with optimal capacity to induce specific health-beneficial gene expression pathways and/or to adapt food processing procedures to make products that optimally retain bioactivity.

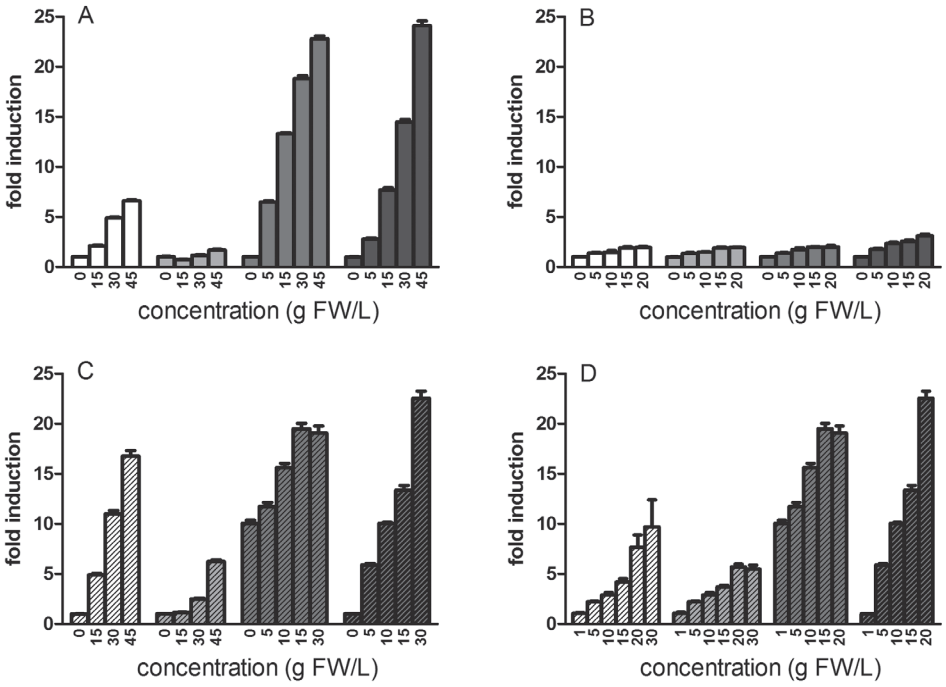

Figure 1. Luciferase activity is induced in EpRE-LUX cells (left panels, A and C) and PPARγ CALUX cells (right panels, B and D) upon exposure to nonhydrolyzed extracts (upper panels, A and B) and enzymatically hydrolyzed extracts (lower panels, C and D) of onion (white), Braeburn apple (light grey), broccoli (mid-grey) and Brussels sprouts (dark grey). The concentration of the extracts is given as gram fresh weight (FW) per liter.

In chapter 6, 97 different tomato varieties were tested for their capacity to induce EpRE-mediated gene expression using the EpRE-LUX assay. By combining these EpRE-LUX data with metabolic profiles of the same tomato extracts and performing multivariate analysis, the flavonoid aglycone quercetin was identified as a major compound responsible for the ability of tomato extracts to induce EpRE-mediated gene expression. As shown in chapter 5,

the induction of PPARα-mediated gene expression found with enzymatically hydrolyzed tomato extracts cannot be fully explained by the presence of flavonoid aglycones in these extracts. Presumably, there are more, yet unidentified bioactive compounds present in the enzymatically hydrolyzed tomato extracts. Testing more tomato varieties in the PPARα CALUX cell line and combining these data with metabolomic profiles of these tomato varieties may be of use in the identification of tomato compounds contributing to the induction of PPARα-mediated gene expression. In the same way, the methods described in chapter 6 may be of use in identifying phytochemicals contributing to the induction of EpRE-mediated and/or PPARγ-mediated gene expression observed with extracts of broccoli, Brussels sprouts, Braeburn apple and onion.

This thesis focusses on the beneficial health effects related to EpRE-mediated, PPARγ-mediated and PPARα-mediated gene expression. However, activation of the Nrf2/EpRE system and of PPARγ also have been related to adverse effects for human health.

Nrf2 has been related to cancer prevention by protecting against cancer cell formation, but also to cancer promotion. Permanent activation of Nrf2 has been reported to promote the survival of cancer cells and resistance to chemotherapy (*33, 34*). In some cancer types – including cancers in lung, gall bladder and neck – mutations of Keap1 and Nrf2 leadingto a permanent activation of the Nrf2/EpRE system were found, which was related to increased survival of tumour cells and resistance of these cancer cells to chemotherapy (*33, 34*). Bioactive compounds from foods, however, will presumably lead to transient activation of Nrf2 and not to permanent activation.

Besides the beneficial health effects of PPARγ activation, PPARγ activation by thiazolidinediones (TZDs) has also been related to adverse effects. TZDs are a group of PPARγ agonists shown to improve insulin sensitivity in patients suffering from diabetes mellitus type 2 (*35, 36*). Besides their effect on insulin sensitivity, well-known adverse effects of TZDs have been reported. First of all, compound specific adverse effects have been reported. Troglitazone and rosiglitazone were both taken from the market; troglitazone for its liver toxicity (*37*), and rosiglitazone due to concerns about its cardiovascular safety (*38*). Pioglitazone is still prescribed in most European countries, but in France and Germany it was taken from the market due to increased risk of bladder cancer (*39*). Furthermore, more general side effects have been reported. Full-PPARγ agonists – including TZDs – have clear therapeutic effects on insulin sensitivity and hyperglycemia, but are also related to adverse effects like edema, fluid retention and weight gain (*40*). Currently, it is believed that selective PPARγ modulators, SPPARMs, may have the same clinical benefits as TZDs, but without their adverse effects (*41, 42*). The SPPARM concept may be promising in the continuing search for new, safer PPARγ-activating drugs.

Besides the degree of PPARγ activation, also the duration of PPARγ activation plays an important role. It is to be expected that bioactive compounds from food lead to a transient activation of PPARγ maintaining metabolic flexibility. In long-term, or even permanent PPARγ activation (which can for example be caused by mutations), this metabolic flexibility may be compromised.

The market for functional foods is still growing and, given the EU requirements, there is a major interest to scientifically support beneficial effects of functional foods and their bioactive ingredients. In this thesis, it was shown that reporter gene assays provide a reliable and useful tool to screen extracts of fruits and vegetables for their capacity to induce transcription factor-mediated gene expression. The next question is if and how reporter gene assays can be useful in supporting nutrition and health claims for functional foods.

Health claims are defined by the European Food Safety Authority (EFSA) as "any claim that states, suggests or implies that a relationship exists between a food category, a food or one of its constituents and health." EU regulation 1924/2006 became effective in January 2007, stating that only health claims approved by the European Commission are allowed (*43*). Health claims are divided into article 13 claims and article 14 claims. Article 14 claims refer to a reduction of disease risk or to the growth, development and health of children. These claims need individual authorisation based on extensive scientific support. Article 13 claims refer to the role of a food compound in growth, development and functions of the body; psychological and behavioural functions; or slimming, weight reduction and satiety. Article 13 claims are subdivided into two categories: article 13.1 claims and article 13.5 claims. Article 13.1 claims are supported by generally accepted scientific data, while article 13.5 claims are based on new scientific data. Once an article 13 claim is approved it can be generally used without the necessity of approval for each product (*43*).

