Towards functional effects of polyphenols
Modulation of energy metabolism revealed

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"Perhaps down at the molecular level there's been a chemical accident…. – something like a spilled tray of drinks, prompting dopamine-like receptors to initiate a kindly cascade of intracellular events…"

Ian McEwan - Saturday

ABSTRACT

A diet rich in fruits and vegetables contains high levels of polyphenols (up to 1 gram per day). Epidemiological studies suggest that a high dietary intake of selected polyphenols can be protective against development of cardiovascular heart diseases in humans. In addition, mechanistic studies demonstrate that polyphenols possess beneficial properties in *in vitro* and animal model systems. Due to the possible beneficial health effects of polyphenols, they are currently being sold extensively as food supplements. However, the basis for most of the health claims attributed to polyphenols in food supplements is often very small. Our objective was to elucidate relevant mechanisms of action of selected polyphenols. We studied the tissue distribution and *in vivo* physiological effects of quercetin (a polyphenol abundant in the human diet) after chronic dietary exposure, followed by *in vitro* elucidation of possible biological mechanisms. We revealed lungs as novel tissue target of quercetin and demonstrated that dietary quercetin alters fatty acid catabolism pathways in rats. In addition, dietary quercetin lowered tumor incidence in the colon of rats in a model of colon carcinogenesis. Furthermore, a major *in vivo* metabolite of quercetin, quercetin 3-O-glucuronide, opposed the effect of quercetin aglycone on SIRT1 activation *in vitro*, whereas quercetin 3-O-glucuronide attenuated glucose utilization in cultured adipocytes in a similar fashion as quercetin aglycone. Although we used high dietary dosages of quercetin and further studies should elucidate physiological effects of a normal dietary intake of polyphenols, the experiments described in this thesis point to a possible beneficial effect of dietary polyphenols. However, as long as the molecular mechanisms in humans are unknown and the risk of increasing dietary intakes of polyphenols via food supplements is not thoroughly investigated, there is no scientific justification for supplementing the diet with large amounts of polyphenols. Nevertheless, our approach successfully identified modulation of energy metabolism by polyphenols as an important process involved in mediating the possible health effects associated with dietary polyphenol intake.

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CHAPTER 1

General Introduction

BACKGROUND

Organisms are continuously influenced by environmental changes and stimuli. Adaptation to these changing conditions is essential for maintaining homeostasis and survival of the organism. Scarcity of food, for example, either leads to a pause in development and reproduction or activates a mechanism to attract or acquire more food sources. Both processes are tightly controlled by genetic and enzymatic processes that are triggered by small molecules. Pheromonal signaling in response to starvation in *C. elegans* was recently discovered to be regulated by steroidal hormones [1, 2] that induce a "dauer state", where the organism does not eat, grow, reproduce and shifts its metabolic processes from glucose utilization to utilization of internal lipid stores [3, 4]. The hormonal signals trigger a transcriptional cascade that leads to the onset of the dauer formation [1, 2].

In plants, availability of food sources is also regulated through chemical interaction. To utilize nitrogen from the soil, leguminous plants are using a symbiotic interaction with nitrogen-fixing bacteria (*Rhizobia*) [5]. The interaction between plant and bacteria starts with a small molecule signal produced and secreted by plant roots. The signal attracts bacteria to the roots and symbiotic root nodules are formed that can convert nitrogen from the soil into ammonia used for amino acid production for plant growth [6]. The small molecule signal that initiates the interaction is a polyphenol [7]. The biochemical mechanism by which polyphenols initiate the interaction was discovered to be via interaction of the polyphenol with a transcription factor in bacteria that transcribes a large set of nodule forming genes (*nod* genes). The transcription of the *nod* genes induces the onset of the *rhizobium* – plant interaction to develop into a nitrogen fixing root nodule [8].

Polyphenols are produced by plants in a process known as secondary metabolism. Secondary metabolites are a large class of compounds that enable plants to interact with their environment and have physiological functions different from those of the primary metabolites, like carbohydrates, proteins and lipids [9]. Polyphenols are not only used by plants to establish symbiotic interactions with other organisms, polyphenols are also used for other types of communication with their environment and for defense. For example, they protect plants from UV radiation and give plants there coloring patterns [10, 11].

The human diet consists for a large part of plant derived products, like vegetables, fruits, and tea. These food products contain relatively high levels of polyphenols. Therefore, the total average intake of polyphenols in a

healthy human diet has been estimated to be around 1 g per day [12]. Whether polyphenols can induce physiological effects in humans has been a subject of study and controversy for several decades. Studies using animal and *in vitro* models that try to identify the mechanisms of action of polyphenols in for example cardiovascular heart diseases (CHD), cancer, inflammation and neurodegenerative diseases, have led to a plethora of possible explanations of both beneficial and adverse effects of polyphenols. However most of the time, these studies do not give a conclusive answer on whether and how dietary polyphenols decrease disease risk and affect human health.

Already at the beginning of the 20th century the finding that an intravenously injected polyphenol fraction isolated from citrus could decrease capillary fragility in both guinea pigs [13] and humans [14] led to a scientific debate about possible misinterpretations of results [15, 16]. The *in vivo* beneficial biological effect attributed to the polyphenol fraction was either not analyzed correctly or the polyphenol fraction was contaminated with other non-polyphenolic compounds [17-19]. Indeed it was confirmed by the authors of the original paper, that vitamin C was needed in addition to the polyphenol fraction to reproduce the observed effect on vascular fragility [20]. This indicated that the chemical stability of the polyphenol fraction or synergism was an important factor for the action of the polyphenolic fraction. Since then mechanisms of action of polyphenols have become subject of a large variety of studies, several proposing beneficial effects on health [21-23] others suggesting unfavorable effects [24]. Limited knowledge on bioavailability of polyphenols could be one of the causes of these contradictions. To shed some light, this introduction describes (1) the current state of knowledge on the bioavailability of polyphenols to tissue targets, (2) *in vivo* studies elucidating mechanisms of action of polyphenols that take into account bioavailability and (3) *in vitro* studies that use relevant human polyphenol metabolites. This overview is primarily focused on the polyphenols that are used in the experiments described in this thesis: quercetin, resveratrol and epigallocatechin galate (EGCg).

BIOAVAILABILITY OF POLYPHENOLS

In vivo bioavailability studies

The first determinant of the oral bioavailability of a polyphenol is the extent of its absorption from the gastro-intestinal tract. Most polyphenols are

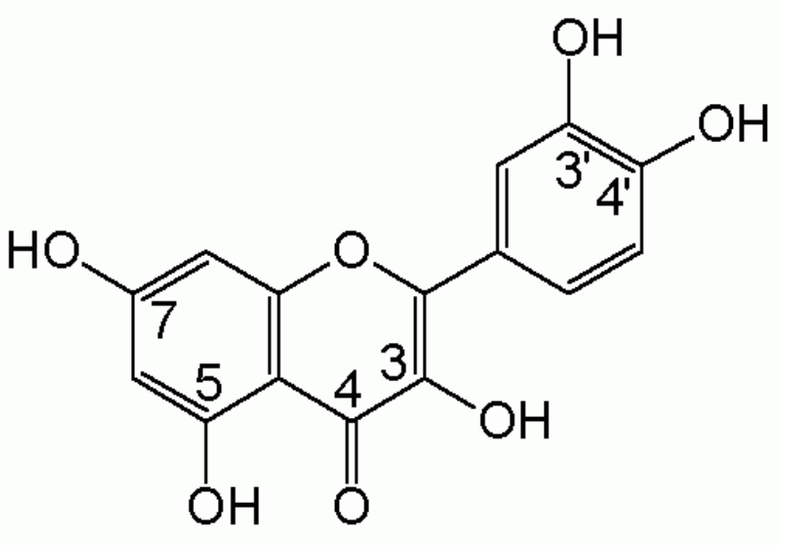

Figure 1.1
Molecular structure and atom numbering of quercetin.

stored in plants conjugated to a glycosidic group. The glycosidic group can be one of a variety of saccharides, like glucose, galactose, and rhamnose, and disaccharides like rhamnoglucoside. In addition, acylation may occur [25]. Although uptake of polyphenols from the stomach has been reported in rats [26], the predominant site of absorption of polyphenols in humans is the intestine. The extent of the intestinal absorption of quercetin for instance, is determined by the type of sugar group (see figure 1.1 for molecular structure of quercetin). Plasma and urine levels of quercetin after an oral dose of quercetin 3-O-glucoside or quercetin 4'-O-glucoside were higher than after ingestion of quercetin aglycone or quercetin 3-O-rhamnoglucoside (rutin) [27, 28]. Also quercetin 3-O-galactoside and quercetin 3-O-rhamnoside were shown to be much less bioavailable than the 3-O-glucoside and 4'-O-glucoside in a rat intestinal perfusion model [29]. However, glycosidated polyphenols are generally not found in plasma, urine and tissues, because in most cases deglycosylation is required for uptake in the small intestine. Deglycosylation of polyphenols occurs through enzymes with glycosidase activity. Extracellular hydrolases, like lactase phloridzin hydrolase (LPH) and intracellular glycosidases in the small intestine were shown to deconjugate glycosidic polyphenols, facilitating their uptake [29-31]. Extracellular LPH was shown to be of particular importance for selectively taking up quercetin glycosides [29]. Quercetin glycosides that were not a substrate for LPH (quercetin 3-O-galactoside and quercetin 3-O-rhamnoside) were not absorbed, whereas good LPH substrates (quercetin 3-O-glucoside and 4'-O-glucoside) were

readily absorbed [29]. Polyphenols not absorbed in the small intestine can be deconjugated and degraded to phenolic acids by the colonic microflora [32, 33]. These phenolic acids are found in urine and plasma after oral polyphenol exposures [34, 35].

The second determinant of polyphenol bioavailability is the degree and type of metabolism upon and after absorption. Most polyphenols are immediately metabolized intracellularly in the small intestine or in the liver by phase II enzymes, UDP-glucuronosyl transferases (UDPGT), sulfotransferases (ST) and catechol-O-methyl transferases (COMT). The resulting plasma metabolites of polyphenols are more hydrophilic than polyphenol aglycones and are therefore easily eliminated through bile and urine [36-38]. Chronic administration of selected polyphenols could possibly increase the systemic exposure considerably. Flavonols have plasma half lives of ~20h, whereas catechins and anthocyanins are eliminated 5-10 times faster [39]. Therefore, repeated ingestion of flavonols could result in accumulation of flavonols in plasma and tissues, whereas catechins and anthocyanins are not likely to accumulate [37].

Murota and Terao [40] demonstrated a novel route for the absorption of polyphenols via the lymph system. The authors cannulated the thoracic lymph duct (the largest lymphatic vessel in the body) for lymph fluid sampling and administered a single dose of quercetin (10 mg/kg BW in propylene glycol) into the stomach of unanesthetized rats. Over a period of 7 hours, quercetin metabolite concentrations were analyzed. Quercetin conjugate levels peaked 30 minutes after administration at 2.5 μM and decreased gradually leaving 1 μM quercetin conjugates in lymph fluid 7 hours after administration. Quercetin aglycone was also detected at 0.04 – 0.11 μM only in the first 30 minutes [40]. This study established the presence of a novel transport pathway through the lymphatic system for polyphenols.

The third determinant of polyphenol bioavailability is their uptake in tissues. Because most polyphenols are present in plasma conjugated to glucuronides and sulfates, the penetration of polyphenols into tissues is difficult, due to the hydrophilic nature of conjugated polyphenols. However, several short term studies have shown now that after oral administration of polyphenols they can be recovered from most analyzed tissues (table 1.1). Most single dose studies recovered polyphenols from liver, kidney and not surprisingly, the gastro-intestinal tract. With repeated dosing other tissues contained considerable levels of polyphenols as well. Radioactivity from radiolabeled EGCg ingestion (0.05% EGCg in drinking water) accumulated in lung, brain, pancreas and bladder to a level that was more than three

Table 1.1 Overview of tissue distribution studies with dietary polyphenols.

Polyphenol	Animal	Dosing type	Dose	Sampling	Tissues	Analysis method	Reference
Quercetin (glucosides)							
Quercetin 4'-O-glucoside	Male Rowett Hooded Lister strain rats	Single dose in diet	3.26 mg in 1g of diet	1h after feeding	Liver, brain, heart, kidneys, lungs, muscle, spleen, testes	Radioactivity, HPLC-PDA, LC-MS/MS	Mullen *et al.* (2002) [45]
Quercetin 4'-O-glucoside	Male Rowett Hooded Lister strain rats	Single dose in diet	3.26 mg in 1g of diet	0.5, 1, 2, 5h after administration	Liver, brain, heart, kidney, lung, muscle, spleen, testes, GI tract	Radioactivity, LC-MS/MS	Graf *et al.* (2005) [46]
Quercetin 3-O-glucoside	Male Wistar rats	Single dose in diet	10g diet containing 10g quercetin 3-glucoside/kg diet	2, 5, 24h after administration	Stomach, cecum, small intestine, intestinal mucosa	HPLC-PDA, LC-MS	Gee *et al.* (2004) [47]
Quercetin	Male F-344 rats	Continuously in diet for 6 weeks	0.45% (58.5 mg/day)	6 weeks after start of administration	GI tract, liver, kidney	LC-MS/MS	Graf *et al.* (2006) [48]
Quercetin	Male Holtzman strain rats	Single dose gastric gavage	5 mg ^{14}C-quercetin	12h after gastric gavage	Small intestine, large intestine, kidney, lungs, liver, spleen, brain, heart	Radioactivity	Petriakis *et al.* (1959) [49]
Quercetin	Male ACI strain rats	Single dose gastric gavage	630 mg ^{14}C quercetin/kg	6h after administration	Liver, kidney, heart, lung, muscle	Radioactivity	Ueno *et al.* (1983) [50]
Quercetin	Male Rowett Hooded Lister strain rats	Continuously in diet for 2 weeks	5 g /kg diet	2 weeks after start of administration	Liver, kidney, heart	HPLC – fluorescence	Morrice *et al.* (2000) [43]

Table 1.1 (continued)

Polyphenol	Animal	Dosing type	Dose	Sampling	Tissues	Analysis method	Reference
Stilbenes							
Resveratrol	Male Balb/c mice	Single dose gastric gavage	5 mg/kg or 50 mg/kg ^{14}C resveratrol	1.5, 3 or 6h post gavage	Duodenum, colon, liver, kidneys, heart, spleen, lungs, testis, brain, carcass	Radioactivity, HPLC-fluorescence	Vitrac *et al.* (2003) [51]
Resveratrol	Male Sprague Dawley rats	Single dose gastric gavage	50 mg/kg BW ^{3}H-resveratrol	2 or 18h following gavage	Liver, kidneys, spleen, heart, small intestine, large intestine, lung, brain	Radioactivity, HPLC - PDA, LC-MS	Abd El Mohsen *et al.* (2006) [44]
Catechins							
Catechins	In utero fetuses from female Sprague Dawley rats	Single dose gastric gavage	green tea extract (unknown composition, 0.55 g/kg in 0.5 ml water) to mother dams	0.5, 1, 2, 3, 5, 8h after administration	Fetal organs: brain, eye, lung, kidney, liver	HPLC-Coularray	Chu *et al.* (2006) [52]
Catechins	C57BL/6 mice	Continuously in diet for 2 weeks	Decaffeinated black tea extract (50 mg/g diet, containing 75.7, 7.2, 6.9, 12.5 mg/g tea solids EGC, EG, EGCG, ECG).	2 weeks after start of administration	Liver, small intestine, prostate, colon	HPLC-Coularray	Henning *et al.* (2006) [53]
Catechins	Male humans	Daily consumption of 5 cups of tea for 5 days	green tea or black tea (containing 25.8, 9.9, 57.7, 30.6 mg EGC, EC, EGCg, ECg /cup of brewed tea)	1 day after the 5 day intake period	prostate	HPLC-Coularray	Henning *et al.* (2006) [53]

Table 1.1 (continued)

Polyphenol	Animal	Dosing type	Dose	Sampling	Tissues	Analysis method	Reference
Catechins (EGC, EGCg, EC)	Male Sprague Dawley rats	Continuously in drinking fluid for 8 days	*Ad libitum* 0.6% w/v (green tea polyphenols containing 560, 76, 86 mg EGCg, EGC, EC per gram)	8 days after start of administration	Bladder, heart, large intestine, esophagus, prostate, spleen, lung, kidney, liver, heart, thyroid	HPLC-Coularray	Kim *et al.* (2000) [42]
Catechins (EGC, EGCg, EC)	Female A/J mice	Continuously in drinking fluid for 8 days	Adl libitum 0.6% w/v in miliQ (green tea polyphenols containing 560, 76, 86 mg EGCg, EGC, EC per gram)	1, 4, 8 and 12 days after start of administration	Liver, lung	HPLC-Coularray	Kim *et al.* (2000) [42]
EGCg	Male CF-1 mice	Single dose gastric gavage	163.8 μmol /kg	20, 50, 90, 180, 300 and 720 min after administration	Lung, liver, spleen, kidneys, colon, small intestine, prostate, brain	HPLC-Coularray	Lambert *et al.* (2003) [54]
EGCg	Female and Male CD-1 rats	Single and duplicate dose via gastric gavage with 6 hours interval	200 μl of 0.05% EGCg solution (3.7 MBq ^{3}H EGCG)	1, 6 and 24h after administration	Brain, lung, heart, liver, kidney, spleen, pancreas, uterus, ovary, mammary gland, bladder, bone, skin, testes	Radioactivity, HPLC-UVVIS	Suganuma *et al.* (1998) [41]
EGCg	Male CF-1 mice	Single dose gastric gavage	50-2000 mg/kg	50 min and 180 min after administration	Lung, liver, colon, small intestine, prostate	HPLC-Coularray	Lambert *et al.* (2006) [55]
Epicatechin	Male wistar rats	Continuously in diet for 1 day	100 mg/kg BW per day	1 day after start of administration	Brain	HPLC-PDA, LC-MS	Abd El Mohsen *et al.* (2002) [56]

Table 1.1 (continued)

Polyphenol	Animal	Dosing type	Dose	Sampling	Tissues	Analysis method	Reference
Isoflavones							
Isoflavones	Female Sprague Dawley rats	Continuously in diet for 4 days	16.8 mg genistein and 12.8 mg daidzein/kg BW daily	4 days after start of administration	Liver, uterus, mammary glands, brain	LC-MS	Gu *et al.* (2005) [57]
Genistein	Female and Male Sprague Dawley rats	Continuously in maternal diet and in postnatally in diet for 140 days	4, 100, 500 µg genistein/g feed (18.5, 370, 1852 µmol/kg BW) continuously in utero	140 days post natal	Mammary gland, uterus, ovary, testes, prostate, thyroid, liver, brain	LC-MS/MS	Chang *et al.* (2000) [58]
Genistein	Male and female wistar rats	Single dose gastric gavage	4 mg / kg BW	2, 7 and 24h after dosing	Heart, lung, thymus, spleen, liver, kidney, abdominal adipose tissue, skeletal muscle (thigh), bone (femur), brain, ovary, testis, uterus, prostate, vagina, stomach wall, jejunum, cecum	Radioactivity, LC/MS	Coldham *et al.* (2000) [59]
Daidzein	C57/Bl-6 mice	Continuously in diet for 12 weeks	200 mg /kg diet	12 weeks after start of administration	Bone (tibia)	Time resolved fluoroimmuno assay kits	Fonseca & Ward (2006) [60]
Daidzein 8-C-glucoside	Male Sprague Dawley rats	Four doses via gastric gavage in 72 hours interval	50 mg/kg BW	24h after final administration	Heart, brain	LC-MS/MS	Prasain *et al.* (2004) [61]

Table 1.1 (continued)

Polyphenol	Animal	Dosing type	Dose	Sampling	Tissues	Analysis method	Reference
Flavones							
Flavones (apigenin or trycin)	C57BL/6J mice	Continuously in diet for 7 days	300 mg/kg	7 days after start of administration	Liver, small intestinal mucosa	HPLC-UVVIS	Cai *et al.* (2006) [68]

Table 1.2 Phase II metabolites of quercetin and resveratrol identified in plasma, urine and tissues.