PPARγ activation may be an interesting target for health claims, as PPARγ activation is related to many beneficial effects in several diseases and physiological processes (table 1 in the general introduction of this thesis) and PPARγ can be activated by bioactive ingredients in food. A possible health claim related to PPARγ could be: "This product contains PPARγ agonists; PPARγ agonists increase insulin sensitivity". This possible health claim would be an article 13.1 health claim, which means that once this health claim is approved, any product containing PPARγ agonists is allowed to contain this claim (*43*). The PPARγ2 CALUX cell line can then be used as a fast and low-cost tool to identify functional foods or food constituents functioning as PPARγ agonists. For the approval of such a health claim, it is important to provide data showing a causal relationship between consumption of the food product or food constituent and the beneficial health effect, preferably in human studies. Activation of PPARγ by thiazolidinediones (TZDs), a class of synthetic PPARγ agonists which were able to induce PPARγ-mediated gene expression in our newly developed PPARγ assay, improves insulin sensitivity in human (*44-47*), showing a cause-effect relationship between TZDs and insulin sensitivity. This mere fact may by itself not prove that the effect is PPARγ-dependent. It has, however, been shown that the minimum effective dose (MED) of TZDs for antihyperglycemic activity in *ob/ob* mice is closely related to their EC_{50} value for PPARγ activation in an *in vitro* reporter gene assay (*48*). These findings suggest that the influence of TZDs on insulin sensitivity is mediated by PPARγ. Human intervention studies showing an effect of natural PPARγ agonists on insulin sensitivity are presently not available. There is evidence, however, showing that insulin resistant volunteers had lower plasma concentrations of β-carotene (*49*) and that plasma concentrations of β-carotene and

lycopene are lower in subjects with impaired glucose tolerance or diabetes (*50*). Besides evidence for a cause-and-effect relationship, it is important to provide evidence showing that the claimed effect is relevant for human health in the target population (*43*). In their opinion on earlier health claim applications, insulin sensitivity was considered a beneficial physiological effect by the NDA Panel of EFSA (*51*).

It was shown in this thesis that reporter gene assays may give insight in the effects of fruit and vegetable extracts on the activation of specific transcription factors and subsequent modulation of gene expression patterns. By testing fruit and vegetable extracts in multiple reporter gene assays, a more complete picture of the overall health effect will be generated. For example, it would be of interest to include an assay for RXR, the dimerizing partner of PPARs. Including a reporter gene assay for PPARβ/δ would be useful to discriminate between specific agonists for one PPAR isotype and dual/pan PPAR agonists activating two (dual) or all three (pan) PPAR isotypes. Furthermore, reporter gene assays for other health-related transcription factors could be included. Insulin sensitivity is for example not only influenced by PPARγ, but also fork head box O1 (FOXO1) plays a role. FOXO1 is a major target of insulin and supresses insulin sensitivity (*49*). Agonists of FOXO1 may counteract the effects of PPARγ activation and antagonists of FOXO1 may amplify the effects of PPARγ agonists. Therefore, a reporter gene assay for FOXO1 may be an interesting addition to the presently described panel of reporter gene assays.

Combining the results of multiple reporter gene assays with metabolomic profiling of these fruit and vegetable extracts may give insight in compounds contributing to the effects on transcription factor activation and subsequent modulation of gene expression. This approach of combining multiple reporter gene assays and metabolomic profiling, is very useful in fast screening of many food items and food compounds. Therefore, the use of reporter gene assays as a first tier in a tiered approach aiming at supporting health claims will limit the number of animal studies and human studies needed, by enabling the ranking and selection of highly promising food items or constituents for further *in vivo* testing.

In this thesis, we focussed mainly on the polyphenols and carotenoids present in tomato. Flavonoids are present in tomato mainly in the form of glycosides. In EpRE-LUX, PPARγ CALUX and PPARα CALUX reporter gene assays, it was observed that flavonoid glycosides failed to induce luciferase expression, and this also holds for the nonhydrolyzed tomato extracts containing (amongst others) flavonoid glycosides (*19, 26, 52*). This may be a consequence of poor uptake of flavonoid glycosides into the reporter cells. Once the tomato extract was enzymatically hydrolyzed in order to remove glycosyl groups, its capacity to induce EpRE-mediated, PPARγ-mediated and PPARα-mediated gene expression drastically increased. In addition, it was shown in chapters 2, 4 and 5 that flavonoid aglycones, unlike flavonoid glycosides, were able to induce EpRE-mediated, PPARγ-mediated and PPARα-mediated gene expression. Deglycosylation of flavonoid glycosides also occurs *in vivo*: before or during uptake flavonoid glycosides are enzymatically deglycoslated in the intestine by β-glucosidase enzymes (*53, 54*). Once taken up, the flavonoid aglycones

are quickly metabolized, mainly into flavonoid glucuronides and other conjugates (*54*). Carotenoids are metabolised as well after uptake (*55-57*). As metabolism of flavonoids and carotenoids may affect their bioactivity, future studies focussing on metabolites of flavonoids and carotenoids would be useful. For example, liver cells and/or intestinal cells may be exposed to (mixtures of) food compounds and fruit and vegetable extracts. During exposure, metabolism of the bioactive compounds takes place and metabolites are excreted into the culture medium. This metabolite-containing culture medium may then be used to expose the reporter cells with. Lee-Hilz and colleagues showed that after 24 h incubating liver cells with quercetin, the culture medium contains various metabolites (mainly glucuronides) and that these metabolites are able to induce EpRE-mediated gene expression (*58*).

CONCLUSION

In conclusion, the work presented in this thesis reveals that tomato extracts as well as phytochemicals present in tomato are able to induce gene expression controlled by the EpRE or mediated by the transcription factors PPARγ or PPARα. Methods were presented to generate extracts of fruits and vegetables which are suitable for investigation in reporter gene assays. In addition, three reporter gene assays, PPARγ1 CALUX assay, PPARγ2 CALUX assay and PPARα CALUX assay were developed and validated within this project. Last but not least, this thesis showed that reporter gene assays are able to pick up differences in modulation of transcription factor-mediated gene expression by different varieties of tomato. This indicates that reporter gene assays can be used as fast and useful tools to select crop varieties with health-beneficial traits or food processing methods that lead to an improved content of possible health-beneficial phytochemicals.