QUERCETIN					
Human		**Mouse**	**Rat**		
Plasma	*Urine*	*Plasma*	*Plasma*	*Urine*	*Tissue*
Quercetin 3'-sulfate [117] Quercetin 3-glucuronide [117]	Quercetin diglucuronide [117] Isorhamnetin 3-glucuronide [117]		Methylated quercetin glucuronide sulfate [46] Quercetin glucuronide sulfate [46] Methylated quercetin glucuronide [47]		Quercetin diglucuronide [46] Methylated Quercetin diglucuronide [45] Methylated quercetin glucuronide sulfate [48]
RESVERATROL					
Human		**Mouse**	**Rat**		
Plasma	*Urine*	*Plasma*	*Plasma*	*Urine*	*Tissue*
Resveratrol 3-glucuronide [118] Resveratrol 4'-glucuronide [118] Resveratrol sulfate [122]	Resveratrol sulfate [119] Resveratrol glucuronide [119]	Resveratrol 3-glucuronide [120] Resveratrol 3-sulfate [120]	Resveratrol 3,4'-disulfate [121] Resveratrol trisulfate [121] Resveratrol 3-glucuronide [121]	Resveratrol 3-glucuronide [120, 121] Resveratrol 3,5-disulfate [121] Resveratrol 3-sulfate [120]	Resveratrol 4'-sulfate [121] Resveratrol disulfate [121] Resveratrol 3-glucuronide [121]

times higher after duplicate administration than after single administration in mice (administered and sampled at 6h intervals) [41]. *Ad libitum* administration of a green tea extract in drinking water (0.6% w/v) to rats demonstrated that EGCg levels in lung tissue were higher than in liver tissue over a period of two weeks [42]. Furthermore, after a 0.25% quercetin diet for two weeks, heart tissue contained detectable levels of quercetin metabolites [43]. An interesting observation by Abd El Mohsen [44] pointed to a higher retention of polyphenols in tissues than in plasma. At two different time points after dosing (2h and 18h), tissues and plasma were collected. No resveratrol metabolites or aglycones were detected in plasma and kidney, whereas lung, brain, heart and liver contained both free and conjugated forms of resveratrol [44]. An overview of studies describing the analysis of polyphenols in tissues is given table 1.1.

Of particular interest is the fact that the nature of the conjugates differs between tissues and plasma. Five hours after a single dose administration of quercetin 4'-O-glucoside (7.6 mg/kg BW), rat plasma contained predominantly methylated quercetin glucuronide sulfates, whereas rat liver and kidney predominantly contained quercetin diglucuronides [46]. In addition, anthocyanidin conjugate profiles in tissues after orally administrating a single dose of bilberry extract (400 mg/kg BW) were different from those in plasma: rat tissues contained methylated anthocyanidin glucuronides, whereas plasma did not [63]. Not only differences in conjugation patterns exist between tissues and plasma, the extent to which polyphenol aglycones are present in tissues and plasma differs as well. Quercetin and methylated quercetin aglycones were not recovered from plasma, while liver, kidney and intestinal tissues contained considerable levels of aglycones after 6 week feeding a 0.45% quercetin diet [48]. Also after a single oral dose of quercetin 4'-O-glucoside (7.6 mg/kg BW), aglycone was detected in liver tissue but not in plasma [46]. Naringenin, resveratrol and pelargonidin were all shown to be present in their aglycone form in lung, liver and kidney, 18 hours after a single oral dose (50 mg/kg BW) [44, 64, 65]. Moreover, genistein aglycone levels in plasma were only 1-5%, whereas in tissues 10-100% of total genistein levels were in the aglycone form [58].

To summarize, orally administrated polyphenols can reach tissue targets, but polyphenols are largely recovered conjugated to glucuronides, sulfates and/or methoxy groups. Nevertheless, differences in the extent and nature of conjugation between plasma and tissues point to active processing of polyphenols in tissues and possibilities for polyphenol aglycone appearance at the target site. Furthermore, most studies used single doses and short-

term applications, whereas chronic administration experiments would probably give different tissue bioavailability data generating new hypotheses for effects in targeted tissues.

IN VITRO BIOAVAILABILITY

Cellular uptake of polyphenols

Intracellular concentrations of polyphenols after cellular exposures have been determined, either as total cell-associated concentrations of polyphenols or polyphenol concentrations in separated cellular organelles, like cytosol, nucleus and cell membrane. Aglycones of polyphenols are most of the time taken up very quickly by cells. Cell-associated concentrations of quercetin in HepG2, myocardioblasts and Caco-2 cells already peaked within 10-60 minutes after exposure [69-71]. The slightly hydrophobic nature of most polyphenols, probably allows most polyphenols to diffuse easily into the cell. Besides the passive diffusion process, active processes might be involved in transporting polyphenols over cell membranes. Indications for active transport of resveratrol, genistein, morin, and epicatechin galate (ECg) have been found. Resveratrol and genistein were taken up more slowly at 4 ^{o}C than at 37 ^{o}C [72, 73], uptake of morin (a trihydroxy flavonol) was increased when ATP was extracellularly added [74] and ECg was taken up at reduced levels when ECg exposure was combined with inhibitors of monocarboxylate transporters (MCT) [75].

Limited knowledge is available on the uptake of polyphenol metabolites. The more hydrophilic character of polyphenol glucuronides and sulfates makes them less likely to enter cells. However, several studies showed that polyphenol glucuronides are taken up by cells. In a study by Shirai *et al.* it was shown that quercetin 3-O-glucuronide (quercetin 3-O-glucuronic acid, Q3GA) could enter the cell, because intracellularly formed 3'-methoxy Q3GA was detected in the medium after a 4h exposure to Q3GA in 3T3 fibroblast cells [76]. In dermal fibroblasts, exposure to Q7GA showed no cell-associated quercetin metabolites, whereas the concentration of Q7GA in the exposure medium declined from 10 μM to 3.5 μM [77]. This might indicate either an intracellular conversion of Q7GA to other quercetin conjugates or instability of the original compound. Direct evidence for intracellular (or cell associated) levels of Q3GA came from a study using differentiated PC12 neuronal cells [78]. Shirai *et al.* demonstrated low levels (1.5 pmol/million cells) of Q3GA in extracts of PC12 cells exposed for 4 hours to 10 μM Q3GA [78]. In addition, exposure of endothelial cells to

hesperetin glucuronides and naringenin glucuronides resulted in intracellular accumulation of the glucuronides of hesperetin and naringenin [79]. In contrast, epicatechin glucuronides were not taken up in endothelial cells, neuronal cells and fibroblasts [79, 80].

Following uptake of polyphenol glucuronides into the cell, metabolism of the polyphenol glucuronides is still a very dynamic process. Extracellular exposure of Q3GA or Q7GA to liver HepG2 cells revealed further metabolism via two separate pathways [81]. The first metabolic pathway was methoxylation at the 3'-position and the second pathway was deglucuronidation by intracellular beta-glucuronidases followed by sulfation at the 3'-position. This conversion was shown to be specific for Q3GA and Q7GA, Q4'GA was not metabolized intracellulary [81]. Additional evidence for intracellular deglucuronidation was given for endothelial cells exposed to hesperetin glucuronides; aglycones were recovered from cell lysates after exposure of the cells to glucuronides [79]. In addition to intracellular metabolism of polyphenols, conjugated polyphenols are transported very efficiently out of the cell by several different transport proteins as well [82, 83]. For example, transport proteins of the MRP and BCRP family mediate the efflux of quercetin glucuronides from intestinal cells and liver cells [84].

Chemical stability of polyphenols in cell culture studies

The chemical stability of polyphenols is a major problem when *in vitro* experiments are performed. Polyphenols with anti-oxidative properties can oxidize rapidly under aqueous experimental conditions. Cell culture media used for exposure of cells to polyphenols contain several components, like iron or copper salts, which can accelerate the oxidation process [85]. Quercetin oxidizes in cell culture media at physiological conditions (pH 7.4 and 37^{o}C) to form quercetin oxidation products [86, 87]. Quercetin levels (10 μM) in DMEM culture medium supplemented with 5% FBS decreased after 4 hours of incubation (pH 7.4 and 37^{o}C) to <1% of the initial quercetin level (figure 1.2). In other cell culture media similar findings have been described [70, 88]. On the other hand, when culture medium was supplemented with 1 mM vitamin C, quercetin was relatively stable over a period of 48 hours (figure 1.2). Oxidation of quercetin results in the formation of numerous different compounds, like quercetin dimers, trimers and phenolic acids (summarized by Awad *et al.* [89]). In addition, during oxidation of quercetin in cell culture media, considerable levels of H_2O_2 and other reactive oxygen species (ROS) are formed [90]. EGCg is also a very unstable compound at physiological *in vitro* conditions. EGCg forms dimers and isomerizes to gallocatechin galate (GCg), and concurrently

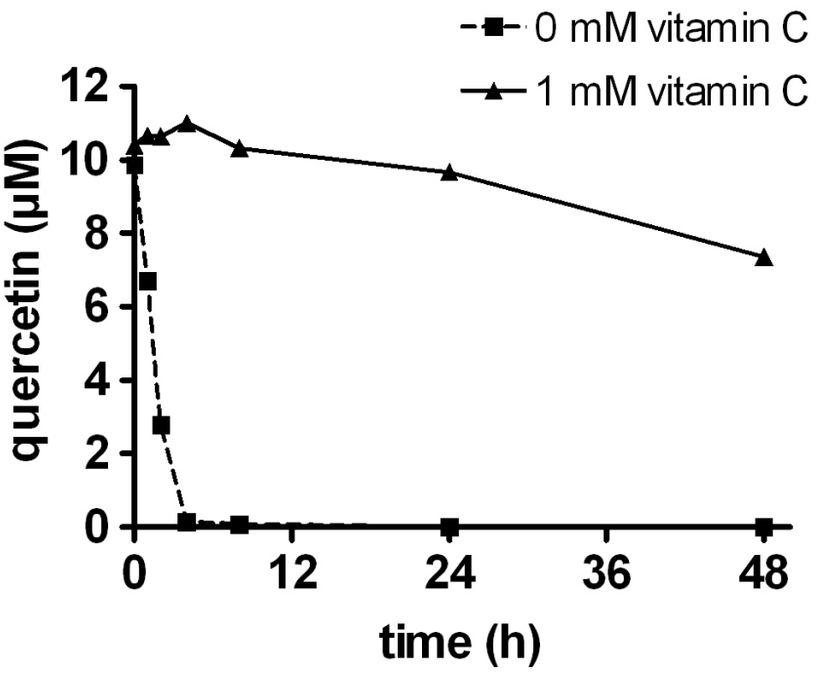

Figure 1.2
Stability of quercetin (10 µM) in DMEM cell culture medium supplemented with 5% FBS over a period of 48 hours with and without vitamin C at pH 7.4 and 37oC. Levels of quercetin in cell culture medium were quantitated using HPLC coulometric analysis as described in [127].

produces H_2O_2 in cell culture medium [91, 92]. Because it is not known whether the described oxidation processes of polyphenols take place at *in vivo* cellular targets, *in vitro* exposure of cells to EGCg and quercetin, without oxidation preventing conditions, could result in non-physiological exposures. Furthermore, the side effects of *in vitro* polyphenol exposure, like formation of ROS and polyphenol oxidation products, give rise to major interpretation difficulties of *in vitro* exposure studies [93].

IN VIVO EFFECTS OF POLYPHENOLS

The first evidence from epidemiological studies that polyphenols could have beneficial effects in humans came from a study by Hertog *et al.* in 1993 [94], where the intake of flavonols was correlated with the risk of developing cardiovascular heart diseases (CHD). Hertog *et al.* found that a high dietary flavonol intake was protective with respect to CHD risk [94]. At present, four additional studies show similar protective effect of a high flavonol intake [95]. Although evidence is less strong, high intake of other polyphenols, like catechins, are suggested to be protective against CHD as well [95]. These data from epidemiological studies indicate that flavonols

can possibly have physiological effects in humans. Mechanisms reported in literature to explain the beneficial effects of polyphenols on CHD are improvement of endothelial function and inhibition of platelet aggregation [21, 22].

Several other physiological processes and disease states, like energy metabolism, tumor formation, inflammation, neurodegeneration, and angiogenesis have been shown to be altered by polyphenol intervention. *In vivo* evidence for these effects of polyphenols from animal models is extensively reviewed [96-106]. Although *in vivo* physiological changes induced by polyphenols are most of the time very well described, the molecular mechanisms how polyphenols bring about these changes are often not so clear. Furthermore, the multitude of effects generated by *in vitro* studies that try to explain the physiological findings can be misleading when the *in vitro* effects are not validated in *in vivo* models. Therefore, elucidation of molecular mechanisms based on *in vivo* analysis of changes in gene expression, protein expression, protein activity and metabolite profiles could generate crucial explanations. In the following section, studies that use this approach to reveal the effects of polyphenols on energy metabolism[1] are used as examples to demonstrate the possibilities of this strategy.

Polyphenolic alterations of energy metabolism

Catechins from green tea, especially EGCg, have been shown to increase energy expenditure and fat oxidation in humans and animals [105]. Feeding mice a high fat (HF) diet for 4 wks, followed by a 4 wk HF diet supplemented with 0%, 0.5% or 1% EGCG, dose dependently decreased body fat accumulation [107]. In similar HF obesity inducing experiments a 1% EGCg diet for 5 months normalized body fat levels to that of mice that were not on a HF diet [108]. Furthermore, mice fed a catechin diet (0.5%) in combination with a HF diet for 15 wks reduced body weight gain by 18% as compared to mice fed a control HF diet. Interestingly, exercise had similar effects on body weight gain reduction as the catechin diet [109]. Other polyphenols were shown to have effects on fat accumulation and/or oxidation as well. Genistein supplementation (0.2%) for 12 wks in mice attenuated the increases in body weight and fat content [110]. Resveratrol treatment (0.4% resveratrol in diet, 400 mg/kg BW/day) for 15 wks

[1] Energy metabolism is used in this thesis to define the integrated manner in which the different tissues in the body regulate the utilization of energy. At the molecular level this implies those pathways that are involved in the sensing, use, storage, transport and synthesis of energy-rich substrates, from ATP and NADH to lipids, carbohydrates and proteins.

normalized body fat content of HF diet fed mice to the body fat content of control non-HF diet mice [111]. Moreover, in a study in which one year old mice were chronically administered a HF diet in combination with resveratrol (0.04% resveratrol in diet, 22.4 mg/kg BW/day), the mortality rate decreased as compared to mice that were fed a HF diet without resveratrol [112]. Blood parameter profiles were analyzed in all of the feeding studies. In general, free fatty acid, triglyceride, glucose, insulin and cholesterol levels were altered by the polyphenol intervention.

In all of the above described studies, the food intakes between polyphenol HF intervention and HF control groups were similar. In one experiment, part of the effect could be explained by decreased fat uptake from the intestines [107], but in all others different molecular mechanisms were responsible for the observed effects on energy metabolism. Lagouge *et al.* [111] demonstrated that resveratrol decreased the acetylation status of PGC1α in muscle, a regulator of mitochondrial biogenesis and mitochondrial function, thereby increasing PGC1α activity. Upregulation of PGC1α activity concomitantly induced expression of down stream target genes, like fatty acid oxidation enzyme (medium chain acyl CoA dehydrogenase, MCAD) and uncoupling protein 3 (UCP3). Microarray analysis of RNA isolated from muscle of resveratrol treated and non-treated HF diet mice revealed that genes related to oxidative phophorylation, electron transport chain and ATP synthesis were upregulated by the 15 wk resveratrol treatment [111]. Similar effects on gene expression were found in the liver of genistein and EGCg treated mice. Genistein upregulated expression levels of a number of mitochondrial fatty acid oxidation genes, which was accompanied by a decrease in body fat accumulation induced by a HF diet [110]. Supplementation of EGCg to a HF diet increased the gene expression of UCP2, whereas fatty acid synthesis genes were downregulated [107].

The above mentioned experiments demonstrate the strength of elucidating mechanisms of action of polyphenols by profiling animal physiology, gene expression and protein activity. However, all studies used an alteration of steady-state animal physiology (a HF diet) to elucidate the mechanism of effects of polyphenols. More research is required to identify the *in vivo* effects of chronic polyphenol intervention using low fat dietary conditions. Large-scale gene expression analysis methods are ideally suited for unbiased analysis of mechanisms of action of polyphenols *in vivo*.

IN VITRO EFFECTS OF POLYPHENOLS

"*Quercetin: An inhibitor of phosphatidyl inositol 3-kinase (PI3K, IC_{50} = 3.8 μM) and phospholipase (2.0 μM). Also inhibits mitochondrial ATPase, phosphodiesterases, and protein kinase C.* " (Merck Biosciences "Protein Kinase and Related Tools Brochure" 2006).

This description from a company selling biochemical products embodies the plethora of *in vitro* biological effects identified for quercetin. Furthermore, a German biochemical company adds to this: "*It induces apoptosis, blocks cells at the G0/G1 interface and activates human deacetylase SIRT1. It is also an inhibitor of fatty acid synthase.*" [http://www.proteinkinase.de/]. Numerous lengthy reviews focused on summarizing the effects of polyphenols (for example: [100, 113, 114]). On the other hand, effects of the most important *in vivo* metabolites are generally neglected or not studied. This section will focus only on effects of physiological relevant polyphenol metabolites. In addition, experimental artifacts arising from *in vitro* exposure to polyphenol aglycones will be described.

In vitro effects of quercetin metabolites

The major metabolites of polyphenols detected in plasma and tissues after polyphenol administration are polyphenol glucuronides, sulfates and methoxylates (table 1.2). Therefore, *in vitro* studies should include those metabolites for assessing relevant mechanisms of action of polyphenols. Direct inhibitory effects of quercetin glucuronides on enzyme activity were demonstrated for purified xanthine oxidase and lipooxygenase [115]. Quercetin 4'-O-glucuronide (Q4'GA) inhibited xanthine oxidase as strongly as quercetin aglycone (Ki = 0.2 μM for both compounds). Q3'GA had similar effects, whereas Q3GA inhibited the enzyme with considerably higher Ki values (160 μM) [115]. Effects on lipooxygenase were similar. This indicated that quercetin glucuronides could on the protein level be just as active as quercetin, yet inhibitory activity was strongly dependent on the conjugation position. Opposing effects of quercetin 3-O-glucuronide (Q3GA), quercetin 3'-sulfate (Q3'S) and quercetin aglycone on VEGF signaling pathways were demonstrated in cultured endothelial cell lines [116]. Q3'S increased phosphorylation of ERK1/2, PI3K and VEGF receptor, resulting in increased proliferation and migration of cultured endothelial cells. Moreover, angiogenesis was shown to be slightly enhanced when VEGF was administered in combination with Q3'S, as compared to VEGF alone [116]. On the other hand, quercetin aglycone and Q3GA were both ineffective in inducing angiogenesis, cell proliferation and migration. Even inhibition of the VEGF mediated effects was demonstrated [116].

Furthermore, extremely low concentrations of Q3'S, Q3GA and quercetin (in the nanomolar range) could elicit the observed effects [116]. In similar experiments using cultured smooth muscle cells, Q3GA dose dependently inhibited the angiotensin induced activation of JNK [123]. Because effects of VEGF and angiotensin are mediated though extracelllular binding to cellular membrane receptors, the effects on intracellular signaling could be induced without intracellular uptake of the polyphenols. However, these studies demonstrate that quercetin metabolites are bioactive compounds. Moreover, *in vivo* observed physiological effects after polyphenol administration can possibly be attributed to polyphenol metabolites.

In vitro oxidative stress

Exposure of cells under *in vitro* conditions to polyphenol aglycones can possibly generate oxidative stress and auto-oxidation of the studied polyphenol [88, 93]. Several studies have indicated that the *in vitro* biological effect of polyphenol aglycones was eliminated when incubation conditions were altered so that oxidative stress was reduced. For example, EGCg was shown to decrease the expression of phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase) in liver H4IIE cells. However, preincubation with either N-acetylcysteine (NAC) or superoxide dismutase (SOD) reversed the EGCg mediated downregulation of PEPCK and G6Pase [124]. In addition, the inhibitory effect of quercetin and myricetin on thioredoxin reductase, was eliminated when SOD was added or exposures where performed under anaerobic conditions [125]. More examples are described by Halliwell [93]. The stability of polyphenols at the *in vivo* cellular target is probably not a problem, due to efficient intracellular or extracellular anti-oxidizing enzyme systems [126]. Experimental conditions for *in vitro* exposures should therefore be controlled to prevent auto-oxidation and generation of ROS.

AIM AND OUTLINE OF THIS THESIS

The experiments described in this thesis are focused on identifying relevant mechanisms of action of selected polyphenols. Our approach was to elucidate *in vivo* targets and effects of quercetin and to clarify the *in vivo* findings with *in vitro* studies using both polyphenol aglycones and metabolites (outlined in figure 1.3).

In vivo studies using chronic dietary supplementation were performed to elucidate the effects of quercetin in specific target tissues. For this, the bioavailability of quercetin in tissues was assessed by analyzing the tissue distribution of quercetin after chronic dietary exposure in rats and pigs

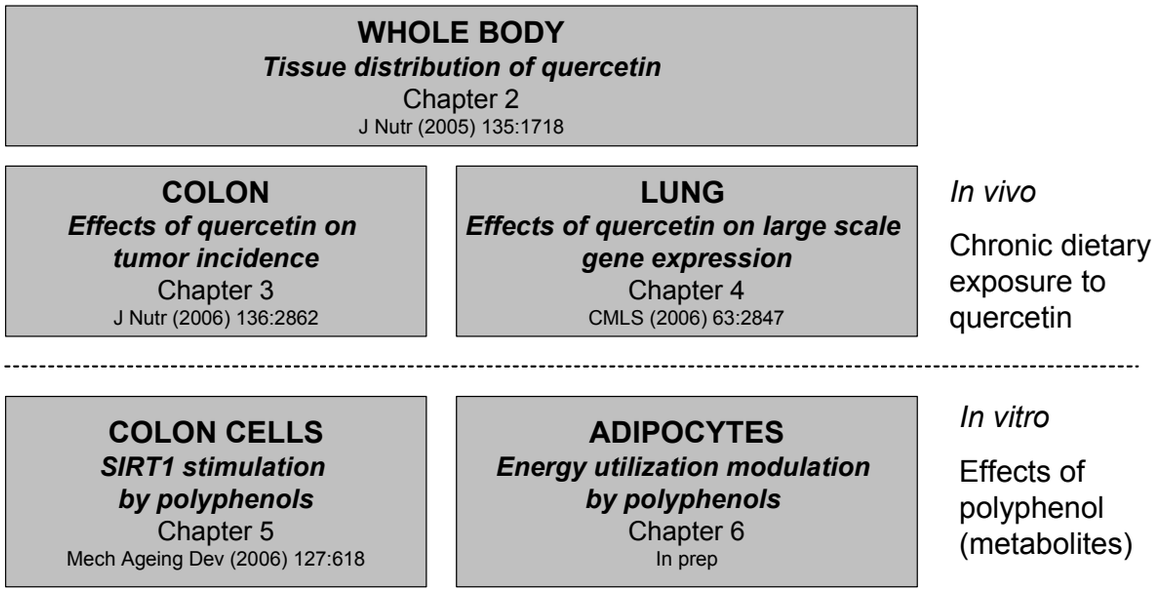

Figure 1.3
Schematic overview of *in vivo* and *in vitro* studies described in this thesis.

(**chapter 2**). A method was set-up and validated to accurately analyze the levels of quercetin (metabolites) in rat and pig tissues. Quercetin (metabolite) levels were the highest in lung tissue. Therefore, lungs were chosen as the primary target tissue for subsequent studies. **Chapter 4** describes the unbiased analysis of large-scale gene expression changes induced by the chronic quercetin diet in lung. Pathway analysis and literature mining were combined to understand the mechanisms of effect of quercetin in the lung. The major physiological process affected by the chronic quercetin diet was fatty acid catabolism. Because levels of polyphenols are the highest in the gastro-intestinal tract, the probability that polyphenols elicit effects in this organ is high. Therefore, the effect of quercetin and rutin (a quercetin glycoside) on colonic tumor formation was studied and discussed in **chapter 3**.

In vitro studies were performed to explain the finding that quercetin alters fatty acid catabolism. Firstly, since SIRT1 (a nutrient sensing deacetylase protein involved in energy metabolism) was previously shown to be stimulated by polyphenols, intracellular and recombinant SIRT1 activity was analyzed when challenged with polyphenols or their metabolites. A novel SIRT1 activity assay to specifically analyze polyphenolic stimulation was developed and the importance of stability and metabolism of polyphenols was highlighted (**chapter 6**). Secondly, polyphenolic changes in carbohydrate and lipid metabolism parameters in differentiated 3T3-L1

cells were evaluated and described in **chapter 5**. The effect of glucuronidation is emphasized in both chapters.

Finally, the results and findings are summarized in **chapter 7** and molecular mechanisms that possibly underlie the *in vivo* findings will be explained and discussed. Implications for further research and future perspectives are given in this chapter as well.

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CHAPTER 2

Tissue distribution of quercetin in rats and pigs

ABSTRACT

Quercetin is a dietary polyphenolic compound with potentially beneficial effects on health. Claims that quercetin has biological effects are mainly based on *in vitro* studies with quercetin aglycone. However, quercetin is rapidly metabolized and availability to tissues is hardly known. To assess the long-term tissue distribution of quercetin, 2 groups of rats were given a 0.1% or 1% quercetin diet (~50 or ~500 mg/kg body weight (BW)) for 11 wk. In addition, a 3-d study was done with pigs fed a 500 mg/kg BW diet. Tissue levels of quercetin and quercetin metabolites were analyzed with an optimized extraction method. Quercetin and quercetin metabolites were widely distributed in rat tissues, with the highest levels in lungs (0.1% quercetin diet: 3.98 nmol/g tissue and 1%: 15.3 nmol/g tissue) and the lowest in brain, white fat and spleen. In the short-term pig study, liver (5.87 nmol/g tissue) and kidney (2.51 nmol/g tissue) contained high levels of quercetin and quercetin metabolites, whereas brain, heart and spleen had low levels. These studies have for the first time identified target tissues of quercetin, which may help to understand the mechanisms of action of quercetin *in vivo*.

Keywords: quercetin, glucuronides, tissue distribution, flavonoids, polyphenols

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INTRODUCTION

Quercetin is a plant-derived, dietary compound with potentially beneficial effects on cardiovascular diseases [1, 2]. It belongs to a group of polyphenolic compounds termed flavonoids. This is a large group of secondary metabolites from plants consisting of ~6000 different compounds [3]. Vegetables and fruits are rich sources of flavonoids in our diet, but tea and red wines also contain flavonoids. The main dietary sources of quercetin are onions, apples and tea [4]. Furthermore, the intake of food supplements containing quercetin is becoming increasingly common [5].

Although quercetin is not the most predominant flavonoid in our diet [6], it is one of the most studied flavonoids. Most research has focused on the anti-oxidant properties of quercetin, its effects on several enzyme systems and effects on biological pathways involved in carcinogenesis, inflammation and cardiovascular diseases [7-12]. However, most of these studies were done *in vitro* with quercetin aglycone (the unconjugated molecule), which is not present in the plasma of humans due to its very efficient phase II metabolism [13]. Because the properties of quercetin aglycone and quercetin metabolites differ, studies with the aglycone have limited value. For example, effects of quercetin aglycone on neuronal apoptosis mediated through the MAPK pathway do not occur when quercetin is conjugated to a glucuronic acid [14].

Upon absorption in the small intestine, quercetin is metabolized immediately by enzymes in the epithelial cells and further metabolized by the liver. The catechol group of quercetin is methylated at the 3' or 4' position by catechol-O-methyl transferase (COMT), resulting in the formation of isorhamnetin (3'OCH_3-quercetin) or tamarixetin (4'OCH_3-quercetin). Both of these metabolites and quercetin can be conjugated at several hydroxyl groups with glucuronic acid or sulfate by UDP-glucuronosyltransferase or sulfotransferase, respectively [15]. The plasma half-life of quercetin in humans is between 17 and 28h [16, 17].

The tissue distribution of quercetin and its metabolites has been assessed by a number of authors. Ueno *et al.* [18] analyzed the tissue distribution of a single oral dose of ^{14}C-labeled quercetin aglycone in rats 6h after administration. The highest radioactivity levels were found in the gastrointestinal tract, liver and kidneys with much lower levels in other organs. A similar distribution was seen in rats 1 h after administration of an oral dose of radiolabeled quercetin 4'-O-glucoside, with high levels in the intestines and the major metabolizing organs [19]. Analysis of the quercetin

metabolites with LCMS-MS resolved 18 different metabolites in plasma and tissues. Free quercetin aglycone could be detected only in low levels in liver, but not in plasma and kidneys [19]. These studies with radioactively labeled quercetin were done with a single oral dose and rats were killed shortly after ingestion. In a longer study with rats fed a 0.25% quercetin diet for 2 wk, the livers contained quercetin and/or quercetin metabolites [20]. Furthermore, in a different study with rats fed a 0.5% quercetin diet for 2 wk, in addition to liver and kidney, heart tissue also appeared to contain detectable concentrations of quercetin and/or quercetin metabolites. The presence of quercetin and/or quercetin metabolites in other organs is not known because liver, kidney, and heart tissues were the only organs analyzed [21].

To improve our understanding of the mechanism of action of quercetin, it is necessary to know which metabolites are present in the body and in which tissues and organs they are found. Because only limited information on the tissue distribution is available at present, we conducted feeding studies in 2 species and determined quercetin and quercetin metabolites in tissues using an optimized extraction method. We analyzed the tissue distribution of quercetin and quercetin metabolites in rats after an 11-wk exposure to a 0.1% or 1% quercetin diet (~50 or ~500 mg/[kg BW · d]) and in pigs after a 3-d exposure to a high quercetin diet [500 mg/(kg BW · d)].

MATERIAL AND METHODS

Chemicals

Quercetin and β-glucuronidase/sulfatase (*Helix pomatia*, G1512) were obtained from Sigma-Aldrich (St Louis, MO, USA). Isorhamnetin (3'OCH_3-quercetin) and quercetin 3-O-β-glucoside were obtained from Roth (Karlsruhe, Germany). Tamarixetin (4'OCH_3-quercetin) was obtained from Extrasynthese (Genay, France). Quercetin 3-O-β-glucuronide was purchased from Apin Chemicals (Abingdon, UK) and verified by NMR. All other chemicals used were of analytical grade.

Animals and diets

Rats

The experimental protocol was approved by the Animal Welfare Committee of Wageningen University, Wageningen, The Netherlands. Inbred male Fisher 344 rats [age 7 wk (4 wk of age at arrival followed by 3 wk acclimatization) 134 +/- 5 g; mean +/- SD BW, Charles River

Laboratories, Inc., Sulzfeld, Germany] were housed in pairs in cages in a room with controlled temperature (20-22 °C), controlled relative humidity (50-70%) and a 12-h light:dark cycle (lights on at 0600 h). The rat diet was a RM3[E]FG SQC breeding diet[1] (Special Diets Services, Witham, UK) and did not contain any detectable concentrations of quercetin. The diet was supplemented with 0.1% quercetin or 1% quercetin (6 rats per group) for 11 wk. Rats consumed their food *ad libitum* and had unlimited access to water; their weight and food consumption were determined weekly. Calculated quercetin intake for rats fed a 1% quercetin diet during the 11 wk ranged from ~800 mg/kg BW per day in wk 1 to ~500 mg/kg BW per day during wk 11. The quercetin intake of the 0.1% quercetin group was 10% that of the 1% quercetin group. In addition to the rats fed a quercetin diet, 6 control rats were given the same diet without the addition of quercetin; their weight and food intake did not differ from the quercetin intake groups.

After consuming the quercetin diet for 11 wk, rats were anesthetized in the morning, without overnight fasting, by inhalation of isoflurane, using N_2O-O_2 (1:1, v/v) as a carrier. Rats were fully bled via the abdominal aorta. Blood (8 – 12 mL) was collected in EDTA-tubes and plasma was subsequently prepared in Leucosep Centrifuge tubes (Greiner Bio-one, Longwood, FL, USA) by centrifuging for 20 min at 1000×g and 4 °C. Plasma samples were stabilized with ascorbic acid (5.6 mmol/L final concentration) before storage at -80 °C. After blood collection, the following tissues were dissected, weighed, and immediately frozen in liquid nitrogen: testes (3.14 +/- 0.14 g wet weight, means +/- SD, n = 12), spleen (0.74 +/- 0.04 g), kidneys (1.92 +/- 0.10 g), abdominal white adipose tissue (4.16 +/- 0.74 g), liver (10.41 +/- 0.57 g), bone (sternum + bone marrow) (2.11 +/- 0.36 g) , thymus (0.26 +/- 0.03 g), heart (0.87 +/- 0.07 g), lungs (1.34 +/- 0.10 g), muscle (quadriceps) (4.39 +/- 0.79 g), brown adipose tissue (0.28 +/- 0.07 g) and brain (1.73 +/- 0.24 g). The emphasis in this study was on non-gastrointestinal tract organs, because gastrointestinal organs are predominantly exposed directly to quercetin from the diet and therefore gastrointestinal organs were not collected.

Pigs

Two cross-bred castrated male pigs (PIC, Schleswig, Germany) weighing 122.4 kg and 138.9 kg, were restrictively fed (80% of the demand for maintenance) a high-quercetin diet (500 mg quercetin/kg BW per day,

[1] The commercial diet contained (g/kg) 223.0 crude protein, 43.0 crude oil, 45.0 crude fiber, and 77.0 ash.

divided into 3 meals a day) for 3 consecutive days. The diet consisted mainly of wheat and defatted soybean meal. After 3 d, the pigs were food deprived for about 8 h before killing. Then tissues (liver, kidney, spleen, brain, heart) and blood were collected. Tissue samples were collected by taking 3 pieces of the organ, each piece weighing approximately 100 g. Whole blood was collected in heparinized tubes and plasma was prepared by centrifugation.

Preparation of samples

All tissues were lyophilized before further processing. Subsequently, all pig tissues were chopped into small pieces in liquid nitrogen and milled using a Janke & Kunkel Analysenmühle A10 (IKA labortechnik, Staufen, Germany). Rat tissues were pooled per intake group (6 rats per group) and ground and homogenized with a mortar and pestle. Liver, bone, kidney, lung, muscle, and brain tissues required additional homogenization in the mill. Samples were stored in airtight containers at -20 ^{o}C.

Extraction: procedure

For extraction, 0.5 +/- 0.02 gram pig tissue or 0.1 +/- 0.01 gram of rat tissue except for spleen (0.06 g), brown adipose tissue (0.04 g) and thymus (0.04 g) were weighed in 50 mL tubes for pig tissues or 15 mL tubes for rat tissues. The dry samples were homogenized in 14 mL of 0.5 mol/L sodium acetate buffer (NaAc, pH 5.0, with 28 mmol/L ascorbic acid) per gram tissue with a vortex and sonicated for 10 min. Each sample was either hydrolyzed with 3 mL *Helix pomatia* enzyme mix (7500 U β-glucuronidase and ~750 U sulfatase activity in 0.5 mol/L NaAc, pH 5.0, with 28 mmol/L ascorbic acid) per gram tissue for 2 h in an incubator at 37 ^{o}C, or not hydrolyzed but processed immediately with the addition of the same volume of NaAc buffer without enzyme mix. Subsequently, all samples were deproteinized with 2 vol acetonitrile and 1 vol of 20%-H_3PO_4 (with 17 mmol/L ascorbic acid) and centrifuged for 10 min at 2300×g and 10 ^{o}C. After centrifugation 1 mL supernatant was filtered through a 0.22 μm acrodisc filter (Pall Corporation, MI, USA) and injected into the HPLC system. Plasma samples were analyzed by a similar method as described previously [22].