REFERENCES

1. Katan, M. B.; Roos, N. M., Promises and Problems of Functional Foods. *Critical Reviews in Food Science and Nutrition* **2004,** *44*, 369-377.
2. Law, M., Plant sterol and stanol margarines and health. *BMJ* **2000,** *320*, 861-864.
3. Menrad, K., Market and marketing of functional food in Europe. *Journal of Food Engineering* **2003,** *56*, 181-188.
4. Arvanitoyannis, I. S.; Van Houwelingen-Koukaliaroglou, M., Functional Foods: A Survey of Health Claims, Pros and Cons, and Current Legislation. *Critical Reviews in Food Science and Nutrition* **2005,** *45*, 385-404.
5. Hasler, C. M., Functional Foods: Benefits, Concerns and Challenges—A Position Paper from the American Council on Science and Health. *The Journal of Nutrition* **2002,** *132*, 3772-3781.
6. Giudice, A.; Montella, M., Activation of the Nrf2–ARE signaling pathway: a promising strategy in cancer prevention. *Bioessays* **2006,** *28*, 169-181.
7. Talalay, P.; Dinkova-Kostova, A. T.; Holtzclaw, W. D., Importance of phase 2 gene regulation in protection against electrophile and reactive oxygen toxicity and carcinogenesis. *Advances in Enzyme Regulation* **2003,** *43*, 121-134.
8. Kwak, M.-K.; Wakabayashi, N.; Kensler, T. W., Chemoprevention through the Keap1–Nrf2 signaling pathway by phase 2 enzyme inducers. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **2004,** *555*, 133-148.
9. Lee, J. S.; Surh, Y. J., Nrf2 as a novel molecular target for chemoprevention. *Cancer Letters* **2005,** *224*, 171-184.
10. Barak, Y.; Nelson, M. C.; Ong, E. S.; Jones, Y. Z.; Ruiz-Lozano, P.; Chien, K. R.; Koder, A.; Evans, R. M., PPAR[gamma] Is Required for Placental, Cardiac, and Adipose Tissue Development. *Molecular Cell* **1999,** *4*, 585-595.
11. Tontonoz, P.; Graves, R. A.; Budavari, A. I.; Erdjument-Bromage, H.; Lui, M.; Hu, E.; Tempst, P.; Spiegelman, B. M., Adipocyte-specific transcription factor ARF6 is a heterodimeric complex of two nuclear hormone receptors, PPAR7 and RXRa. *Nucleic Acids Research* **1994,** *22*, 5628-5634.
12. Tontonoz, P.; Spiegelman, B. M., Fat and Beyond: The Diverse Biology of PPARÎ³. *Annual Review of Biochemistry* **2008,** *77*, 289-312.
13. Kersten, S.; Seydoux, J.; Peters, J. M.; Gonzalez, F. J.; Desvergne, B.; Wahli, W., Peroxisome proliferator–activated receptor α mediates the adaptive response to fasting. *The Journal of Clinical Investigation* **1999,** *103*, 10.
14. Reddy, J. K.; Hashimoto, T., Peroxisomal beta-oxidation and peroxisome proliferator-activated receptor alpha: an adaptive metabolic system. *Annual review of nutrition* **2001,** *21*, 193-230.
15. Idzior-Walus, B.; Sieradzki, J.; Rostworowski, W.; Zdzienicka, A.; Kawalec, E.; Wójcik, J.; Żarnecki, A.; Blane, G., Effects of comicronised fenofibrate on lipid and insulin sensitivity in patients with polymetabolic syndrome X. *European Journal of Clinical Investigation* **2000,** *30*, 871-878.
16. Koh, K. K.; Han, S. H.; Quon, M. J.; Yeal Ahn, J.; Shin, E. K., Beneficial Effects of Fenofibrate to Improve Endothelial Dysfunction and Raise Adiponectin Levels in Patients With Primary Hypertriglyceridemia. *Diabetes Care* **2005,** *28*, 1419-1424.
17. Remick, J.; Weintraub, H.; Setton, R.; Offenbacher, J.; Fisher, E.; Schwartzbard, A., Fibrate Therapy: An Update. *Cardiology in Review* **2008,** *16*, 129-141 10.1097/CRD.0b013e31816b43d3.
18. Kumaraguruparan, R.; Chandra Mohan, K. V. P.; Abraham, S. K.; Nagini, S., Attenuation of N-methyl-N'-nitro-N-nitrosoguanidine induced genotoxicity and oxidative stress by tomato and garlic combination. *Life Sciences* **2005,** *76*, 2247-2255.
19. Gijsbers, L.; van Eekelen, H. D. L. M.; Nguyen, T. H.; de Haan, L. H. J.; van der Burg, B.; Aarts, J. M. M. J. G.; Rietjens, I. M. C. M.; Bovy, A. G., Induction of electrophile-responsive element (EpRE)-mediated gene expression by tomato extracts in vitro. *Food Chemistry* **2012,** *135*, 1166-1172.
20. Gijsbers, L.; Man, H.-Y.; Kloet, S. K.; de Haan, L. H. J.; Keijer, J.; Rietjens, I. M. C. M.; van der Burg, B.; Aarts, J. M. M. J. G., Stable reporter cell lines for peroxisome proliferator-activated receptor γ (PPARγ)-mediated

modulation of gene expression. *Analytical Biochemistry* **2011,** *414*, 77-83.

21. Engelhard, Y. N.; Gazer, B.; Paran, E., Natural antioxidants from tomato extract reduce blood pressure in patients with grade-1 hypertension: A double-blind, placebo-controlled pilot study. *American Heart Journal* **2006,** *151*, 100.e6-100.e1.
22. Paran, E.; Novack, V.; Engelhard, Y.; Hazan-Halevy, I., The Effects of Natural Antioxidants from Tomato Extract in Treated but Uncontrolled Hypertensive Patients. **2009,** *23*, 145-151.
23. Parvin, R.; Akhter, N., *Protective effect of tomato against adrenaline-induced myocardial infarction in rats*. 2009; Vol. 34.
24. Silaste, M.-L.; Alfthan, G.; Aro, A.; Antero Kesäniemi, Y.; Hörkkö, S., Tomato juice decreases LDL cholesterol levels and increases LDL resistance to oxidation. *British Journal of Nutrition* **2007,** *98*, 1251-1258.
25. Harry, G. J.; Billingsley, M.; Bruinink, A.; Campbell, I. L.; Classen, W.; Dorman, D. C.; Galli, C.; Ray, D.; Smith, R. A.; Tilson, H. A., In Vitro Techniques for the Assessment of Neurotoxicity. *Environmental Health Perspectives* **1998,** *106*, 131-158.
26. Gijsbers, L.; van Eekelen, H. D. L. M.; de Haan, L. H. J.; Swier, J. M.; Heijink, N. L.; Man, H.-Y.; Bovy, A. G.; Keijer, J.; Aarts, J. M. M. J. G.; van der Burg, B.; Rietjens, I. M. C. M., Induction of peroxisome proliferator-activated receptor γ (PPARγ)-mediated gene expression by tomato (Solanum lycopersicum L.) extracts. **Submitted to Journal of Agricultural and Food Chemistry**.
27. Liu, R. H., Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. *The American Journal of Clinical Nutrition* **2003,** *78*, 517S-520S.
28. Jeurissen, S. M. F.; Punt, A.; Delatour, T.; Rietjens, I. M. C. M., Basil extract inhibits the sulfotransferase mediated formation of DNA adducts of the procarcinogen 1'-hydroxyestragole by rat and human liver S9 homogenates and in HepG2 human hepatoma cells. *Food and Chemical Toxicology* **2008,** *46*, 2296-2302.
29. Berger, J.; Moller, D. E., The mechanisms of action of PPARs. *Annual Review of Medicine* **2002,** *53*, 409-435.
30. Michalik, L.; Wahli, W., Peroxisome proliferator-activated receptors: three isotypes for a multitude of functions. *Current Opinion in Biotechnology* **1999,** *10*, 564-570.
31. Hayes, J. D.; Kelleher, M. O.; Eggleston, I. M., The cancer chemopreventive actions of phytochemicals derived from glucosinolates. *European journal of nutrition* **2008,** *47 Suppl 2*, 73-88.
32. Vermeulen, M.; Boerboom, A.-M. M. J. F.; Blankvoort, B. M. G.; Aarts, J. M. M. J. G.; Rietjens, I. M. C. M.; van Bladeren, P. J.; Vaes, W. H. J., Potency of isothiocyanates to induce luciferase reporter gene expression via the electrophile-responsive element from murine glutathione S-transferase Ya. *Toxicology in Vitro* **2009,** *23*, 617-621.
33. Hayes, J. D.; McMahon, M., NRF2 and KEAP1 mutations: permanent activation of an adaptive response in cancer. *Trends in Biochemical Sciences* **2009,** *34*, 176-188.
34. Lau, A.; Villeneuve, N. F.; Sun, Z.; Wong, P. K.; Zhang, D. D., Dual roles of Nrf2 in cancer. *Pharmacological Research* **2008,** *58*, 262-270.
35. Bennett, S. M. A.; Agrawal, A.; Elasha, H.; Heise, M.; Jones, N. P.; Walker, M.; Wilding, J. P. H., Rosiglitazone improves insulin sensitivity, glucose tolerance and ambulatory blood pressure in subjects with impaired glucose tolerance. *Diabetic Medicine* **2004,** *21*, 415-422.
36. Raji, A.; Seely, E. W.; Bekins, S. A.; Williams, G. H.; Simonson, D. C., Rosiglitazone Improves Insulin Sensitivity and Lowers Blood Pressure in Hypertensive Patients. *Diabetes Care* **2003,** *26*, 172-178.
37. Gale, E. A. M., Lessons from the glitazones: a story of drug development. *The Lancet* **2001,** *357*, 1870-1875.
38. Krentz, A. J., Rosiglitazone: Trials, Tribulations and Termination. *Drugs* **2011,** *71*, 123-130.
39. Mamtani, R.; Haynes, K.; Bilker, W. B.; Vaughn, D. J.; Strom, B. L.; Glanz, K.; Lewis, J. D., Long-term therapy with thiazolidinediones and the risk of bladder cancer: A cohort study. *ASCO Meeting Abstracts* **2012,** *30*, 1503.
40. Yamanouchi, T., Concomitant therapy with pioglitazone and insulin for the treatment of type 2 diabetes. *Vascular health and risk management* **2010,** *6*, 189-97.
41. Higgins, L. S.; DePaoli, A. M., Selective peroxisome proliferator-activated receptor γ

(PPARγ) modulation as a strategy for safer therapeutic PPARγ activation. *The American Journal of Clinical Nutrition* **2010,** *91*, 267S-272S.

42. Zhang, F.; Lavan, B. E.; Gregoire, F. M., Selective Modulators of PPAR-γ; Activity: Molecular Aspects Related to Obesity and Side-Effects. *PPAR Research* **2007,** *2007*.
43. Verhagen, H.; Vos, E.; Francl, S.; Heinonen, M.; van Loveren, H., Status of nutrition and health claims in Europe. *Archives of Biochemistry and Biophysics* **2010,** *501*, 6-15.
44. Derosa, G.; Salvadeo, S. A. T.; D'Angelo, A.; Fogari, E.; Ragonesi, P. D.; Ciccarelli, L.; Piccinni, M. N.; Ferrari, I.; Gravina, A.; Maffioli, P.; Cicero, A. F., Rosiglitazone Therapy Improves Insulin Resistance Parameters in Overweight and Obese Diabetic Patients Intolerant To Metformin. *Archives of Medical Research* **2008,** *39*, 412-419.
45. Kahn, S. E.; Utzschneider, K. M., What's Next for Diabetes Prevention? *Diabetes Care* **2011,** *34*, 1678-1680.
46. Miyazaki, Y.; Mahankali, A.; Wajcberg, E.; Bajaj, M.; Mandarino, L. J.; DeFronzo, R. A., Effect of Pioglitazone on Circulating Adipocytokine Levels and Insulin Sensitivity in Type 2 Diabetic Patients. *Journal of Clinical Endocrinology & Metabolism* **2004,** *89*, 4312-4319.
47. Yamasaki, Y.; Kawamori, R.; Wasada, T.; Sato, A.; Omori, Y.; Eguchi, H.; Tominaga, M.; Sasaki, H.; Ikeda, M.; Kubota, M.; Ishida, Y.; Hozumi, T.; Baba, S.; Uehara, M.; Shichiri, M.; Kaneko, T.; Ad- Glucose Clamp Study Group, J., Pioglitazone (AD-4833) Ameliorates Insulin Resistance in Patients with NIDDM. *The Tohoku Journal of Experimental Medicine* **1997,** *183*, 173-183.
48. Willson, T. M.; Cobb, J. E.; Cowan, D. J.; Wiethe, R. W.; Correa, I. D.; Prakash, S. R.; Beck, K. D.; Moore, L. B.; Kliewer, S. A.; Lehmann, J. M., The Structure−Activity Relationship between Peroxisome Proliferator-Activated Receptor γ Agonism and the Antihyperglycemic Activity of Thiazolidinediones. *Journal of Medicinal Chemistry* **1996,** *39*, 665-668.
49. Facchini, F. S.; Humphreys, M. H.; DoNascimento, C. A.; Abbasi, F.; Reaven, G. M., Relation between insulin resistance and plasma concentrations of lipid hydroperoxides, carotenoids, and tocopherols. *The American Journal of Clinical Nutrition* **2000,** *72*, 776-779.
50. Ford, E. S.; Will, J. C.; Bowman, B. A.; Narayan, K. M. V., Diabetes Mellitus and Serum Carotenoids: Findings from the Third National Health and Nutrition Examination Survey. *American Journal of Epidemiology* **1999,** *149*, 168-176.
51. NDA, E. p., Opinion on the substantiation of health claims related to alpha-lipoic acid and "protection of the nerve system" (ID 3157) and increase in insulin sensitivity (ID 3158) pursuant to Article 13(1) of Regulation (EC) No 1924/20061. *EFSA journal* **2011,** *9*, 2202.
52. Gijsbers, L.; Man, H.-Y.; van Eekelen, H. D. L. M.; de Haan, L. H. J.; Keijer, J.; Aarts, J. M. M. J. G.; Rietjens, I. M. C. M.; van der Burg, B., Induction of peroxisome proliferator-activated receptor α (PPARα)-mediated gene expression by extracts of tomato. **in preparation**.
53. Day, A. J.; DuPont, M. S.; Ridley, S.; Rhodes, M.; Rhodes, M. J. C.; Morgan, M. R. A.; Williamson, G., Deglycosylation of flavonoid and isoflavonoid glycosides by human small intestine and liver β-glucosidase activity. *FEBS Letters* **1998,** *436*, 71-75.
54. Walle, T.; Otake, Y.; Walle, U. K.; Wilson, F. A., Quercetin Glucosides Are Completely Hydrolyzed in Ileostomy Patients before Absorption. *The Journal of Nutrition* **2000,** *130*, 2658-2661.
55. During, A.; Harrison, E. H., Intestinal absorption and metabolism of carotenoids: insights from cell culture. *Archives of Biochemistry and Biophysics* **2004,** *430*, 77-88.
56. Khachik, F.; Carvalho, L.; Bernstein, P. S.; Muir, G. J.; Zhao, D.-Y.; Katz, N. B., Chemistry, Distribution, and Metabolism of Tomato Carotenoids and Their Impact on Human Health. *Experimental Biology and Medicine* **2002,** *227*, 845-851.
57. Parker, R. S., Absorption, metabolism, and transport of carotenoids. *The FASEB Journal* **1996,** *10*, 542-51.
58. Lee-Hilz, Y. Y.; Stolaki, M.; van Berkel, W. J. H.; Aarts, J. M. M. J. G.; Rietjens, I. M. C. M., Activation of EpRE-mediated gene transcription by quercetin glucuronides depends on their deconjugation. *Food and Chemical Toxicology* **2008,** *46*, 2128-2134.