Extraction: optimization

Extraction of quercetin, isorhamnetin, tamarixetin and their metabolites from tissues was optimized with 2 different methods. Method 1 (described in detail above) was based on the extraction of quercetin metabolites from plasma [22] and method 2 was based on the extraction of quercetin

metabolites from rat tissues by Mullen *et al.* [19]. Method 2 consisted of the following steps: samples were extracted for 1 h in 60% methanol (with 5.6 mmol/L ascorbic acid) at 60 °C in a shaking water bath. The extract was transferred to a 15 mL tube and centrifuged for 10 min at 2300×g and 10 °C. The supernatant was transferred to a clean tube and the residue was extracted 2 more times. The organic solvent from the supernatant was evaporated with a Zymark Turbo vap LV Evaporator (Zymark Corp., Hopkinton, MA, USA) at 60 °C, under a mild nitrogen flow (~5 psi). Tubes were weighed before and after evaporation of extraction solvent. Subsequently, 350 µL of the remaining water phase was hydrolyzed enzymatically for 2 h in an incubator at 37 °C with 150 µL *Helix pomatia* enzyme mixture in 0.5 mol/L NaAc (pH 5, with 28 mmol/L ascorbic acid). After hydrolysis the samples were deproteinized with 2 vol acetonitrile and 1 vol of 20%-H_3PO_4 (with 17 mmol/L ascorbic acid), centrifuged for 10 min at 10000×g and 10 °C and injected into the HPLC.

All optimizations were performed with pig liver tissue samples. Method 1 had already been optimized for plasma samples [22]. Further optimizations for freeze-dried tissue samples included: variation of enzyme incubation time (0 - 6 h) and homogenization volume (3.5, 7, and 10.5 mL). For method 2 the following extraction conditions were optimized: type of extraction solvent (methanol or acetonitrile), volume fraction of extraction solvent [20% - 80% (v/v) solvent in water], extraction temperature (room temperature and 60 °C), extraction time (a single time for 1 h or 3 times 1 h) and extraction pH (2 and 7). Recovery for both methods was calculated by spiking ground chicken liver (from a local grocery store), pig liver or rat liver samples with a known amount of quercetin, isorhamnetin, and tamarixetin, or Q3G standard. Standards were added before the addition of extraction solvent, at 50%, 100% and 200% of the original concentration in the tissue sample.

HPLC analysis

The HPLC system consisted of 2 Merck Hitachi LaChrom Elite L2130 pumps (Hitachi Ltd, Tokyo, Japan) and a Merck Hitachi LaChrom Elite L2200 injector. Separation was achieved by injecting 50 µL sample onto a Chromolith RP-18e column (100.0 x 4.6 mm, Merck, Darmstadt, Germany), protected by a NewGuard RP18 guard column (15.0 mm x 3.2 mm, 5 µm, Perkin Elmer, Shelton CT, USA). Columns were housed in a column heater (ESA, Chelmsford, MA, USA) set at 30 °C. The solvents for elution were 5% acetonitrile in 25 mmol/L citrate buffer (pH 3.7) (solvent A) and 70% acetonitrile in 25 mmol/L citrate buffer (pH 3.7) (solvent B).

The elution program at a flow rate of 2.5 mL/min was as follows: 0 – 4 min: linear gradient from 0% to 28% B, 4 – 16 min: isocratic at 28% B, 16 to 17 min: linear from 28% to 100% B, 17 to 18.5 min: isocratic at 100% B, 18.5 – 19 min: linear return to 0% B. Total run time was 22 min. Peaks were detected with a coulometric array detector (Coularray detector model 6210; ESA, Chemsford, MA, USA) set at 75, 250, and 500 mV (Pd as reference). Quercetin was quantified at the lowest potential (75 mV). Because baseline separation of isorhamnetin and tamarixetin could not be achieved, the 3 different oxidation potentials were used to quantify isorhamnetin and tamarixetin. Tamarixetin did not oxidize at 75 mV; therefore isorhamnetin was quantified at this potential. Tamarixetin was quantified at 500 mV after subtracting the area of the small isorhamnetin peak at 500 mV. The latter was calculated from the isorhamnetin peak at 75 mV, using the ratio of the peak height of isorhamnetin at 75 mV and the peak height at 500 mV of an isorhamnetin standard.

The injection peak was discarded to the waste by using a Multiport Streamswitching system (model 790 MUST, Spark Holland, Emmen, The Netherlands). At 4 min after injection the mobile phase was redirected to the detector and at 17 min after injection the port was switched back to the waste, resulting in the lowest possible contamination of the detector cell. To prolong the lifetime of the Coularray detector cell, eluent was continually pumped through the cell with a Merck Hitachi L6000 pump when the detector was out of use.

Correction for residual blood

Because tissues of rats and pigs had not been perfused after excision, they contained residual blood containing quercetin, isorhamnetin and their metabolites. Correction for residual blood was done by comparing the hemoglobin (Hb) content of freeze-dried tissues to the Hb content of freeze-dried whole blood determined with a spectrophotometric method [23]. Whole-blood samples from pigs were collected from the same pigs as the tissues. Rat whole blood was taken from a 6- mo-old Wistar rat. Freeze dried tissues, ~0.01 g for pooled rat tissues and ~0.1 g for pig tissues, were homogenized in 14 mL Tris-HCl pH 7.4, with 50 mmol/L EDTA, per gram tissue and centrifuged at 10000×g for 10 min at 10 ^{o}C. Then, 2 μL of the supernatant was used for spectrophotometric determination of the hemoglobin content with a Nanodrop (ND-1000 UV-Vis Spectrophotometer, Wilmington, DE, USA). An absorbance spectrum was taken from 220 nm to 750 nm and all samples were analyzed in duplicate. The fraction of residual blood (f_{bl}) in each tissue was calculated by

dividing the peak maximum at 540 nm of each tissue extract (Hb_{tissue}) with the peak maximum at 540 nm of a whole blood extract (Hb_{bl}) (Eq. 1).

$$f_{bl} = \frac{Hb_{tissue}}{Hb_{bl}} \quad \textbf{(1)}$$

The linearity of the method was verified by spiking freeze-dried pig liver samples with increasing concentrations of freeze-dried whole blood from pig. The quercetin or isorhamnetin concentrations that were found in each tissue extract (C_{tot}) were corrected for blood contamination by subtracting the quercetin or isorhamnetin concentrations that were found in plasma samples (C_{pl}) multiplied by the fraction of residual blood in that tissue (f_{bl}) (equation 2).

$$C_{corr} = C_{tot} - f_{bl} \cdot C_{pl} \quad \textbf{(2)}$$

where C_{corr} is the corrected concentration for quercetin or isorhamnetin. Plasma concentrations of quercetin and isorhamnetin were assumed to be equal to whole-blood concentrations. Tamarixetin could not be detected in plasma samples, therefore tamarixetin concentrations in tissue extracts were not corrected.

Statistical methods

All samples were extracted in duplicate. Quercetin, isorhamnetin and tamarixetin concentrations were expressed in nmol/g wet wt (nmol/g tissue), and represent total concentrations of aglycones plus hydrolyzed conjugates, unless indicated otherwise. Tissues of 6 rats were pooled before analysis. Tissues of pigs were analyzed separately and individual values for both pigs are given.

RESULTS

Extraction method and residual blood

Two different extraction methods were tested and optimized. Optimization of method 1 for tissue samples resulted in an optimal homogenization volume of 7 mL buffer for a 0.5-g sample and a 2-h incubation period with *Helix pomatia* β-glucuronidase/sulfatase at 37 °C. These conditions gave the highest yield for quercetin with the least variation: 14 +/- 1.35 nmol/g dry wt (mean +/- SD, n = 8 different extractions on 4 different days). The recoveries for quercetin aglycone, isorhamnetin aglycone, tamarixetin aglycone and quercetin 3-O-β-glucoside were determined by addition of standard compounds and ranged from 72 to 91% (Table 2.1).

Table 2.1
Characteristics of the method used for analyzing pig and rat tissues (method 1).

	Quercetin	Isorhamnetin	Tamarixetin	Q3G[5]
Recovery (mean +/- SD %)[1]	81 +/- 9	85 +/- 8	91 +/- 22	72 +/- 3
LOD (nmol/g tissue)[2]	0.12	0.14	0.19	-[6]
CV within day (%)[3]	5	9	-	-
CV between day (%)[4]	9	15	5	-

[1] Recovery was determined at 3 levels of addition of the flavonoid (50%, 100% and 200% of the original level in the sample).
[2] LOD is the limit of detection defined as the concentration producing a peak height 3 times the baseline noise and calculated for uncorrected tissue levels in nmol per gram wet weight (nmol/g tissue).
[3] The within day CV was determined by comparing different injections (n=3) of the same extract within the same run.
[4] The between day CV was determined by comparing different extractions of the same pig liver samples over a period of 3 mo of at least 5 different analyses.
[5] Q3G = quercetin 3-O-β-glucoside.
[6] (-) = not analyzed.

Optimization of method 2 resulted in large differences in yield under different extraction conditions. The optimal solvent concentration was 60% (v/v) methanol in water. Extraction with 70% (v/v) acetonitrile gave similar quercetin concentrations. When the pH of the extraction solution during methanolic extraction was lowered to pH 2, the quercetin concentrations decreased with 50%, as compared to methanolic extraction at pH 7. Extracting at room temperature resulted in concentrations that were 20% lower than extracting at 60°C. The optimized method 2 gave approximately 20% lower quercetin concentrations than method 1. The recoveries of quercetin aglycone and quercetin 3-O-β-glucoside for method 2 were 86% +/- 4% and 103% +/- 3% (mean +/- SD, n = 2 different extractions) respectively. Because method 1 gave higher quercetin values than method 2 and was less time-consuming, method 1 was chosen for analysis of the tissues from pigs and rats. The analytical characteristics for method 1 are shown in Table 2.1.

To verify the validity of the blood correction method, pig liver tissue was spiked with freeze-dried whole blood. No additional peaks appeared in the absorbance spectrum of Hb and the peak maximum at 540 nm increased linearly with increased spiked blood content (data not shown). The fraction of residual blood in the tissues ranged from 0.01 to 0.25 (Table 2.2).

Table 2.2
Fraction of residual blood in rat and pig tissues[1].

	Rats		Pigs	
	0.1% quercetin diet	1% quercetin diet	Pig1	Pig2
blood	1	1	1	1
liver	0.06	0.07	0.06	0.05
kidney	0.10	0.09	0.04	0.06
muscle	0.01	0.01	-[2]	-
heart	0.13	0.12	0.06	0.09
lung	0.13	0.15	-	-
brain	0.02	0.02	0.03	0.02
testes	0.03	0.04	-	-
spleen	0.25	0.13	0.26	0.18
thymus	0.02	0.03	-	-
bone	0.02	0.02	-	-
brown fat	0.01	0.01	-	-
white fat	0.01	0.02	-	-

[1] Tissues of 6 rats were pooled before analysis.
[2] (-) = tissue not collected.

Tissue distribution of quercetin, isorhamnetin and tamarixetin in rats

Analysis of tissues and plasma of rats fed a 0.1% or 1% quercetin diet showed that the tissue and plasma concentrations of quercetin, isorhamnetin and tamarixetin were ~4 times higher in rats fed the 1% quercetin diet than those fed the 0.1% (Tables 2.3, and 2.4). Thus a 10-fold increase in the dose of dietary quercetin resulted in a 4-fold increase in concentrations of quercetin, isorhamnetin and tamarixetin in plasma and tissues. In contrast to the difference in the concentrations of quercetin, isorhamnetin and tamarixetin between the rats fed the 0.1% and 1% diet, the distribution of quercetin, isorhamnetin and tamarixetin over the different tissues was similar for the 2 groups of rats. The highest concentration of the sum of quercetin, isorhamnetin and tamarixetin was found in the lungs of both the 0.1% (3.98 nmol/g tissue) and 1% (15.3 nmol/g tissue) quercetin intake groups. The lowest concentrations were found in the brain, white fat and spleen (tables 2.3 & 2.4). The major metabolizing organs (liver and kidney) contained intermediate quercetin, isorhamnetin and tamarixetin concentrations. For liver this was 1.79 nmol/g tissue (0.1% group) and 6.34 nmol/g tissue (1% group) and for kidney it was 2.85 nmol/g tissue (0.1% group) and 11.6 nmol/g tissue (1% group) (Tables 2.3, and 2.4).

Table 2.3
Quercetin, isorhamnetin and tamarixetin concentrations in tissues of rats fed a 0.1% quercetin diet for 11 wk[1,2].

	Sum of quercetin, isorhamnetin and tamarixetin	Quercetin		Isorhamnetin		Tamarixetin	
	nmol/g tissue[3]	nmol/g tissue	% of sum	nmol/g tissue	% of sum	nmol/g tissue	% of sum
plasma (nmol/mL)	23.4	7.7	33	15.7	67	<LOD[3]	
lung	3.98	1.04	26	2.84	71	0.10	2
testes	2.98	0.82	27	2.07	69	0.10	3
kidney	2.85	0.93	33	1.88	66	0.04	1
heart	2.20	0.50	23	1.38	63	0.32	14
liver	1.79	0.52	29	1.18	66	0.09	5
brown fat	1.53	0.15	10	1.12	73	0.25	17
thymus	1.37	0.15	11	1.22	89	0.00[5]	0
muscle	1.10	0.12	11	0.77	69	0.21	19
bone	0.92	0.14	16	0.78	84	0.00	0
brain	0.33	<LOD		0.19	57	0.14	43
spleen	0.02	<LOD		<LOD		0.02	100
white fat	<LOD	<LOD		<LOD		<LOD	

[1] All quercetin, isorhamnetin and tamarixetin concentrations represent total concentrations of aglycones plus hydrolyzed conjugates and are corrected for residual blood.
[2] Tissues of 6 rats were pooled before analysis.
[3] Data are expressed as nmol per gram wet weight.
[4] <LOD = below detection limit. LODs are quercetin = 0.12 nmol/g tissue, isorhamnetin = 0.14 nmol/g tissue and tamarixetin = 0.19 nmol/g tissue.
[5] Values can be lower than LOD because of the correction factor for residual blood.

For most of the tissues in both the 0.1% and 1% groups, the concentration of quercetin was ~ 30% (range 23% - 36%) of the total concentration of quercetin metabolites (Tables 2.3, and 2.4). However, in some tissues, the relative contribution of quercetin to the total concentration of quercetin metabolites was lower. In bone, muscle, thymus, and brown fat tissue of the 0.1% group (Table 2.3) the quercetin concentration ranged from 10 to 16% of the total concentration of quercetin metabolites, indicating that the level of methoxylation and/or tissue uptake of methylated metabolites differed between tissues. In addition, tamarixetin was not

Table 2.4
Quercetin, isorhamnetin and tamarixetin concentrations in tissues of rats fed a 1% quercetin diet for 11 wk[1,2].

	Sum of quercetin, isorhamnetin and tamarixetin	Quercetin		Isorhamnetin		Tamarixetin	
	nmol/g tissue[3]	nmol/g tissue	% of sum	nmol/g tissue	% of sum	nmol/g tissue	% of sum
plasma (nmol/mL)	107.5	40.4	38	67.2	62	<LOD[4]	
lung	15.3	5.02	33	9.34	61	0.98	6
testes	14.4	4.37	30	7.65	53	2.37	16
kidney	11.6	3.67	32	6.38	55	1.57	14
thymus	10.4	2.66	26	6.42	62	1.26	12
heart	7.94	2.46	31	5.14	65	0.34	4
liver	6.34	2.25	36	3.68	58	0.40	6
brown fat	5.55	1.53	28	3.13	56	0.89	16
bone	5.36	1.83	34	2.80	52	0.72	13
muscle	4.16	1.21	29	2.58	62	0.37	9
white fat	1.22	<LOD		0.56	46	0.66	54
brain	0.68	0.06[5]	8	0.46	67	0.17	25
spleen	0.11	<LOD		<LOD	0	0.11	100

[1] All quercetin, isorhamnetin and tamarixetin concentrations represent total concentrations of aglycones plus hydrolyzed conjugates and are corrected for residual blood.
[2] Tissues of 6 rats were pooled before analysis.
[3] Data are expressed as nmol per gram wet weight.
[4] <LOD = below detection limit. LODs are quercetin = 0.12 nmol/g tissue, isorhamnetin = 0.14 nmol/g tissue and tamarixetin = 0.19 nmol/g tissue.
[5] Values can be lower than LOD because of the correction factor for residual blood.

detected in the plasma of either group, whereas moderate concentrations of tamarixetin were detected in most of the tissues. Tamarixetin concentrations were always lower than quercetin or isorhamnetin concentrations.

Tissue distribution of quercetin, isorhamnetin and tamarixetin in pigs

In pigs, the plasma concentration of quercetin was 1.1 nmol/mL and that of isorhamnetin 0.15 nmol/mL (Table 2.5). Tamarixetin was not detected in pig plasma. In contrast to what was observed in rats, the tissue concentrations in the liver and kidney of pigs were higher than the plasma

concentrations. The quercetin concentrations in liver and kidney were 3.8 nmol/g tissue and 1.8 nmol/g tissue, respectively. The other tissues (brain, heart, and spleen) contained only low concentrations of quercetin; these were at the limit of detection (LOD) which is 0.12 nmol/g tissue for quercetin. Furthermore, isorhamnetin and tamarixetin could not be detected in brain, heart, and spleen.

Quercetin and isorhamnetin aglycone concentrations in rat tissues

For analysis of aglycone levels in tissue samples the enzymatic hydrolysis step was omitted from the extraction method. Tissue samples were immediately deproteinized and injected into the HPLC after homogenization in sodium acetate buffer. In both the 0.1% and 1% quercetin groups, several tissue extracts contained high concentrations of free quercetin and isorhamnetin. The aglycone concentrations in lung extracts ranged from 15% to 22%, liver 25-40%, kidney 12-17% and testes 4-10%. On the contrary, relative concentrations of aglycones in plasma extracts were only 0.4-0.8%. The other rat tissue extracts did not contain detectable aglycone.