8

SUMMARY

SUMMARY

The consumption of food products with health-promoting properties, such as margarines with plant sterols, fruit juice enriched with calcium and cereals with (soluble) fibre, has increased rapidly during the last years. The present thesis provides proof-of-principle that reporter gene assays are effective tools to investigate the effects of functional foods and food compounds on transcription factor-mediated gene expression.

In order to test fruits and vegetables for selected functions in reporter gene assays, extraction methods for vegetables and fruits are needed which result in extracts suitable for testing in reporter gene assays. In **chapter 2**, methods are described to prepare three tomato extracts:

- a nonhydrolyzed tomato extract containing apolar compounds such as isoprenoids, further referred to as isoprenoid-containing tomato extract,
- a nonhydrolyzed extract containing semi-polar compounds, further referred to as nonhydrolyzed tomato extract,
- an extract of enzymatically hydrolyzed tomato, containing deglycosylated semi-polar compounds and further referred to as hydrolyzed tomato extract.

All three tomato extracts were compatible with cell-physiological and cell culture conditions and tested in the EpRE-LUX assay. Both the isoprenoid-containing chloroform extract of tomato fruit and most individual isoprenoids (except for neurosporene) were unable to induce EpRE-mediated gene expression. The nonhydrolyzed tomato extract as well as the flavonoid glycosides were not able to induce EpRE-mediated gene expression. The enzymatically hydrolyzed tomato extract, containing deglycosylated polyphenols, was able to induce EpRE-mediated luciferase expression at both mRNA level and protein level. In line with this finding, the flavonoid aglycones quercetin, kaempferol, naringenin and naringenin chalcone also induced EpRE-mediated gene expression and these four flavonoid aglycones may therefore contribute to the gene expression induction seen with the enzymatically hydrolyzed tomato extract. Together, the results presented in chapter 2 suggest that induction of EpRE-regulated genes, such as detoxifying phase II and antioxidant enzymes, may contribute to the beneficial health effects of tomato.

Chapter 3 of this thesis describes the development and validation of two newly developed stable reporter gene assays; the PPARγ1 CALUX assay and the PPARγ2 CALUX. It was shown that the newly developed stable reporter cell lines PPARγ1 CALUX and PPARγ2 CALUX specifically respond to agonists for PPARγ, and that these reporter cell lines provide a reliable and useful tool to screen (bio-)chemicals for PPARγ agonist or antagonist activity.

As beneficial effects related to PPARγ activation partially overlap with those associated with tomato consumption, tomato components and extracts of tomato fruits were tested for their capacity to induce PPARγ-mediated gene expression in **chapter 4**. Thirty tomato compounds were selected based on their presence in tomato fruit and were investigated using the PPARγ2 CALUX cells. Of these compounds, three fatty acids, seven carotenoids and three polyphenols were able to induce PPARγ2-mediated gene expression. In line

with these findings, both the isoprenoid-containing tomato extract and the enzymatically hydrolyzed extract, containing deglycosylated phenolic compounds, also induced PPARγ-mediated gene expression. These data indicate that induction of PPARγ-mediated transcription by tomato compounds may contribute to the beneficial effects associated with tomato consumption.

In **chapter 5**, the development and validation of a newly developed PPARα CALUX reporter assay is described. Tomato extracts as well as individual tomato components, including flavonoids and carotenoids, were tested for their capacity to activate PPARα-mediated gene expression using the PPARα CALUX assay. The isoprenoid-containing tomato extract was able to induce PPARα-mediated gene expression, which is consistent with our findings that β-carotene, γ-carotene and lycopene also induced PPARα-mediated gene expression. In addition, it was shown that the nonhydrolyzed tomato extract, containing semi-polar compounds, is only a weak inducer of PPARα-mediated gene expression, but that enzymatic hydrolysis of the tomato homogenate before extraction greatly increased the inducing potency. This is in line with our findings that none of the flavonoid glycosides is able to induce PPARα-mediated gene expression, but that the flavonoid aglycones kaempferol and naringenin are.

To assess differences between tomato extracts, enzymatically hydrolyzed extracts of seven different tomato varieties were generated and tested for their capacity to induce PPARγ-mediated **(chapter 4)** and PPARα-mediated **(chapter 5)** gene expression. The extracts of these varieties were all able to induce both PPARγ-mediated gene expression and PPARα-mediated gene expression. For PPARγ-mediated gene expression, a 1.6-fold difference was found between the least potent and the most potent variety, and for the PPARα-mediated gene expression this was 1.7-fold. In both the PPARγ2 CALUX assay and the PPARα CALUX assay, the two most potent varieties had a high flavonoid content, while the two least potent varieties had a low flavonoid content. This may suggest that the differences between these extracts of tomato varieties in potency to induce PPARγ-mediated gene expression or PPARα-mediated gene expression may be partly explained by differences in levels of kaempferol and naringenin and/or other flavonoids. However, kaempferol and naringenin showed relatively low induction (up to 5-fold for PPARγ-mediated gene expression and up to 1.5-fold for PPARα-mediated gene expression) and cannot fully explain the high induction (up to 10-fold for PPARγ-mediated gene expression and up to 9-fold for PPARα-mediated gene expression) found with the hydrolyzed tomato extract. Presumably there are more, yet unidentified PPARγ-activating and PPARα-activating compounds present in the hydrolyzed tomato extract.

In **chapter 6**, extracts of 97 different tomato accessions were screened for their capacity to induce EpRE-mediated gene expression using the EpRE-LUX assay. A 3-fold difference in EpRE-mediated luciferase activity between the least potent tomato variety and the most potent tomato variety was observed. Metabolomic profiles of the 97 extracts were generated in order to identify phytochemicals responsible for the differences in potency of the 97 tomato varieties to induce EpRE-mediated gene expression. The flavonoid aglycone quercetin was identified as one of the main compounds responsible for the ability of

tomato extracts to induce EpRE-mediated gene expression, using multivariate analysis combining the reporter gene assay data with metabolite profiles of the same tomato extracts. In addition, also yet unidentified compounds correlated with the response in the EpRE-LUX assay and these compounds may also contribute to EpRE induction. The results presented in chapter 6 indicate that combining reporter gene assays and metabolomic profiling of tomatoes provides a powerful tool towards the identification of its health beneficial constituents.

Future studies to verify that the extraction methods presented in chapter 2 of this thesis are also applicable to other fruits and vegetables and to processed foods, would be useful. As metabolism of flavonoids and carotenoids may affect their bioactivity, future studies may also focus on the bioactivity of flavonoid and carotenoid metabolites. A further issue to consider in future research is under which conditions the increases in EpRE-, PPARγ- or PPARα-mediated gene expression may turn out to reflect adverse instead of beneficial health effects. In addition, *in vivo* validation of the possible health effects of the increased EpRE-, PPARγ- or PPARα-mediated gene expression would be of importance and could focus on the compounds and varieties defined as being most active in our present work using the reporter gene assays.

In conclusion, the work presented in this thesis provides proof-of-principle that reporter gene assays can be implemented for screening bioactive food compounds as well as whole fruit and vegetable extracts for their capacity to induce transcription factor-mediated health-related gene expression. It was shown that reporter gene assays are able to pick up differences in transcription factor-mediated gene expression induced by different varieties of tomato. Furthermore, this thesis provides proof-of-principle that active ingredients contributing to the activity of the whole tomato extracts could be identified by combining reporter gene assays and metabolomic profiling. Our results indicate that combining multiple reporter gene assays and metabolomic profiling, is useful in fast screening of larger numbers of food items and food compounds. Therefore, the use of reporter gene assays as a first tier in a tiered approach aiming at supporting health claims, will limit the number of animal studies and human studies needed, by enabling the ranking and selection of highly promising food items or constituents for further *in vivo* testing.

9

SAMENVATTING

SAMENVATTING

De consumptie van voedingsmiddelen met gezondheidsbevorderende effecten, zoals margarine met plantensterolen, vruchtensap met toegevoegd calcium en ontbijtgranen met voedingsvezels, is de afgelopen jaren snel gestegen. Het is echter lastig de effectiviteit van dergelijke extra toevoegingen vast te stellen. Dit proefschrift laat zien dat reportergen-assays effectieve instrumenten zijn om dergelijke functionele effecten van voedingsmiddelen en bioactieve stoffen uit voedingsmiddelen te onderzoeken door gebruik te maken van genexpressie gemedieerd door verschillende transcriptie factoren.

Om groenten en fruit te kunnen testen in reportergen-assays, zijn extractiemethoden nodig om groente- en fruitextracten te maken die geschikt zijn voor gebruik in reportergen-assays. In **hoofdstuk 2** worden methoden beschreven om drie verschillende tomatenextracten te bereiden:

- een ongehydrolyseerd tomatenextract dat apolaire stoffen zoals isoprenoïden bevat en verder isoprenoïden-bevattend tomatenextract zal worden genoemd,
- een ongehydrolyseerd tomatenextract dat semi-polaire stoffen bevat en verder ongehydrolyseerd tomatenextract genoemd zal worden,
- en een enzymatisch gehydrolyseerd tomatenextract dat gedeglycosyleerde semi-polaire stoffen bevat en verder gehydrolyseerd tomatenextract genoemd zal worden.

Alle drie de tomatenextracten verstoorden de celfysiologische en celkweekcondities niet en werden in een van de gebruikte gen-expressieassays, de nrf2-gemedieerde EpRE-LUX reportergen-assay, getest. Zowel het isoprenoïden-bevattend tomatenextract als de meeste individuele isoprenoïden (met uitzondering van neurosporeen) waren niet in staat EpRE-gemedieerde genexpressie te induceren. Ook het ongehydrolyseerde tomatenextract en flavonoïd-glycosiden waren niet in staat om EpRE-gemedieerde genexpressie te induceren. Het enzymatisch gehydrolyseerde extract, dat gedeglycosyleerde polyfenolen bevat, was wel in staat EpRE-gemedieerde genexpressie te induceren, zowel op mRNA-niveau als op eiwitniveau. In lijn met deze bevindingen bleken ook de flavonoid-aglyconen quercetine, kaempferol, naringenine en naringenine chalcone EpRE-gemedieerde genexpressie te induceren. Deze vier flavonoïden zouden dus bij kunnen dragen aan de inductie die waargenomen werd met het enzymatisch gehydrolyseerde tomatenextract. Samen wijzen de resultaten, gepresenteerd in hoofdstuk 2, erop dat de inductie van EpRE-gereguleerde genen, zoals ontgiftende fase II-enzymen en antioxidant-enzymen, zouden kunnen bijdragen aan de gezondheidsbevorderende effecten van tomaten.

Hoofdstuk 3 van dit proefschrift beschrijft de ontwikkeling en validatie van twee nieuwe, stabiele transcriptiefactor-gemedieerde gen expressie assays: de PPARγ1 CALUX-assay en de PPARγ2 CALUX-assay. De data in dit hoofdstuk laten zien dat deze reportercellijnen specifiek reageren op agonisten voor PPARγ en dat deze reportercellijnen een betrouwbaar en bruikbaar instrument zijn om bioactieve stoffen te screenen op PPARγ-agonistische of -antagonistische activiteit.

Omdat de gezondheidsbevorderende effecten gerelateerd aan PPARγ-activatie deels overlappen met effecten geassocieerd met tomatenconsumptie, zijn in **hoofdstuk 4**

tomatenextracten en tomateninhoudsstoffen getest op hun capaciteit om PPARγ-gemedieerde genexpressie te induceren. Dertig stoffen werden geselecteerd op basis van hun aanwezigheid in tomaat en werden onderzocht in de PPARγ2 CALUX-assay. Van deze tomatenstoffen waren drie vetzuren, zeven carotenoïden en drie polyfenolen in staat PPARγ2-gemedieerde genexpressie te induceren. In lijn met deze bevindingen induceerden ook het isoprenoïden-bevattende tomatenextract en het enzymatisch gehydrolyseerde tomatenextract de PPARγ-gemedieerde genexpressie. Deze resultaten wijzen erop dat de inductie van PPARγ-gemedieerde genexpressie door tomatenstoffen zou kunnen bijdragen aan de gezondheidsbevorderende effecten die geassocieerd zijn met tomatenconsumptie.

In **hoofdstuk 5** wordt de ontwikkeling en validatie van de PPARα CALUX transcriptiefactor-gemedieerde genexpressie-assay beschreven. Vervolgens zijn tomatenextracten en individuele tomateninhoudsstoffen met behulp van deze nieuwe PPARα CALUX-assay getest op hun capaciteit om PPARα-gemedieerde genexpressie te induceren. Het isoprenoïd-bevattende extract was in staat PPARα-gemedieerde genexpressie te induceren, wat overeenkomt met de bevinding dat β-caroteen, γ-caroteen en lycopeen ook in staat zijn om PPARα-gemedieerde genexpressie te induceren. Daarnaast gaf het ongehydrolyseerde tomatenextract een zwakke inductie van PPARα-gemedieerde genexpressie, maar enzymatische hydrolyse van tomaat voorafgaande aan extractie, leidde tot een aanzienlijke verhoging van de inductiecapaciteit. Dit is in overeenstemmingmet onze bevindingen dat geen van de flavonoïd-glycosiden in staat was PPARα-gemedieerde genexpressie te induceren, maar dat de flavonoïd-aglyconen kaempferol en naringenine daartoe wel in staat waren.

Om verschillen tussen tomatenextracten te onderzoeken, werden enzymatisch gehydrolyseerde extracten gemaakt van zeven verschillende tomatenvariëteiten en getest op hun capaciteit om PPARγ-gemedieerde **(hoofdstuk 4)** en PPARα-gemedieerde **(hoofdstuk 5)** genexpressie te induceren. De extracten van deze variëteiten waren allemaal in staat om zowel PPARγ-gemedieerde als PPARα-gemedieerde genexpressie te induceren. Voor PPARγ-gemedieerde genexpressie werd een verschil van 1.6-fold waargenomen tussen de meest potente en de minst potente variëteit, en voor PPARα-gemedieerde genexpressie was dat 1.7-fold. Zowel in de PPARγ2 CALUX assay als in de PPARα CALUX assay hadden de twee meest potente tomatenvariëteiten ook een hoog gehalte aan flavonoïden, terwijl de twee minst potente tomatenvariëteiten een laag gehalte aan flavonoïden hadden. Dit zou erop kunnen wijzen dat de verschillen in capaciteit om PPARγ-gemedieerde genexpressie of PPARα-gemedieerde genexpressie te induceren, deels verklaard kunnen worden door verschillen in de gehaltes aan kaempferol, naringenine en andere flavonoïden. Kaempferol en naringenine lieten echter een relatief lage inductie zien (tot vijfvoudige inductie voor PPARγ-gemedieerde genexpressie en tot 1.5-fold inductie voor PPARα-gemedieerde genexpressie) en kunnen de hoge inductie (tot tienvoudige inductie voor PPARγ-gemedieerde genexpressie en tot negenvoudige inductie voor PPARα-gemedieerde genexpressie) waargenomen met het gehydrolyseerde tomatenextract, niet volledig verklaren. Waarschijnlijk zijn er meer – tot nu toe ongeïdentificeerde – stoffen aanwezig in tomaat die PPARγ en/of PPARα kunnen activeren.