To determine whether quercetin aglycone was released from quercetin conjugates during extraction, tissues were spiked with a known amount of

Table 2.5
Quercetin, isorhamnetin and tamarixetin concentrations in tissues of pigs fed a high quercetin diet (500 mg/kg BW per day) for 3 d[1].

	Sum of quercetin, isorhamnetin and tamarixetin		Quercetin		Isorhamnetin		Tamarixetin	
	nmol/g tissue[2]		nmol/g tissue		nmol/g tissue		nmol/g tissue	
	Mean	Δ	Mean	Δ	Mean	Δ	Mean	Δ
plasma	1.25	0.35	1.1	0.30	0.15	0.05	<LOD[3]	
liver	5.87	0.42	3.78	0.07	1.39	0.02	0.70	0.32
kidney	2.51	0.54	1.84	0.50	0.33	0.05	0.35	0.02
brain	0.22	0.07	0.22	0.07	<LOD		<LOD	
heart	0.11	0.04	0.11	0.04	<LOD		<LOD	
spleen	0.07	0.07	0.07	0.07	<LOD		<LOD	

[1] All quercetin, isorhamnetin and tamarixetin concentrations represent total concentrations of aglycones plus hydrolyzed conjugates and are corrected for residual blood.
[2] Data are expressed as nmol per gram wet weight.
[3] <LOD = below detection limit. LODs are quercetin = 0.12 nmol/g tissue, isorhamnetin = 0.14 nmol/g tissue and tamarixetin = 0.19 nmol/g tissue.

quercetin 3-O-β-glucuronide standard. Three high-aglycone tissues rats fed 1% quercetin (lung, kidney and liver) and 3 tissues without any detectable levels of aglycone (brain, muscle and bone) were spiked with 0, 20 or 37 nmol quercetin 3-O-β-glucuronide/g dry wt. Liver, lung, and kidney extracts contained high levels of free quercetin aglycone after addition of the glucuronide standard, whereas brain, muscle, and bone extracts only showed low deconjugation of quercetin 3-O-β-glucuronide standard to free aglycone (Fig. 2.1). These results suggest that in some tissues, deconjugation of quercetin glucuronides takes place during extraction.

DISCUSSION

This study shows for the first time that quercetin and its metabolites are widely distributed in rat tissues after long-term oral exposure to quercetin. The highest concentrations of quercetin, isorhamnetin and tamarixetin were found in the lungs and the lowest in brain and white fat. The tissue distribution of quercetin, isorhamnetin and tamarixetin in both intake groups showed a comparable pattern, but the concentrations in the 0.1% group were about 4 times lower than those in the 1% group.

The high concentrations of quercetin metabolites in lungs of rats may be a result of the 11-wk chronic exposure of the rats. In short-term studies in which rats were given quercetin (or its glycoside) no such marked distribution was seen. Mullen *et al.* [19] showed that 1 h after administration of a single oral dose of radioactively labeled quercetin 4'-O-β-glucoside (~ 8 mg/kg BW) most of the radioactivity was recovered, apart from the gastrointestinal tract, in the plasma, liver, and kidney of rats. Only low levels of radioactivity were found in lungs, heart, testes, spleen, and muscle, and the lowest levels of radioactivity were found in the brain. Ueno *et al.* [18] also did not find high concentrations of quercetin in tissues other than the gastrointestinal tract, liver and kidney, after a single oral dose of 630 mg radioactively labeled quercetin/kg BW. Lung, muscle, and heart levels of radioactive quercetin were significantly lower than those in kidney and liver. A 2-wk exposure to a 0.5% quercetin diet in rats resulted in high concentrations not only in kidney and liver, but also in heart [21], indicating that longer exposures cause quercetin and its metabolites to reach tissues other than liver and kidney.

The lowest concentrations of quercetin, isorhamnetin and tamarixetin were found in brain tissues of rats. Some flavonoids were shown to enter the brain after i.v. or oral exposure. Naringenin [24], puearin [25], tangeretin [26], genistein [27, 28], and epicatechin [29] were detected in

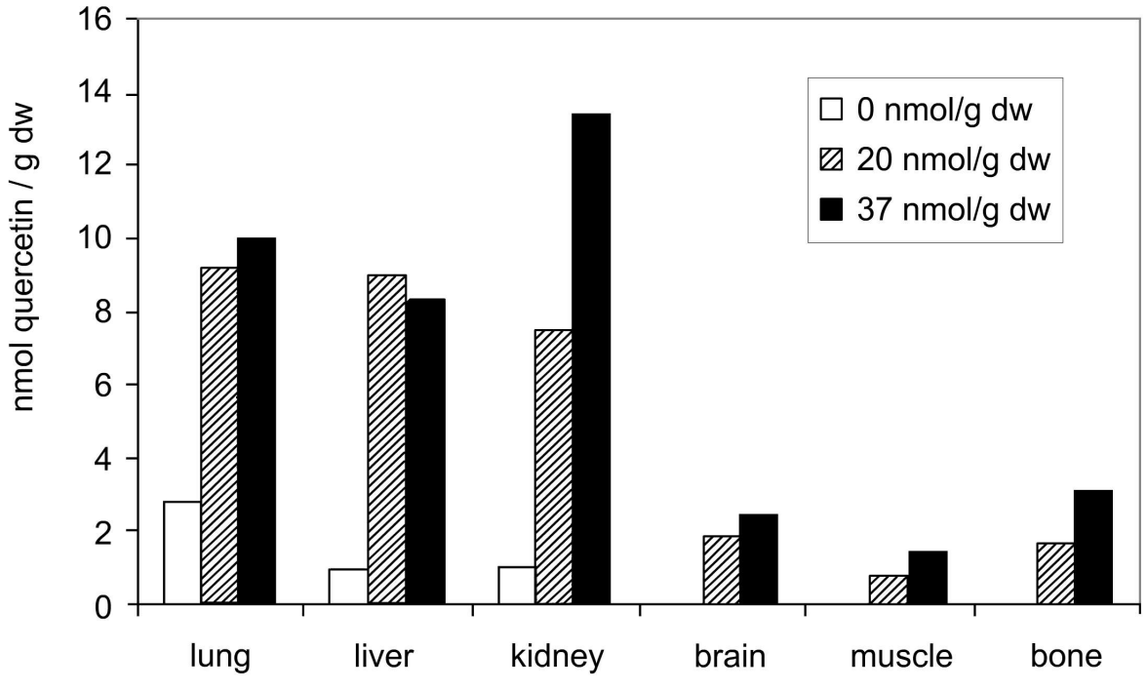

Figure 2.1
Quercetin aglycone concentrations in rat tissue extracts after a standard addition of 0 nmol, 20 nmol or 37 nmol quercetin 3-O-β-glucuronide per gram dry wt.

brain tissue. However, as noted by Youdim *et al.* [30] most of these studies did not correct for residual blood in the brain. The importance of correcting for residual blood was described by several authors [23, 31-35]. Although for some tissues, like spleen, the residual blood levels are probably overestimated, because of its role in the breakdown of erythrocytes, the residual blood concentrations reported in literature [31, 32, 35] agree with the concentrations of blood we found in tissues (table 2.2). In our study we corrected for residual blood, thus the quercetin, isorhamnetin and tamarixetin that we found in the brain were located intracellularly or interstitially and not intravascularly.

The difference between quercetin concentrations in the testes and brain is striking. Like the brain and placenta, the testes have a specific barrier [36], but the quercetin concentrations in the testes were among the highest of all organs. This indicates that the influx and efflux of quercetin conjugates is regulated differently in brain and testis tissue. Although most of the transporters in the brain are also expressed in the testis, the location of efflux transporters in the brain is mainly in the endothelium cell layer, whereas efflux pumps in the testis are expressed mainly in the sertoli cells [37]. This may result in higher interstitial concentrations of quercetin in the testis than in brain.

The tissue and plasma concentrations of quercetin, isorhamnetin and tamarixetin in pigs differed from the concentrations in tissues and plasma of rats. The plasma concentrations in pigs were lower than those in liver and kidney of pigs, and the concentrations in pig heart tissue were similar to those in pig brain and spleen (table 2.5), whereas in rats, plasma concentrations were at least 6 times higher than tissue concentrations; heart tissue was similar to liver and kidney (Table 2.3, and 2.4). This may be explained by several factors that differ between the 2 feeding studies. The administration of the diet differed in the 2 feeding studies. Pigs were given the quercetin diet in 3 separate meals each day, whereas the rats consumed their food *ad libitum*. In addition, the pigs were food deprived for 8 h before they were killed, whereas the rats were not food deprived. Pigs and rats also differ in the way they eliminate quercetin from their body. Pigs are much less effective in converting quercetin to isorhamnetin. The activity of COMT in pigs is probably much lower than in rats; 1 h after a single dose of quercetin 4'-O-β-glucoside more than half of the total quercetin metabolite concentrations consisted of methoxy derivatives in rats [19], whereas in pigs the concentrations of methoxy derivatives were always much lower than quercetin derivatives [38, 39], which is in accordance with our data. The plasma half-life for quercetin, isorhamnetin and tamarixetin in pigs is only 4 h [38], whereas the plasma half-life for quercetin, isorhamnetin, and tamarixetin in rats is much longer than 4 h (> 8 h, as derived from [40]). Thus, quercetin is eliminated at least twice as fast from the blood in pigs as in rats.

Although quercetin is present primarily in tissues conjugated to methoxy, sulphate, and glucuronic acid groups, it cannot be excluded that quercetin appears in tissues in the unconjugated form. We found that especially lung, liver, and kidney of rats possess a high deconjugation activity, probably due to enzymes with β-glucuronidase activity. This high enzyme activity resulted in an *ex vivo* conversion of conjugated quercetin to free aglycone during the extraction procedure (see Fig. 2.1). However this high β-glucuronidase activity of tissues may also cause *in vivo* conversion of conjugated quercetin to free aglycone. Enzymes with β-glucuronidase activity can either be released under certain physiological conditions, such as inflammation and neoplasm [41, 42] or microsomal β-glucuronidase enzymes can convert glucuronides directly to the aglycone. O'Leary *et al.* [43] showed that quercetin 3-O-β-glucuronide and quercetin 7-O-β-glucuronide were deconjugated in hepatocytes and further metabolized to quercetin 3'-sulfate, indicating that quercetin glucuronides can enter tissue cells and that

microsomal enzymes with β-glucuronidase activity are able to convert quercetin glucuronides intracellularly to the quercetin aglycone.

In conclusion, these studies have demonstrated that long-term exposure to quercetin in rats results in a wide distribution of quercetin metabolites to most of the organs. A short exposure in pigs did not result in high concentrations of quercetin metabolites in tissues other than kidney and liver. In addition, this study does not exclude the presence of free aglycone in tissues, but when aglycone concentrations are analyzed, attention should be paid to deconjugation reactions during extraction. These experiments have identified target tissues of quercetin, which may help to understand the mechanisms of action of quercetin *in vivo*.

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ABBREVIATIONS

BW, body weight; COMT, catechol-O-methyl transferase; Hb, hemoglobin; LOD, limit of detection; Q3G, quercetin 3-O-β-glucoside;

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CHAPTER 3

Quercetin, but not its glycosidated conjugate rutin, inhibits azoxymethane-induced colorectal carcinogenesis in F344 rats

ABSTRACT

The effect of the flavonoid quercetin and its conjugate rutin was investigated on (biomarkers of) colorectal cancer (CRC). Male F344 rats (n = 42/group) were fed 0, 0.1, 1, or 10 g quercetin/kg diet or 40 g rutin/kg diet. Two wk after initial administration of experimental diets, rats were given 2 weekly subcutaneous injections with 15 mg/kg body wt azoxymethane (AOM). At wk 38 post-AOM, quercetin dose dependently ($P < 0.05$) decreased the tumor incidence, multiplicity, and size, whereas tumor incidences were comparable in control (50%) and rutin (45%) groups. The number of aberrant crypt foci (ACF) in unsectioned colons at wk 8 did not correlate with the tumor incidence at wk 38. Moreover, at wk 8 post-AOM, the number and multiplicity of ACF with or without accumulation of ß-catenin were not affected by the 10 g quercetin/kg diet. In contrast, another class of CRC-biomarkers, ß-catenin accumulated crypts, contained less ß-catenin than in controls ($P < 0.05$). After enzymatic deconjugation, the plasma concentration of 3'-O-methyl-quercetin and quercetin at wk 8 was inversely correlated with the tumor incidence at wk 38 ($r = –0.95$, $P < 0.05$). Rats supplemented with 40 g rutin/kg diet had only 30% of the (3'-O-methyl-) quercetin concentration of 10 g quercetin/kg diet-fed rats ($P < 0.001$). In conclusion, quercetin, but not rutin, at a high dose reduced colorectal carcinogenesis in AOM-treated rats, which was not reflected by changes in ACF-parameters. The lack of protection by rutin is probably due to its low bioavailability.

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INTRODUCTION

Colorectal cancer (CRC) is a highly frequent malignancy in the Western world with a lifetime risk of 5% [1]. Epidemiological studies suggest that intake of fruits and vegetables [2], dietary fiber [3], and flavonoids [4] are inversely correlated with the CRC incidence. One of the major flavonoid representatives in the human diet is quercetin, which in its natural form is glycosylated and can be found in fruits and vegetables, including blueberries, onions, curly kale, broccoli, and leek [5]. Once ingested, conjugated quercetin can be released by intracellular and membrane-bound ß-glycosidases of small intestinal cells [6]. In the colon, on the other hand, quercetin glycosidic bonds can be hydrolyzed by local ß-glycosidases produced by bacteria, leading to colonic release and absorption of quercetin [7]. This is also the case with rutin (quercetin-3-O-ß-rutinoside), the major quercetin glycoside found in tea.

Worldwide, CRC shows a high annual incidence (1,000,000 patients) and mortality (530,000) [8]. Therefore, biomarkers that reveal colorectal carcinogenesis at an early stage are urgently needed for prevention of colorectal tumors. Such biomarkers were first described in 1981 by Shamsuddin *et al.* [9, 10] and designated aberrant crypts in 1987 by Bird [11]. However, the validity of these histo-pathological aberrations as predictors of colon carcinogenesis is still a matter of debate [12–14]. It has been suggested that histological lesions showing accumulation of ß-catenin in colonic crypts may be more relevant indicators of colorectal carcinogenesis [15]. In mice, these ß-catenin accumulated crypts (BCA-C) demonstrate increased cell proliferation and are positively correlated with the development of colorectal tumors. Gene mutations in ß-catenin, among others, may prevent phosphorylation of the ß-catenin protein, which is required for its degradation and can lead to its cytoplasmic accumulation [16]. Consequently, cytoplasmic ß-catenin can migrate into the nucleus and may target cell proliferation, differentiation, and apoptosis [1, 17]. Therefore, accumulation of free ß-catenin is thought to be an early event in colorectal carcinogenesis [16].

Because quercetin supplements are commercially available and claimed to be health promoting, the aim of the present study was to investigate the effects of quercetin aglycone, i.e. the sugar-free flavonoid, and its major dietary source, rutin, on (biomarkers of) colorectal carcinogenesis. Based on previous studies [18, 19], it was hypothesized that quercetin might inhibit development of CRC in azoxymethane (AOM)-treated rats.

MATERIAL AND METHODS

Experimental design

At 4 wk of age, 210 inbred male F344 rats (Charles River Laboratories) weighing 40–90 g were housed (2/cage) in sawdust-covered plastic cages with wire tops. Rats consumed food and tap water *ad libitum*. Experimental protocols were approved by the Ethical Committee of Wageningen University and Research Centre. Rats were first acclimatized for 3 wk on a basal RM3[E] FG SQC diet [20] (SDS Special Diets Services), subsequently allocated to 5 groups of 42 rats each, and fed the control (RM3) diet or enriched diets containing either 0.1, 1, or 10 g quercetin/kg diet or 40 g rutin/kg diet (Sigma). Experimental diets were produced monthly and analyzed for flavonoid contents by HPLC [21]. After 2 wk, 30 rats from each group were injected subcutaneously with 15 mg/kg body wt of the carcinogen azoxymethane (AOM; NCI/MRI) dissolved in a physiological salt solution, whereas the remaining 12 rats/group received the solvent only. One wk thereafter, injections were repeated (wk 0 post-AOM).

Interim autopsy

At 8 wk post-AOM, 8 AOM-treated and 6 untreated rats/diet group were killed in the morning without overnight food deprivation. Rats were anesthetized by inhalation of 5% isoflurane combination with O_2 and N_2O (1:1). After collection of abdominal aortic blood, quercetin and its metabolites in plasma of untreated rats were stabilized by 5.6 mmol/L ascorbate (Merck) [20].

Following excision, colorectums were cut longitudinally and rinsed with 70% ethanol at 4°C. Colorectums of AOM-treated rats were fixed between filter paper and stored in 70% ethanol at 4°C prior to the aberrant crypt foci (ACF) counts and finally embedded in paraffin.

Final autopsy

At wk 38 post-AOM, rats were killed as described above, with modifications. Among tumor-bearing rats, the tumor number and maximum diameter were recorded. Large tumors (diameter 5 mm) were removed and split diagonally [13]. The luminal part, as well as small tumors (diameter <5 mm), were fixed in RNAlater, for mRNA analysis to be reported elsewhere. Therefore, tumor classification as being adenomas or carcinomas was assessed only in large tumors.

ACF in unsectioned colons

To visualize ACF, colorectums of AOM-treated rats killed at the interim autopsy were stained for 10 min with 0.1% (w:v) methylene blue dissolved in 70% ethanol. ACF were recognized as single or multiple enlarged crypts, with altered luminal openings and thickened epithelium, in comparison with surrounding normal crypts [11]. ACF numbers and multiplicity, i.e. the number of aberrant crypts per ACF, were scored.

Quantification of quercetin and its metabolites in plasma

Quercetin metabolites in pooled plasma samples of AOM-untreated rats killed at the interim autopsy were deconjugated by the Helix pomatia enzyme (Sigma) with ß-glucuronidase 7500 kU/L) and sulfatase (500 kU/L) activity [20]. Quercetin and its metabolites were analyzed by HPLC with Coularray detection and quantified with calibration curves for quercetin, 3'-O-methyl-quercetin (isorhamnetin, Roth), and 4'-O-methyl-quercetin (tamarixetin, Extrasynthese). Plasma concentrations of rats fed 1 and 10 g quercetin/kg diet were published previously [20].