In **hoofdstuk 6** zijn extracten van 97 verschillende tomatenvariëteiten met behulp van de EpRE-LUX assay gescreend op hun capaciteit om EpRE-gemedieerde genexpressie te induceren. Er werd een drievoudig verschil in EpRE-gemedieerde genexpressie waargenomen tussen de minst potente variëteit en de meest potente variëteit. Van alle 97 tomatenvariëteiten werden metabolietprofielen gemaakt om stoffen te identificeren die verantwoordelijk zijn voor deze verschillen in genexpressie inducerende capaciteit. Door het combineren van genexpressiegegevens uit de reportergen-assay met de metabolietprofielen in een multivariate analyse werd quercetine geïdentificeerd als een van de belangrijkste stoffen die verantwoordelijk zou kunnen zijn voor de capaciteit van tomatenextracten om EpRE-gemedieerde genexpressie te induceren. Daarnaast lieten ook nog ongeïdentificeerde stoffen een goede correlatie zien met de respons in de EpRE-LUX cellen. De in dit hoofdstuk beschreven resultaten laten zien dat het combineren van reportergen-assays met metabolietanalyse een nuttige aanpak kan zijn in de identificatie van gezondheidsbevorderende stoffen in tomaat.

Toekomstige studies die laten zien of de extractiemethoden, gepresenteerd in hoofdstuk 2, ook toepasbaar zijn op andere groenten en fruit en op bewerkte voedingsmiddelen zouden nuttig zijn. Omdat metabolisme van flavonoïden en carotenoïden hun bioactiviteit kan beïnvloeden, is het van belang dat de bioactiviteit van de metabolieten van flavonoïden en carotenoïden nader wordt onderzocht. Een andere kwestie om nader te onderzoeken, is onder welke condities de inductie van EpRE-, PPARγ-, of PPARα-gemedieerde genexpressie zou kunnen leiden tot nadelige in plaats van heilzame gezondheidseffecten. Daarnaast zou een *in vivo*-validatie van de mogelijke gezondheidseffecten van een toename in EpRE-, PPARγ- of PPARα-gemedieerde genexpressie van belang zijn en daarin zou men kunnen focussen op de stoffen en variëteiten die in dit proefschrift zijn aangeduid als meest actief.

Samenvattend laat het in dit proefschrift gepresenteerde werk zien dat transcriptiefactor-gemedieerde genexpressie-assays kunnen worden ingezet in de effect-screening van bioactieve stoffen en van groente- en fruitextracten. De assays zijn in staat verschillen tussen tomatenvariëteiten ten aanzien van de inductie van transcriptiefactor-gemedieerde genexpressie, zichtbaar te maken. Daarnaast laat dit proefschrift zien dat actieve componenten die bijdragen aan de activiteit van tomatenextracten, kunnen worden geïdentificeerd door het combineren van reportergen-assaydata en metabolietprofielen van die extracten. Onze resultaten wijzen erop dat deze combinatie van reportergen-assays met metabolietprofielen bruikbaar is voor het snel screenen van grotere aantallen voedingsmiddelen en inhoudsstoffen. Het gebruik van reportergen-assays als eerste fase in een stapsgewijze benadering om gezondheidsclaims te onderbouwen, zal het aantal benodigde dierstudies en humane studies beperken, omdat veelbelovende voedingsmiddelen en hun inhoudsstoffen kunnen worden geselecteerd en alleen deze verder getest hoeven te worden.

A

APPENDIX

DANKWOORD

Nu mijn proefschrift klaar is, is het tijd om stil te staan bij alle hulp en steun die ik heb mogen ontvangen. Heel veel mensen hebben op hun eigen manier een rol gespeeld bij de totstandkoming van dit geheel, en ik wil al deze mensen bedanken!

Allereerst wil ik mijn promotoren en begeleiders bedanken. Ivonne, dankjewel voor je efficiënte begeleiding en voor je snelle en constructieve feedback op mijn abstracts en manuscripten. Jaap, hoewel mijn werk bij HAP helaas niet tot het gehoopte resultaat heeft geleid, heb ik wel veel geleerd in deze periode. Bedankt ook voor je kritische blik op mijn artikelen. Jac, je hebt me wegwijs gemaakt in de wondere wereld van de moleculaire biologie, dankjewel daarvoor.

Ook alle mensen die betrokken waren bij het FND project wil ik hartelijk bedanken. Henriëtte, bedankt voor de fijne samenwerking. Bedankt voor al je tomatenextracten en alle LC-MS analyses. Bart en Hai-Yen, ik wil jullie bedanken voor de fijne samenwerking met BDS en voor alle experimenten die bij en door jullie zijn uitgevoerd. Laura, Arnaud en Renate, ook jullie hartelijk bedankt voor jullie inzet en input. Mijn studenten Wouter, Samantha (leuk dat je nu een collega bent!), Jorik en Nienke; het was een genoegen jullie te mogen begeleiden, dank voor jullie bijdragen aan mijn onderzoek.

Zowel op TOX als op HAP heb ik me altijd erg thuis gevoeld, voornamelijk door mijn fantastische collega's. Allemaal heel hartelijk bedankt voor de goede sfeer, jullie hulp en tips en natuurlijk voor de fijne tijd. A special thanks to Alicia, Merel, Nynke and Suzanne, for your friendship and support, for the GG and of course for the wonderful ladies nights.

Henriëtte en Nynke, ik ben blij dat jullie naast me staan op de dag van mijn verdediging. Fijn dat jullie mijn paranimfen willen zijn!

Pap en mam, dank jullie wel voor jullie liefde en vertrouwen. Het is fijn om te weten dat jullie trots op me zijn. Esther, wat was het fijn om de pieken en dalen van een promotietraject met jou te kunnen delen. Over een tijdje is het jouw beurt!

Lieve Jack, afgelopen jaren was jij mijn praatpaal, mijn rots in de branding en mijn rustpunt. Bij jou kan ik altijd terecht met mijn frustraties en successen. En hoe druk het op het werk ook was, thuis kon ik me ontspannen en maakte jij me altijd weer aan het lachen. Dank voor al je steun en liefde, en nu samen op weg naar een prachtige toekomst!