Immuno-histochemistry.

After alcohol fixation and paraffin embedding, we performed immuno-histochemistry on the middle of the distal colon because of the highest ACF frequency. Per rat, 35 colon sections were cut en face at 4 μm [15] for hematoxylin and eosin staining (every 1st slide) as a reference for adjacent slides stained for total ß-catenin (every 2nd slide) and Ki67 (every 3rd slide). After blocking endogenous peroxidase (30 min in 0.3% [v:v] H_2O_2 in methanol) and heat-induced antigen retrieval (15 min in 1 mmol/L EDTA-NaOH, pH 8.0 at 95–98°C), sections were incubated with 25% (v:v) goat serum in PBS (15 min). Subsequently, sections were incubated with primary antibodies against total ß-catenin (Neomarkers) for 30 min (4000x PBS dilution, rabbit polyclonal antibody) or the proliferation marker Ki67 (Neomarkers) for 10 min (200x PBS-dilution, rabbit monoclonal antibody). Sections were then incubated with the secondary Powervision Poly horseradish peroxidase labeled polyclonal antibody (Immuno Vision Technologies) for 30 min. Horseradish peroxidase activity was visualized by incubation with Vector NovaRED (Vector Laboratories) for 10 min. Finally, sections were weakly counterstained with hematoxylin for 45 s.

ß-Catenin accumulated crypts (BCA-C) and ß-catenin accumulated ACF (BCA-ACF) were recognized as crypts or ACF, respectively, showing nucleic and/or cytoplasmic ß-catenin accumulation [15]. The number of lesions, multiplicity, and grade of ß-catenin accumulation was scored, the latter expressed as excessive ß-catenin staining intensity (low, intermediate,

or high) relative to normal crypts. To distinguish ACF in unsectioned and en face sectioned colons, the term en face ACF is used for the latter.

In Ki67-stained slides, the number of positive and negative nuclei were scored per ACF and expressed as the proliferation index [22]. Large colorectal tumors (diameter 5 mm) were classified in hematoxylin and eosin stained slides [13].

Statistics

We analyzed the dose-dependent effect of quercetin on ACF multiplicity and tumor incidence with the Cochran-Mantel-Haenszel test. Tumor incidences among different groups were compared with one another using the chi-square test. We calculated correlations between dietary quercetin and plasma concentrations of quercetin and its metabolites with the Pearson's correlation coefficient. Quercetin-mediated effects on CRC precursors, assessed by immuno-histochemistry in rats fed a 0 or 10 g quercetin/kg diet, were analyzed using the nonparametric Mann-Whitney U test to account for unequal variances. Plasma concentrations of quercetin and 3'-O-methyl-quercetin were compared using the 1-way ANOVA, followed by the t test with the overall error term to compare all groups. Mean values are presented as means ± SD and differences were significant if P 0.05 (2-tailed).

RESULTS

Diets, body wt, dietary intake, and survival

The basal diet contained no quercetin and rutin. For AOM-treated rats fed the diet supplemented with 10 g quercetin/kg, the corrected dietary intake of quercetin was 900 mg/(kg body wt•d) at wk 2, declining to 450 mg/(kg body wt•d) at wk 38 post-AOM. The corrected quercetin intake by untreated rats fed diets containing 1 g/kg and 0.1 g quercetin/kg were 10% and 1%, respectively, of the group fed 10 g quercetin/kg. AOM-treated rats fed the 40 g rutin/kg diet consumed 3500 mg rutin/(kg body wt•d) at wk 2, declining to 1700 mg/(kg body wt•d) at wk 38. In the course of the experiment, the corrected dietary intakes of quercetin and rutin did not differ between AOM-treated and untreated rats.

In total, 4 AOM-treated rats were down in health or moribund as a result of colon tumors (2 rats), a zymbal gland tumor (1 rat), and a small intestinal tumor (1 rat), most likely caused by AOM toxicity. Because these rats were killed 3–4 mo before completion of the study, they were excluded from further analyses to ensure proper time-matched comparisons between groups.

ACF in unsectioned colons

All AOM-treated rats killed at wk 8 post-AOM (n = 8/group) developed ACF found mainly in the distal colon. Quercetin did not affect ACF, because ACF numbers in control rats (45.8 ± 20.0) were comparable to rats fed a 0.1- (59.4 ± 18.3), 1- (42.3 ± 13.8), or 10 g quercetin/kg diet (49.6 ± 17.8). In addition, ACF numbers among rutin-treated rats (42.9 ± 14.9) were comparable with the controls. Quercetin did not affect the ACF multiplicity (range: 1–8), even when ACF were classified as small (3 aberrant crypts/focus) and large (4 aberrant crypts/focus) [23, 24] (data not shown).

ß-Catenin accumulation

Two subpopulations of en face ACF were found that showed either ß-catenin immuno-reactivity restricted to the cell membrane, as for the normal adjacent crypts (Fig. 3.1A), or immuno-reactivity in the nucleus and/or cytoplasm (Fig. 3.1D). The second class of putative CRC biomarkers, BCA-C (Fig. 3.1G), did not show an enlarged crypt lumen or thickened epithelium relative to the surrounding normal crypts, in contrast to ACF and BCA-ACF.

When compared with control rats, the number of BCA-C was not affected by the 10 g quercetin/kg diet. However, BCA-C numbers with an intermediate grade of ß-catenin accumulation were lower ($P < 0.05$) in rats fed the 10 g quercetin/kg diet (Supplemental Table 1[1]).

Compared with controls, the number of en face ACF, regardless of the ß-catenin accumulation status, was not affected by the 10 g quercetin/kg diet (0 vs. 10 g quercetin/kg diet: 10.3 ± 4.4 vs. 10.5 ± 6.7, n = 8/group). The same was true for ACF with (1.4 ± 1.1 vs. 1.8 ± 1.3) and without (9.0 ± 4.1 vs. 9.0 ± 6.2) ß-catenin accumulation, consistent with observations in methylene blue-stained ACF in unsectioned colons (45.8 ± 20 vs. 49.6 ± 17.8).

Multiplicities of ACF, BCA-ACF, and BCA-C did not differ between rats fed the treatment with the 10 g quercetin/kg diet and controls, even when a distinction was made between small (3 aberrant crypts/focus) and large (4 aberrant crypts/focus) lesions (data not shown). This result agrees with methylene blue-stained ACF in unsectioned colons.

[1] Supplemtal table available at The Journal of Nutrition webpages: http://jn.nutrition.org/cgi/data/136/11/2862/DC1/

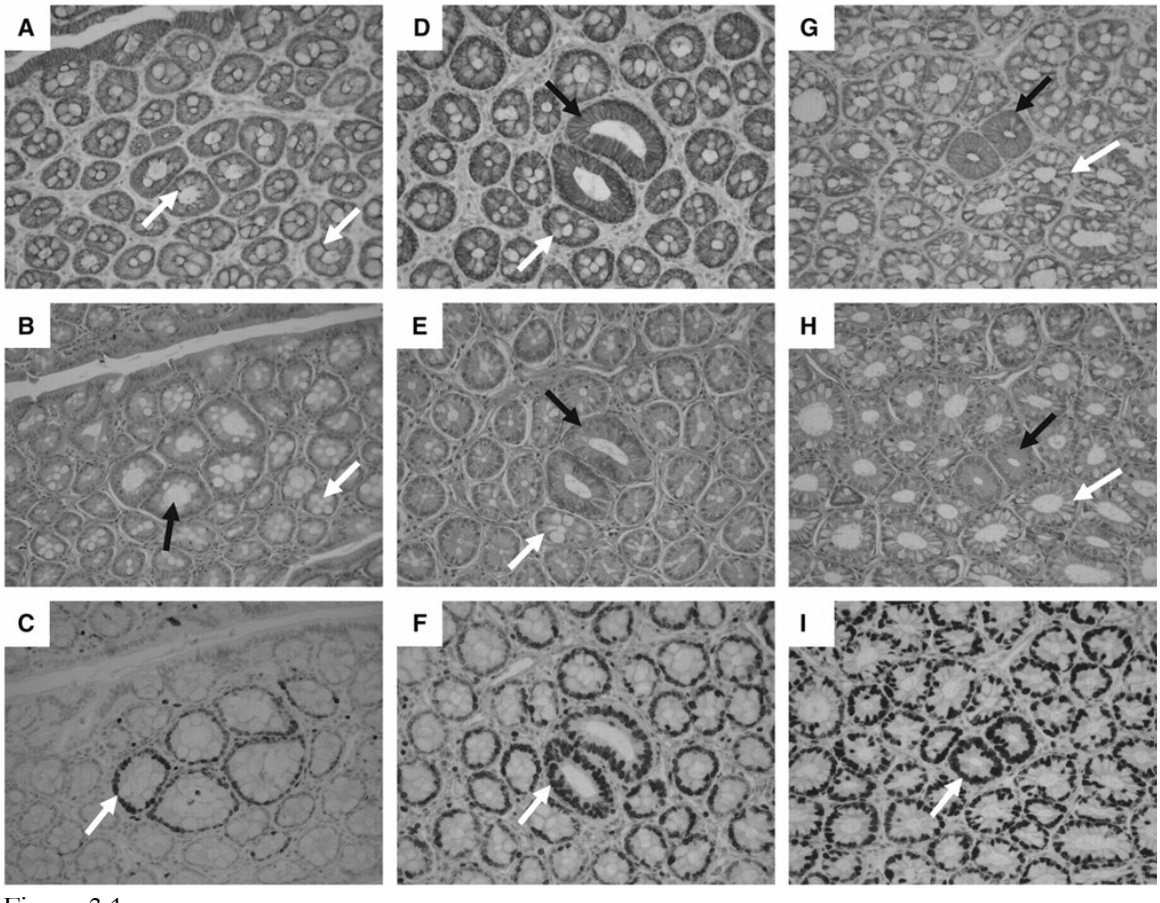

Figure 3.1
ß-Catenin, hematoxylin and eosin, and Ki67 staining in en face-oriented ACF, BCA-ACF, and BCA-C in colons of AOM-treated rats killed at wk 8 post-AOM (200x magnification). (A) ß-catenin staining in "normal" ACF is located at the cell surface, similar to healthy surrounding crypts (white arrows). (B) These ACF show enlarged crypts with wide luminal openings (black arrow) compared with healthy surrounding crypts (white arrow) in combination with (C) a single layer of Ki67 positive nuclei in the crypts. (D) In BCA-ACF, ß-catenin staining occurred in both the cytoplasm and nuclei (black arrow), whereas surrounding healthy crypts showed ß-catenin staining located at the cell surface (white arrow). (E) BCA-ACF showed enlarged crypts (black arrow) relative to the healthy surrounding crypts (white arrow), with (F) multiple layers of Ki67 positive nuclei. (G) BCA-C showed accumulation of ß-catenin in both the cytoplasm and nucleus (black arrow) in contrast to normal cells that showed ß-catenin staining located at the cell membrane (white arrow). (H) BCA-C lack typical morphological features of ACF, including a split enlarged lumen, are similar in size (black arrow) in comparison with the surrounding normal mucosa (white arrow) and show (I) multiple layers of Ki67 positive nuclei. > 1.3) and panel C represents genes with a $p < 0.05$.

Proliferation

The proliferation index within the total number of en face ACF, regardless of the ß-catenin accumulation status, decreased ($P < 0.05$) from 99.0 ± 0.5% (n = 5/group) in control rats to 80 ± 16.5% in 10 g quercetin/kg

diet-treated rats. In ACF without ß-catenin accumulation, the proliferation index tended ($P = 0.060$) to be lower in rats fed the 10 g quercetin/kg diet (79.8 ± 18.5%) than in controls (98.7 ± 1.1%). The relatively few ACF with ß-catenin accumulation also tended to be lower ($P = 0.07$) in rats fed the 10 g quercetin/kg diet (82.1 ± 18.9%) than in controls (99.8 ± 0.4%).

Colorectal tumors

At 38 wk post-AOM, untreated rats fed the control diet or diets supplemented with quercetin or rutin had not developed colorectal tumors. The tumor incidence, i.e. the number of tumor-bearing rats expressed as a percentage of the total number of rats, was 50% in AOM-treated rats fed the control diet and was dose dependently ($P < 0.05$) decreased by dietary quercetin (0 vs. 10 g quercetin/kg diet: $P < 0.05$, Table 3.1). The dose dependent decrease in tumor incidence in quercetin-fed rats at wk 38 did not correlate with the ACF numbers scored in unsectioned colons obtained at wk 8. Both the tumor multiplicity (the mean number of tumors per rat after exclusion of tumor negative rats) and tumor diameter were negatively correlated with the dietary quercetin concentration (both: $r = –0.98$, $P < 0.05$). The tumor incidence, multiplicity, and size were not affected by the 40 g rutin/kg diet.

Plasma concentrations of quercetin and its metabolites

Following enzymatic hydrolysis, plasma concentrations of quercetin and 3'-O-methyl-quercetin at wk 8 (Table 3.2) correlated linearly with dietary concentrations of quercetin (both: r 0.99, $P < 0.01$). Interestingly, rats fed the 40 g rutin/kg diet, which equals 20 g quercetin aglycone/kg diet, had only 30% of quercetin and 3'-O-methyl-quercetin relative to rats fed the 10 g quercetin/kg diet ($P < 0.001$).

Tumor incidences in quercetin-fed rats at wk 38 were negatively correlated with plasma concentrations of both quercetin and 3'-O-methyl-quercetin at wk 8 after enzymatic deconjugation ($r = –0.95$, P 0.05). Plasma concentrations of tamarixetin (4'-O-methyl-quercetin) were below the detection limit for all flavonoid-supplemented groups.

Table 3.1
Tumor characteristics in AOM-treated rats fed control, quercetin, or rutin diets at wk 38 post-AOM administration

	Quercetin, g/kg diet				Rutin, g/kg diet
	0	**0.1**	**1**	**10**	**40**
Rats, *n*	22	22	22	20	20
Tumor bearing rats, *n*	11	9	8	4	9
Tumor incidence[1,2], *%*	50	41	36	20*	45
Tumors, *n*	17	13	11	4	12
Tumor multiplicity[3], *n*	1.55 ± 0.93	1.44 ± 1.01	1.38 ± 0.74	1.00 ± 0.0	1.33 ± 0.50
Tumor size, overall[3], *mm*	5.94 ± 3.83	5.85 ± 4.22	5.64 ± 3.98	5.00 ± 1.83	5.92 ± 3.73
Small (<5 mm) tumors, *% of total*	47	46	45	50	50
Large (5 mm) tumors, *% of total*	53	54	55	50	50
Adenoma, *% of large tumors*	11	-	50	-	50
Carcinoma, *% of large tumors*	89	86	33	100	67

[1] Values are means ± SD or percent. * Different from control, $P < 0.05$.
[2] Quercetin mediated a dose dependent decrease in tumor incidence: $P < 0.05$.
[3] Tumor multiplicity and size were inversely associated with increasing dietary quercetin; both, $r = -0.98$, $P < 0.05$.

DISCUSSION

In this article, quercetin aglycone and its glycoside rutin were investigated for their potential to modulate (biomarkers of) colorectal carcinogenesis. The results of our study indicate that quercetin, but not rutin, is able to reduce AOM-induced colorectal carcinogenesis when administered as of the preinitiation phase. Furthermore, methylene blue-stained ACF in unsectioned colons at wk 8 did not correlate with the CRC incidence at wk 38, even when discriminating between small and large ACF. This finding is in contrast to previous studies, which indicated that especially large ACF (4 crypts/focus) are reliable CRC biomarkers [23, 24]. The lack of a correlation between the number of methylene blue-stained ACF and the tumor incidence has been described [12–14]. Wijnands *et al.* investigated the effect of a 40 g rutin/kg diet in a similar AOM model and found comparable tumor incidences in controls and rutin-treated rats [13], as was the case in our study.

Because we found no relation between ACF and the tumor incidence, we hypothesized that a specific ACF subclass might be related to CRC. Therefore, ACF were immuno-histochemically classified into lesions with

Table 3.2
Plasma concentrations of quercetin and 3'-O-methyl-quercetin in untreated rats fed quercetin or rutin, killed at the interim autopsy at wk 8.

	Quercetin, g/kg diet			Rutin, g/kg diet
	0.1	**1**	**10**	**40**
			μmol/l	
Total quercetin[1]	0.85 ± 0.13[d]	7.66 ± 0.35[c]	37.44 ± 5.06[a]	13.01 ± 0.40[b]
Total 3'-O-methyl-quercetin	1.74 ± 0.21[c]	15.70 ± 1.22[b]	59.91 ± 12.73[a]	17.88 ± 0.96[b]

[1] Values are means ± SD, n = 5/group. Within data series, means without a common letter differ from one another, $0.001 < P < 0.05$.

and without accumulation of the CRC-related protein ß-catenin. However, neither of these ACF subclasses was influenced by quercetin. Taken together, ACF cannot be considered general biomarkers for CRC.

Another putative CRC biomarker analyzed is the BCA-C. Among BCA-C, quercetin significantly decreased ß-catenin accumulation at an intermediate grade, whereas none of the ACF-related variables were affected. Although quercetin only partially affected BCA-C-related variables, these results suggest that the BCA-C might be better biomarkers for CRC than ACF. Hata *et al.* [15] provided evidence that BCA-C, but not ACF, are reliable biomarkers in AOM-induced colorectal carcinogenesis. These authors described increasing ACF numbers but decreasing tumor numbers toward the proximal colon. In contrast, occurrence of BCA-C and colorectal tumors increased toward the rectum and were correlated with one another.

Quercetin not only dose dependently decreased the incidence of colorectal tumors but also reduced the tumor multiplicity and size of AOM-induced colorectal tumors. The quercetin-mediated decrease in tumor incidence is consistent with Deschner *et al.* [18], who fed AOM-treated CF1 mice with 1–20 g quercetin/kg diet for 50 wk. In our study, the protective effect evoked by quercetin might be caused by decreased cell proliferation, as was indicated by the dose-dependent decrease in tumor size and the significant decrease in proliferation index in ACF. Another mechanism involved might be induction of apoptosis by quercetin [19].

Rats were supplemented with a 40 g rutin/kg diet (20 g quercetin aglycone/kg diet) to compensate for rutin's low bioavailability [25, 26]. Therefore, calculated concentrations of quercetin and its metabolites in the 40 g rutin/kg diet-treated group should be twice that of rats fed the 10 g quercetin/kg diet, which is in contrast to our findings. Compared with rats

fed the 10 g quercetin/kg diet, rutin-supplemented rats showed only 30% of the quercetin and 3'-O-methyl-quercetin concentrations. Limiting factors involved in bioavailability of the quercetin moiety in rutin are probably intracolonic quercetin degradation and limited colonic absorption (7), the latter as a result of a relatively low absorptive area in the colon [5].

In this study, corrected quercetin intake by rats supplemented with the lowest quercetin concentration of the 0.1 g quercetin/kg diet was 4.5–9.0 mg quercetin/(kg body wt/d) throughout the experiment. Linear extrapolation of this dietary intake by rats to a human weighing 60 kg would equal daily supplementation with 0.3–0.5 g quercetin, which approximates the daily intake of 1 quercetin supplement of 500 mg. The CRC incidence was significantly decreased in AOM-treated rats fed the 10 g quercetin/kg diet, which after extrapolation to a human weighing 60 kg would equal supplementation with 30–50 g quercetin aglycone/d. This amount is an increase of 2,000–3,000 times the regular dietary quercetin intake of an estimated 16.3 mg/d when expressed as aglycone [27]. One of the major quercetin sources in the human diet is rutin [7], which in this experiment seems to be the inactive quercetin isoform with respect to reduction of colorectal carcinogenesis. Thus, when assuming similarity in AOM-induced development of CRC in rats and colorectal carcinogenesis in humans, inhibition of human colorectal carcinogenesis cannot be achieved by regular dietary intake of conjugated quercetin or by intake of supplements containing quercetin aglycone. Quercetin's beneficial effect might occur only at unrealistically high concentrations equaling daily intakes of 60–100 quercetin pills of 500 mg each.

In conclusion, inhibition of the tumor incidence by quercetin at wk 38 was not associated with the ACF number or multiplicity at wk 8 or with the ACF classification based on the absence or presence of ß-catenin accumulation. Therefore, the ACF are not reliable biomarkers for CRC. On the other hand, ß-catenin accumulation in BCA-C was partially decreased by quercetin. Therefore, BCA-C might be more relevant CRC biomarkers than BCA-ACF, but this requires further research. In contrast to quercetin aglycone, rutin lacks anticarcinogenic activity, probably as a result of its low bioavailability.

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ABBREVIATIONS

AOM, azoxymethane; ACF, aberrant crypt foci; BCA-ACF, ß-catenin accumulated aberrant crypt foci; BCA-C, ß-catenin accumulated crypts; CRC, colorectal cancer.

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CHAPTER 4

Chronic quercetin exposure affects fatty acid catabolism in rat lung

ABSTRACT

Dietary quercetin intake is suggested to be health promoting, but this assumption is mainly based on mechanistic studies performed *in vitro*. Previously, we identified rat lung as a quercetin target tissue. To assess relevant *in vivo* health effects of quercetin, we analyzed mechanisms of effect in rat lungs of a chronic (41 weeks) 1% quercetin diet using whole genome microarrays. We show here that fatty acid catabolism pathways, like beta oxidation and ketogenesis, are upregulated by the long-term quercetin intervention. Upregulation of genes (Hmgcs2, Ech1, Acox1, Pcca, Lpl and Acaa2) was verified and confirmed by qRT-PCR. In addition, free fatty acid levels were decreased in rats fed the quercetin diet, confirming that quercetin affects fatty acid catabolism. This *in vivo* study demonstrates for the first time that fatty acid catabolism is a relevant process that is affected in rats by chronic dietary quercetin.

Keywords: quercetin, microarray, fatty acid metabolism, nutrigenomics, flavonoids, polyphenols

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INTRODUCTION

Flavonoids are polyphenolic compounds of plant origin that are present in our diet. Epidemiological studies suggest that a diet rich in flavonoids is protective against cardiovascular diseases and possibly lung cancer [1]. Mechanistic studies show that flavonoids have a wide range of properties that can contribute to the potential beneficial health effects of flavonoids. For example, they are strong antioxidants [2, 3], inhibit tumor formation [4], improve endothelial function [5, 6] and affect energy metabolism [7]. To elucidate the molecular mechanisms explaining the beneficial effects of flavonoids observed *in vivo*, several studies have employed a systems biology approach with transcriptomics and proteomics techniques, using cell culture systems [8-18]. However, these *in vitro* studies may have generated misleading findings due to the non-physiological exposure conditions mostly used. In the human body, flavonoids are rapidly metabolized and therefore flavonoid aglycones (flavonoid molecules without conjugates), frequently used *in vitro*, are hardly present in plasma and tissues [19]. In addition, flavonoids are rapidly oxidized under cell culture conditions giving rise to oxidation products and H_2O_2 [20]. This, and the observation that flavonoid metabolites have profoundly different bioactivities and pharmacokinetics than flavonoid aglycones [21-23], suggests that reported mechanisms of action of flavonoids based solely on *in vitro* experiments are of limited value.

Quercetin is a flavonol abundant in onions, apples, tea and red wine and one of the most studied flavonoids. Quercetin is taken up in the intestine and immediately metabolized by phase II enzymes in the intestine and liver to methoxy, glucuronic acid and sulfate conjugates [24, 25]. Chronic treatment of rats with a 0.1% and 1% quercetin diet for 11 weeks resulted in appearance of quercetin metabolites in all analyzed tissues. The highest levels of quercetin metabolites were found in the lungs [26]. For that reason, lung is a relevant *in vivo* target tissue for studying molecular mechanisms underlying the quercetin mediated beneficial health effects using a transcriptomics approach. Only one study on quercetin used an animal model in combination with large-scale gene expression techniques. Mutch *et al.* [27] showed that exposure of hepatic cytochrome-P450 oxidoreductase knockout and wild type mice to an acute high dose of quercetin (7 mg/mouse), induced alterations in several biological pathways in jejunum, colon and liver. Although metabolism of quercetin did not differ between wild type and knockout mice, tissue-specific effects on amino acid metabolism, lipid metabolism, glutathione metabolism and antigen

presentation were found [27]. This study used an acute dose in combination with a genetic intervention.

The aim of our study was to identify, in chronically quercetin administered rats, the relevant *in vivo* biological processes that are regulated by long-term quercetin intervention, mimicking the effects of chronic daily intake of quercetin in the human diet. We chose lung tissue as a target tissue of quercetin action, because the highest concentrations of quercetin metabolites were found in lung tissue after chronic dietary supplementation of quercetin [26]. In addition, lung tissue seems to be an important target based on epidemiological studies indicating that a high flavonoid diet is protective against lung cancer [1]. We describe the analysis of large-scale gene expression changes induced by quercetin in lungs of rats fed a 1% quercetin diet (~500 mg quercetin/kg body weight per day) for 41 weeks. We identified pathways affected by chronic quercetin intervention and used quantitative real time PCR (qRT-PCR) and plasma analysis to confirm the effects in these pathways.

METHODS

Animal experiment

The animal experiment was performed as previously described [26]. In short, inbred male Fisher 344 rats (Charles River Laboratories, Inc., Sulzfeld, Germany) were housed in pairs in cages and were fed for 41 weeks a RM3[E]FG SQC breeding diet (Special Diets Services, Witham, UK) supplemented with 0% (control), 0.1% (w/w) quercetin or 1% (w/w) quercetin (6 rats per group). Calculated quercetin intake for rats fed a 1% quercetin diet were ~500 mg quercetin/(kg BW · day) and for rats fed a 0.1% quercetin diet: ~50 mg quercetin/(kg BW · day). The control diet did not contain quercetin or quercetin glycosides (data not shown). Animal weight and food intake did not differ between control and quercetin intake groups during supplementation. After 41 weeks, rats were anaesthetized in the morning, without overnight fasting, by inhalation of 5% isoflurane, using N_2O-O_2 (1:1, v/v) as carrier. Animals were fully bled via the abdominal aorta. Blood (8 – 12 ml) was collected in EDTA-tubes and plasma was subsequently prepared in Leucosep Centrifuge tubes (Greiner Bio-one, Longwood, FL, USA) by centrifuging for 20 minutes at 1000×g and 4 ^{o}C. Plasma samples were stabilized with ascorbic acid (1 mg/ml plasma final concentration) before storage at -80 ^{o}C. After blood collection, lungs were dissected and left lung (*pulmonis sinistri*) and right lung (*pulmonis dextri*) were separately snap frozen in liquid nitrogen and stored at -80 ^{o}C.

The left lung was used for RNA isolation. For the microarray experiment lungs of rats fed a 1% quercetin diet and control diet were used. For plasma parameter and qRT-PCR analysis all treatment groups (control, 0.1% quercetin and 1% quercetin) were used. The experimental protocol was approved by the Animal Welfare Committee of Wageningen University, Wageningen, The Netherlands.

RNA isolation

Freeze dried lung tissue was homogenized in liquid nitrogen and total RNA was isolated with Trizol (Invitrogen) according to the instructions from the manufacturer, with an additional phenol/chloroform/isoamylalcohol (25:24:1, v:v:v) purification step, followed by a second chloroform purification. Integrity and quality of RNA samples were checked by gel electrophoresis and spectrophotometric analysis using a Nanodrop (Isogen Life Science). All RNA samples had OD 260/280 ratios between 1.9-2.1, OD 260/230 ratios higher than 1.8 and displayed excellent visual integrity.

Microarray

A pooled reference design was used to analyze differential lung gene expression in 1% quercetin treated rats vs. control rats. All samples were labeled individually in duplicate. The protocol described in [28] was used with adjustments. In brief, RNA samples (35 μg total RNA per sample) from control and 1% quercetin treated rats were reverse transcribed and directly labeled with Cy5 (Amersham Biosciences) using SuperScript II Reverse Transcriptase kit (Invitrogen). A reference pool was prepared by mixing equal quantities of total RNA from all samples (control group and 1% quercetin group). The reference pool was complemented with additional total RNA isolated from lungs of rats treated with a 1% quercetin diet for 1 week to yield a sufficiently large reference pool. Reference RNA samples (35 μg total RNA per sample) were reverse transcribed and labeled with Cy3 (Amersham Biosciences). Labeled cDNA samples were purified using QIAquick PCR Purification Kit (Qiagen) and ethanol precipitation steps. After purification, samples were denatured and Cy3 labeled cDNA samples were pooled. Equal volumes of Cy5 target sample and Cy3 reference sample were combined, mixed with 2x hybridization buffer (Agilent Technologies) and 10x control targets (Agilent Technologies) and hybridized to a Whole Rat Genome oligo array, containing 44290 60-mer oligo spots (including ~3000 control spots, G4131A, Agilent Technologies), for 17 hours at 60 ^{o}C in Agilent hybridization chambers in an Agilent hybridization oven with rotation (Agilent Technologies). After hybridization, microarrays were washed with

an SSPE wash procedure (Agilent Technologies) according to manufacturers protocol and scanned with a Scanarray Express HT scanner (Perkin Elmer). Signal intensities for each spot were quantified using ArrayVision 8.0 (GE Healthcare life sciences). Saturated spots and spots with signal intensity lower than 2 times the background were discarded, leaving 33241 transcripts for normalization and analysis of differential expression. Quality check was performed for each microarray using both the LimmaGUI package in R [29] from the Bioconductor project and Microsoft Excel. The best microarray from two duplicates, based on MA-plot, normal probability plot and signal intensity distribution [30, 31], was selected for further analysis. Data normalization was performed according to Pellis *et al.* [32] using GeneMaths XT 1.5 (Applied Maths). Pathway analysis was performed using MetaCore (GeneGo). Cluster analysis was performed using GeneMaths XT 1.5. Annotation from Agilent (Version 20050601) and TIGR annotations from Resourcer [33] were used for gene identification. Genes were classified according to gene ontology terminology and literature mining.

Quantitative RT-PCR

Differential expression for individual genes was assessed by qRT-PCR. cDNA was synthesized from 1 μg of total RNA for each sample using the iScript cDNA Synthesis kit (Bio-Rad). Primers were designed for Sybr Green probes with Beacon Designer 4.0 (Premier Biosoft International) (table 4.1). PCR amplification and detection was performed with the iQ SYBR Green Supermix and the MyIQ single-color real-time PCR detection system (Bio-Rad). A standard curve for all genes including reference genes was generated using serial dilutions of a pool prepared from all cDNA samples. The level of mRNA for each gene was normalized using *Hmbs* and *Rps26* as reference genes, chosen on the basis of microarray data which showed similar expression levels for all microarrays. All samples were analyzed within-run in duplicate. PCR amplification and detection for all samples and genes was performed in triplicate on three individual days.

Plasma parameters

Plasma levels of free fatty acids, triglycerides and β-hydroxybutyrate were analyzed using commercial kits (WAKO chemicals). Assays were adjusted so that small sample volumes could be used and analyzed with a microplate reader (BioTec Synergy HT). Free fatty acids and triglyceride assays contained ascorbate oxidase. Therefore, added vitamin C, up to 1 mg/ml, in plasma samples did not interfere in the assays. Vitamin C (1 mg/ml) did not interfere in the assay for β-hydroxybutyrate (data not shown).

Table 4.1
Primers used for quantitative real time PCR. All primers were intron-spanning

Gene symbol	Sequence ID	Forward primer (5' - 3')	Reverse primer (5' - 3')	Product length (bp)
Lpl	NM_012598	TCTGTCACACGTCTAACACATCAC	ACAATAGAAGGCTCCTCACTTTGC	191
Ech1	NM_022594	TGTGGTCTCTGGTGCAGGAAAG	GGTATCGGCTGATGAGGTCTCG	134
Hmgcs2	NM_173094	GCCTTGGACCGATGCTATGC	CTAGGGATTTCTGGACCATCTTGC	145
Acaa2	NM_130433	GGAACACAGGCGACCTTTGAG	GTGGTGGCTGCTGACAATGAC	166
Acox1	NM_017340	ACAGTTCTGAGAGCACAGCATC	CATTCCAGGAGAAAGGTTAAGGC	108
Pcca	XM_341383	AATGGGCAAGGTGAAACTGGTG	GTGGTGACTGAAGGGCTACAAG	104
Hmbs	NM_013168	TCGCTGCATTGCTGAAAGGG	CATCCTCTGGACCATCTTCTTGC	200
Rps26	NM_013224	TCATAGCAAGGTTGTCAGGAATCG	CTTTGGTGGAGGTCGTGGTG	103

Statistical methods and software

For microarray data analysis, p-values for differential expression between the 1% quercetin group (n=6) and control group (n=6) were calculated using t-test statistics on log intensity values. Fold changes were calculated using median linear signal intensity values. The p-value was used to prioritize data based on highest significance between the six control animals and six quercetin animals. T-test statistics were used to test for significant differences in the quantitative RT-PCR experiment and plasma parameter analysis and $p<0.05$ was considered significant.

RESULTS

Quercetin induced global gene expression changes in lungs of rats

We analyzed global gene expression changes in lungs of rats fed a quercetin diet for 41 weeks using microarrays. From the 33421 transcripts with signal intensities above two times the background, 384 genes were differentially expressed with a fold change higher than 1.3 fold. Of these, 34 genes were expressed with a fold change higher than 1.5 fold (figure 4.1). Essentially all of these genes showed a high variation in expression level in both the

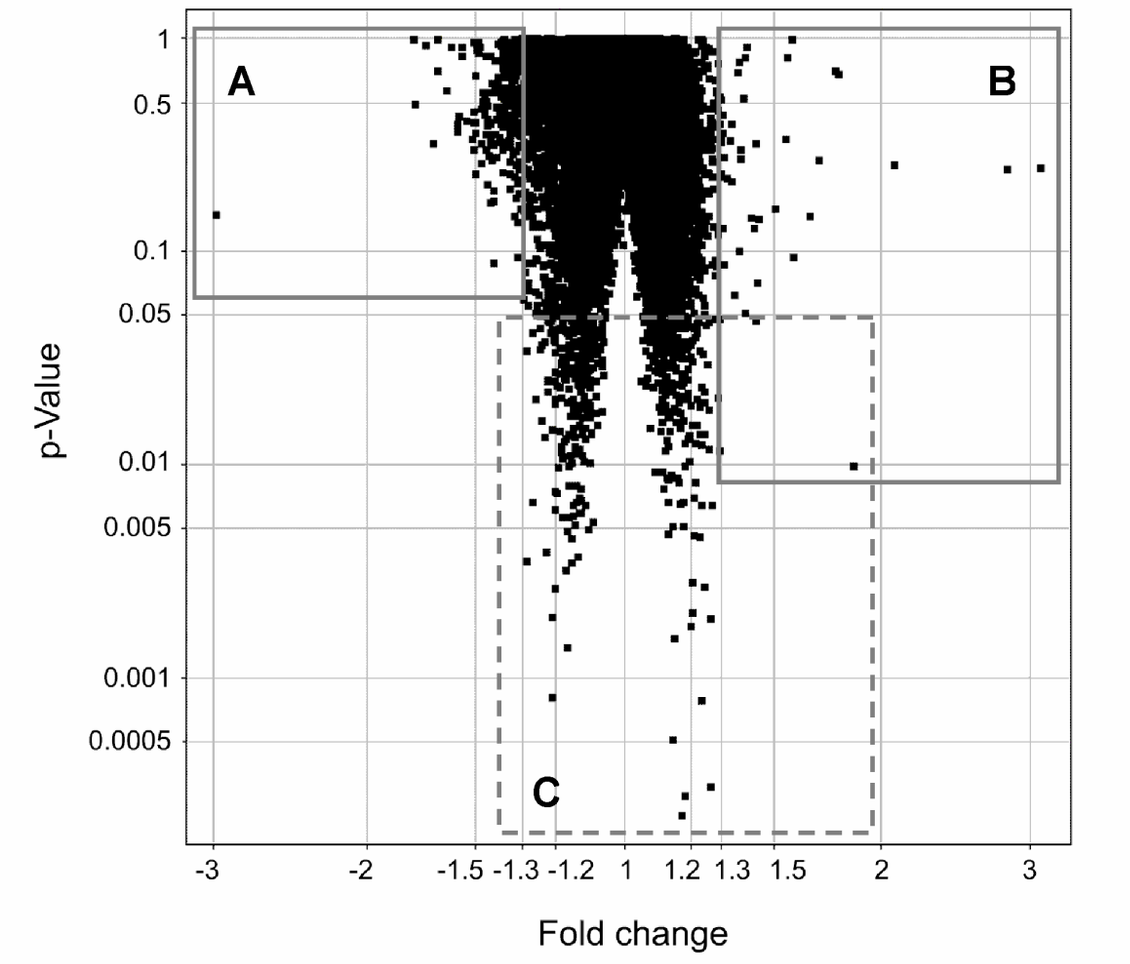

Figure 4.1
Volcano plot of 33421 transcripts showing the t-test statistics p-value plotted against the fold change of each transcript (1% quercetin vs. control diet). Panel A represents genes that are downregulated (fold change < -1.3), panel B represents genes that are upregulated (fold change > 1.3) and panel C represents genes with a $p < 0.05$.

control and quercetin treated group (panel A and B in figure 4.1), and were therefore not significantly different between the two groups. Among the 34 genes with differential expression higher than 1.5 fold, 13 coded for immunoglobulins, whereas the remaining 21 genes did not belong to a common functional category (supplemental table S1[1]). Analysis of the similarities in expression profile of the 384 genes using hierarchical clustering resulted in grouping of 20 immunoglobulin genes upregulated in

[1] Supplemental data is available at www.foodbioactives.nl

Table 4.2
Twenty upregulated immunoglobulin genes (1% quercetin vs control) that grouped in one cluster. Identified by using UPGMA clustering with pearson's correlation coefficient as distance measure (384 genes were used with a fold change higher than 1.3). Genes are sorted based on cluster order.