CURRICULUM VITAE

Linda Gijsbers was born on July 6th, 1983 in Eindhoven and grew up in Duizel. After her graduation from secondary education at Rythovius College in Eersel in 2001, she started her study Nutrition and Health at Wageningen University. In 2004, she obtained her BSc degree. During her master, Linda completed two MSc theses. The first thesis was about peanut allergy and was performed at the UMC Utrecht. The second thesis was performed at the department of Human and Animal Physiology of Wageningen University, where Linda investigated the effects of the oestrous cycle on the acute phase response in rats. For her internship, she went to Instituto Nazionale di Ricerca per Gli Alimenti e la Nutrizione (INRAN) in Rome, to investigate the health status of teenagers. After this internship, she participated in several ecology and plant-related courses, to broaden her knowledge of biology. In 2007, she finalized her study Nutrition and Health and obtained her MSc degree. In that same year, she started the educational master at the IVLOS institute of Utrecht University. In 2008 she graduated and gained the qualification biology teacher (first degree, for teaching at schools for secondary education).

From August 2008 until August 2012, Linda worked as a PhD student on the project presented in this thesis, which was a collaboration between the divisions 'Toxicology' and 'Human and Animal Physiology' of Wageningen University. During this period, she completed the Postgraduate Education in Toxicology.

LIST OF PUBLICATIONS AND ABSTRACTS

Publications

B. van der Burg, R. Winter, M. Heimer, P. Berckmans, G. Suzuki, L. Gijsbers, A. Jonas, S. van der Linden, H. Witters, J. Aarts, J. Legler, A. Kop-Schneider and S. Bremer. Optimization and prevalidation of the *in vitro* ERα CALUX method to test estrogenic and antiestrogenic activity of compounds. *Reproductive Toxicology, 30 (2010), 73-80.*

L. Gijsbers, H. Man, S. Kloet, L. de Haan, J. Keijer, I. Rietjens, B. van der Burg and J. Aarts. Stable reporter cell lines for peroxisome proliferator-activated receptor γ (PPARγ)-mediated modulation of gene expression. *Analytical Biochemistry, 414 (2011), 77-83.*

L. Gijsbers, H. van Eekelen, T. Nguyen, L. de Haan, B. van der Burg, J. Aarts, I. Rietjens and A. Bovy. Induction of electrophile-responsive element (EpRE)-mediated gene expression by tomato extracts *in vitro. Food Chemistry, 135 (2012), 1166-1172.*

L. Gijsbers, H. van Eekelen, L. de Haan, J. Swier, N. Heijink, S. Kloet, A. Bovy, J. Keijer, J. Aarts, B. van der Burg and I. Rietjens. Induction of peroxisome proliferator-activated receptor γ (PPARγ)-mediated gene expression by tomato (*Solanum Lycopersicum L.)* extracts. *Submitted for publication to Journal of Agricultural and Food Chemistry.*

H. van Eekelen, L. Gijsbers, C. Maliepaard, R. Vreeburg, R. Finkers, Y. Tikunov, V. Gomez Roldan, L. de Haan, J. Aarts, I. Rietjens and A. Bovy. Identification by metabolomic screening of compounds from tomato inducing Electrophile-Responsive Element (EpRE)-mediated gene transcription. *Submitted for publication to Food Chemistry.*

L. Gijsbers, H. Man, H. van Eekelen, J. Keijer, J. Aarts, I. Rietjens, B. vann der Burg. Induction of peroxisome proliferator-activated receptor α (PPARα)-mediated gene expression by extracts of tomato. *In preparation.*

Abstracts

L. Gijsbers, I. Rietjens, J. Keijer and J. Aarts. Development of *in vitro* reporter gene bioassays for detection of beneficial health effects of functional foods. *Abstract in "Current Research 2010" by VLAG graduate school.*

L. Gijsbers, H. Man, S. Kloet, L. de Haan, J. Keijer, I. Rietjens, B. van der Burg and J. Aarts. Stable reporter cell lines for PPARγ-mediated modulation of gene expression. *Poster presentation at Benelux Nuclear Receptor Meeting 2010, 28 October2010, Ghent, Belgium.*

L. Gijsbers, H. Man, S. Kloet, L. de Haan, J. Keijer, I. Rietjens, B. van der Burg and J. Aarts. Stable reporter cell lines for PPARγ-mediated modulation of gene expression. *Poster presentation at Annual Meeting of Dutch Society of Toxicology (NVT, Nederlandse Vereniging voor Toxicologie), 17-18 May 2011, Zeist, The Netherlands.*

L. Gijsbers, H. Man, S. Kloet, L. de Haan, J. Keijer, I. Rietjens, B. van der Burg and J. Aarts. Stable reporter cell lines for PPARγ-mediated modulation of gene expression. *Poster presentation at MitoFood Conference, 13-15 April 2011, Wageningen, The Netherlands.*

L. Gijsbers, J. Aarts, I. Rietjens and J. Keijer. An assay toolbox for health effects of functional foods. *Oral presentation during the Toxicology PhD trip 2011, givenat Givaudan, 20 June 2011, Kemptthal, Switserland.*

L. Gijsbers, H. van Eekelen, L. de Haan, T. Nguyen, J. Aarts, I. Rietjens and A. Bovy. Induction of EpRE-mediated gene expression by bioactive ingredients of tomato (Lycopersicon esculentum Mill.) *Poster presentation at 51st Annual Meeting and ToxExpo by Society of Toxicology, 11-15 March, 2012, San Francisco, USA.*

L. de Haan, L. Gijsbers, H. Man, S. Kloet, J. Keijer, I. Rietjens, B. van der Burg and J. Aarts. Development and validation of two stable reporter cell lines for PPARγ-mediated modulation of gene expression. *Poster presentation at 51st Annual Meeting and ToxExpo by Society of Toxicology, 11-15 March, 2012, San Francisco, USA.*

OVERVIEW OF COMPLETED TRAINING ACTIVITIES

Completed courses

General courses
VLAG PhD week (VLAG, 2009)
PhD competence assessment (WGS, 2009)
Effective Behaviour in your Professional Surroundings (WGS, 2009)
Techniques for Writing and Presenting Scientific Papers (WGS, 2009)

Discipline-specific courses
Techniques in Molecular Biology (Groningen, 2008)
Ecotoxicology (Postgraduate Education in Toxicology (PET), 2009)
Toxicological Risk Assessment (PET, 2009)
Organ Toxicology (PET, 2010)
Reproductive Toxicology (PET, 2010)
Immunotoxicology (PET, 2010)
Medical, Forensic and Regulatory Toxicology (PET, 2010)
Mutagenesis and Carcinogenesis (PET, 2011)
Measuring cellular mitochondrial function (WIAS/VLAG, 2011)

Optionals
Preparation Research proposal (2008)
PhD trip division of Toxicology (Switzerland and Italy, 2011)
Attending scientific presentations at Toxicology and at Human and Animal Physiology (2008-2012)

Attended conferences
Benelux Nuclear Receptor Meeting 2009 (Oegstgeest, The Netherlands, 2009)
Benelux Nuclear Receptor Meeting 2010 (Ghent, Belgium, 2010)
Mitofood conference (Wageningen, The Netherlands, 2011)
NVT jaarvergadering 2011 (Zeist, The Netherlands, 2011)
SOT Annual Meeting and ToxExpo (San Francisco, USA, 2012)

Approved by graduate school VLAG

The studies described in this thesis were conducted within a collaborative project of Wageningen University, Plant Research International, BioDetection Systems and Syngenta. The research described in this thesis was financially supported by the Food and Nutrition Delta (grant number FND07007).

Financial support from Wageningen University for printing this thesis is gratefully acknowledged.

Lay out and printing: Off Page, Amsterdam, The Netherlands.

Cover design: Mirian Hendriks

Photo on page 155: Mirian Hendriks