Sequence ID	Gene name
Z75902[a]	Immunoglobulin, epsilon chain, variable region (clone Hg32)
XM_578308[a]	Similar to monoclonal antibody kappa light chain
XM_345754	Similar to Ig heavy chain v region VH558 A1/A4 precursor
AY331040[a]	Anti-SPE7 immunoglobulin E heavy chain variable region
Z93363[a]	Immunoglobulin variable region (clone ERF2.37)
XM_234745[a]	Similar to Ig heavy chain V-I region HG3 precursor
BC092586	Similar to immunoglobulin heavy chain 6 (Igh-6)
XM_345756[a]	Similar to BWK3 (predicted)
XM_234749	Similar to Ig heavy chain precursor V region (IdB5.7)
XM_234686[a]	Similar to immunoglobulin heavy chain
XM_345750	Similar to Ig H-chain V-JH3-region
XM_575534	Similar to NGF-binding Ig light chain
S81289	IgM kappa chain variable region {CDR1 to CDR3 region}
XM_341195	Immunoglobulin joining chain (predicted)
Z93359	Immunoglobulin variable region (clone ERF2.13)
AF217591[a]	Immunoglobulin kappa light chain variable region
X55180[a]	Monoclonal antibody Y13-259 Vk
XM_575533[a]	Similar to IG light chain Vk region Y13-259
M84148[a]	Rat IgK chain VJ1 region
XM_578339	Similar to immunoglobulin kappa-chain

[a] Immunoglobulin genes from supplemental table S1 with fold change higher than 1.5.

the 1% quercetin treated group as compared to the control diet group (table 4.2). Three additional immunoglobulin genes were present in the 384 gene data set, of which two were downregulated and one did not have a comparable profile. The average expression profile of the 20 upregulated immunoglobulins (figure 4.2) illustrated that 4 animals in the control group showed a relatively low expression of immunoglobulin genes, whereas 2 animals showed a relatively high immunoglobulin gene expression. In contrast, in the quercetin diet group, 5 animals showed a relatively high immunoglobulin gene expression, whereas the remaining animal did not. This indicates that the immunoglobulin genes were coordinately regulated.

Genes were also ranked on the basis of highest significance instead of fold change (panel C in figure 4.1). The 35 genes that were most significantly modulated ($p < 0.01$, table 4.3) belonged to various functional

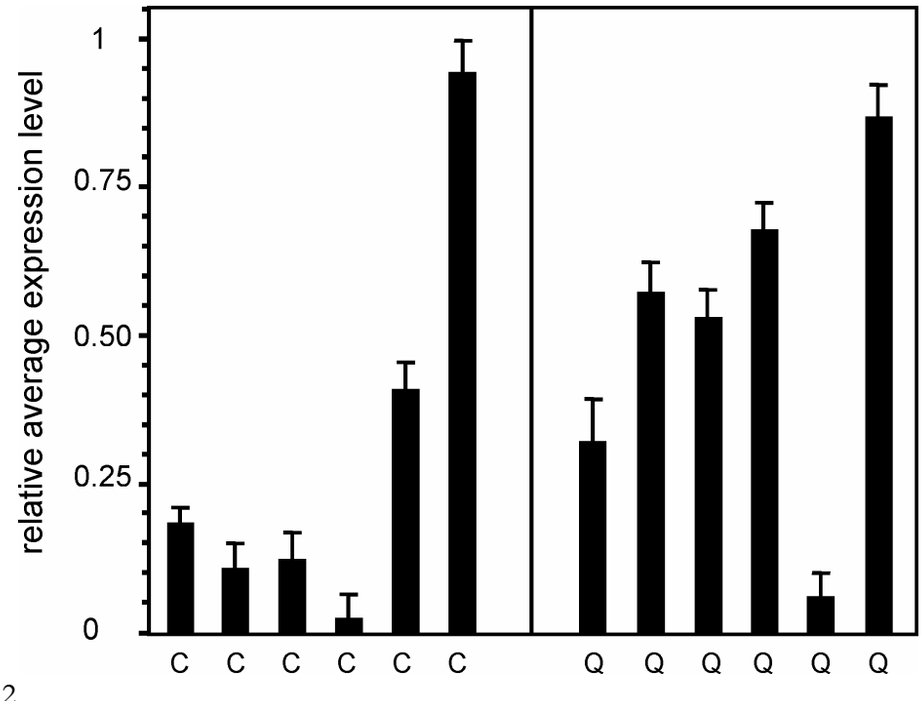

Figure 4.2
Relative average expression profile (mean +/- SEM) of the 20 immunoglobulin genes from table 4.2. Expression level for each gene was normalized using the mean expression level of that gene of all arrays (n=12). C = rat treated with a control diet for 41 weeks. Q = rat treated with a 1% quercetin diet for 41 weeks.

categories. However, a group of 5 genes (*Ech1*, *Hmgcs2*, *Ant2*, *Atp5j2*, *Aco1*) was involved in mitochondrial transport and energy metabolism. To get more insight whether energy metabolism was affected by quercetin intervention we used a wider criterion for selection of genes ($p < 0.05$). Mapping this selection of differentially expressed genes (see supplemental table S2) to the Metacore pathway database, containing over 400 curated pathway maps, showed an over-representation of genes involved in lipid metabolism pathways (data not shown). This indicated that energy metabolism is indeed a major process affected by quercetin.

We identified all genes involved in energy metabolism that were regulated by quercetin and grouped them into the pathway diagram shown in figure 4.3. Most of the regulated genes were shown to be involved in the catabolism of fatty acids. Quercetin induced both peroxisomal and mitochondrial beta-oxidation genes (*Acaa2*, *Ech1*, *Acox1* and *Pcca*). In addition, the ketogenic

Table 4.3
Differentially expressed annotated genes with p< 0.01 in lungs of rats treated with a 1% quercetin diet (n=6) as compared to rats treated with a control diet (n=6). Genes are ordered based on function and within functional category based on p-value.

Gene symbol	Gene name	Sequence ID	p-Value	Fold change
Energy metabolism & mitochondrial transport				
Atp5j2	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit f, isoform 2	Rn.3543	0.0008	-1.22
Ant2	ADP, ATP carrier protein, fibroblast isoform	NM_057102	0.0050	-1.14
Aco1	Aconitase 1	NM_017321	0.0069	1.18
Ech1	Enoyl coenzyme A hydratase 1, peroxisomal	NM_022594	0.0082	1.16
Hmgcs2	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	NM_173094	0.0085	1.16
Nucleotide metabolism				
Dpysl3	Dihydropyrimidinase-like 3	Rn.93365	0.0019	1.26
Tyms	Thymidylate synthase	NM_019179	0.0039	-1.24
Cell adhesion				
Krt2-8	Keratin complex 2, basic, gene 8	BF281337	0.0059	-1.20
Igsf4a_predicted	Immunoglobulin superfamily, member 4A (predicted)	Rn.19928	0.0087	1.13
Asb16_predicted	Ankyrin repeat and SOCS box-containing 16 (predicted)	AW530584	0.0098	-1.16
Cell proliferation/differentiation				
Igfbp3	Insulin-like growth factor binding protein 3	NM_012588	0.0027	1.24
Fhl1	Four and a half LIM domains 1	BC061782	0.0080	1.21
Apoptosis				
Casp14_predicted	Caspase 14 (predicted)	XM_234878	0.0042	-1.15
Nme6	Expressed in non-metastatic cells 6, protein	AI014074	0.0063	-1.12
DNA repair				
Pnkp	Polynucleotide kinase 3 ' -phosphatase	NM_001004259	0.0006	1.14
Mbd4_predicted	Methyl-CpG binding domain protein 4 (predicted)	XM_342742	0.0035	-1.30
Xpc_predicted	Xeroderma pigmentosum, complementation group C (predicted)	XM_232194	0.0067	1.13

Table 4.3 (continued)

Gene symbol	Gene name	Sequence ID	p-Value	Fold change
Immune response				
LOC361454	Chemokine C-C motif receptor-like 1 adjacent	CB546044	0.0014	-1.17
Ccl12_predicted	Chemokine (C-C motif) ligand 12 (predicted)	XM_213425	0.0065	-1.28
LOC366755	Similar to immunoglobulin heavy chain	XM_345742	0.0097	1.86
Protein modification				
Gypc_predicted	Glycophorin C (predicted)	AY234182	0.0015	1.15
BRAP2	BRCA1-associated protein	BF284618	0.0066	-1.15
Hspcb	Heat shock 90kDa protein 1, beta	AI236795	0.0094	-1.20
Pja2	Praja 2, RING-H2 motif containing	NM_138896	0.0096	1.09
Receptor				
Olr1350	Olfactory receptor 1350 (predicted)	NM_001000752	0.0039	-1.14
Olr197	Olfactory receptor 197 (predicted)	NM_001000188	0.0050	-1.10
Signal transduction				
Map2k4_predicted	Mitogen activated protein kinase kinase 4 (predicted)	CA510796	0.0046	1.21
Rit1_predicted	Ras-like without CAAX 1 (predicted)	BF406174	0.0057	-1.13
Sorbs1_predicted	Sorbin and SH3 domain containing 1 (predicted)	AW917667	0.0068	1.21
Protein transport				
Vps39_predicted	Vacuolar protein sorting 39 (yeast) (predicted)	XM_342500	0.0054	1.14
Calcium transport				
Cacng6	Calcium channel, voltage-dependent, gamma subunit 6	NM_080694	0.0065	1.23
Cytoplasmic metabolism				
Ephx2	Epoxide hydrolase 2	BM986667	0.0078	-1.20
Miscellaneous				
Tm7sf1_predicted	Transmembrane 7 superfamily member 1 (predicted)	XM_237907	0.0003	1.26
Otos	Otospiralin	NM_139188	0.0020	-1.21
Tcte1_predicted	T-complex-associated testis expressed 1 (predicted)	XM_236941	0.0077	-1.12

pathway was clearly upregulated: *Hmgcs2* and *Hibadh* expression levels were mutually increased in quercetin treated rats, whereas *Scot-S*, the enzyme responsible for the reverse reaction, was downregulated. Upstream of beta-oxidation, two important genes were modulated by quercetin as well. Lipoprotein lipase (*Lpl*) and the gene coding for the channel importing acyl CoA into mitochondria, *Vdac1*, were both upregulated by quercetin intervention. Another prominent mitochondrial transporter, *Ant2*, was downregulated together with one of the subunits of the ATP synthase protein (*Atp5j2*). These two genes are both part of the last step in the oxidative phosphorylation. Besides regulation of genes involved in the catabolism of fatty acids, a key regulator of glycolysis, phosphofructokinase (*Pfk*) was downregulated and tricarboxylic acid (TCA) cycle gene acotinase (*Aco1*) was upregulated by chronic quercetin treatment. Of the two other adenine nucleotide translocators (*Ant1* and *Ant3*), only Ant1 was expressed in the lung but did not change significantly. In addition, *Ppara*, *Ppard*, *Pparg*, *Pgc1a* and *Sirt1-7* were expressed in the lung but did not pass the significance filtering criteria (data not shown).

Fatty acid catabolism gene expression

To confirm microarray gene expression results, we analyzed quercetin regulated genes related to the fatty acid catabolism pathways with quantitative real time PCR. We selected 6 genes (*Lpl*, *Hmgcs2*, *Ech1*, *Acaa2*, *Acox1*, *Pcca*) on the basis of regulation by quercetin and expression level in the microarray experiment. In addition, RNA isolated from lungs of rats fed a 0.1% quercetin diet for 41 weeks was used for qRT-PCR experiments. The genes with lowest variation within groups and therefore the lowest p-value in the microarray experiment (*Ech1* and *Hmgcs2*) were both significantly upregulated in the 0.1% quercetin group and/or the 1% quercetin group ($p<0.05$) (figure 4.4). *Ech1* was significantly upregulated in both the 0.1% and 1% quercetin diet group, whereas *Hmgcs2* was significantly upregulated only in the 0.1% quercetin diet group. Differential expression of *Acaa2*, *Acox1*, *Pcca* and *Lpl* using qRT-PCR was consistent with differential expression found in the microarray experiment. All genes were upregulated by the 1% quercetin treatment, however sample size ($n=6$) was too small to reach significance using qRT-PCR ($p = 0.06$-0.1).

Fatty acid catabolism plasma parameters

To further investigate the role of quercetin in fatty acid catabolism, analysis of fatty acid catabolism parameters was performed in rat plasma. Consistent with microarray data and qRT-PCR results, chronic quercetin intervention in rats affected fatty acid catabolism plasma parameters. Plasma free fatty

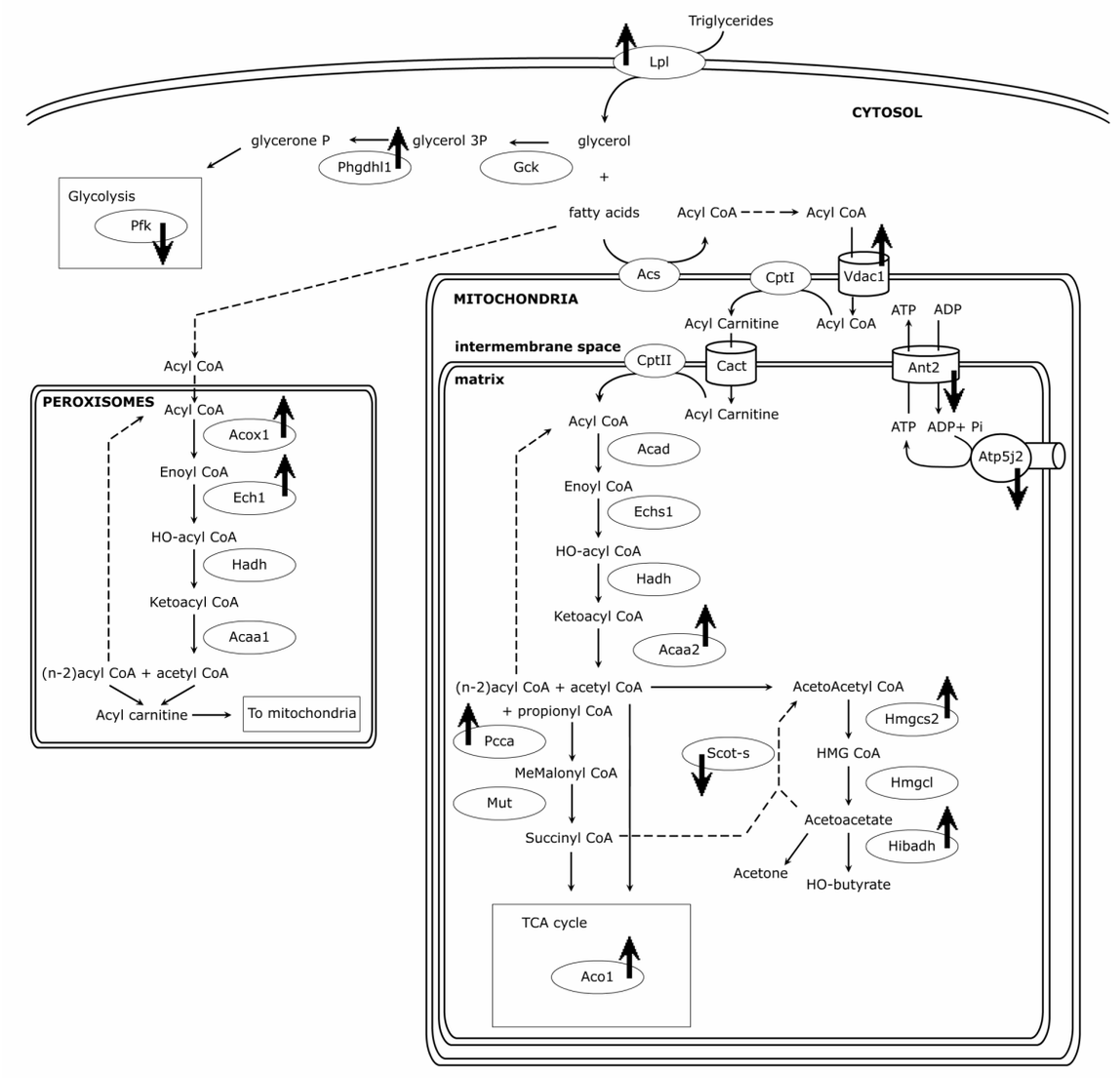

Figure 4.3
Diagram of fatty acid catabolism. Large arrows indicate up- or downregulation by treatment of rats with a 1% quercetin diet for 41 weeks. Genes without an arrow did not change significantly. Ech1 together with Hadh are part of the bifunctional protein. Hadh was upregulated as well, but did not pass the significance filtering criteria (data not shown). Abbreviations: Acaa1; acetyl-Coenzyme A acyltransferase 1, Acaa2; acetyl-Coenzyme A acyltransferase 2, Acad; acetyl-Coenzyme A dehydrogenase, Aco1; aconitase 1, Acox1; acyl-Coenzyme A oxidase 1, Acs; acyl-CoA synthetase, Ant2; adenine nucleotide translocator 2, Atp5j2; ATP synthase (H+ transporting, mitochondrial F0 complex, subunit f, isoform 2), Cact; carnitine-acylcarnitine translocase, CptI; carnitine palmitoyltransferase 1, CptII; carnitine palmitoyltransferase 2, Ech1; enoyl Coenzyme A hydratase 1 (peroxisomal), Echs1; enoyl Coenzyme A hydratase (short chain, 1, mitochondrial), Gck; glucokinase, Hadh; hydroxyacyl-Coenzyme A dehydrogenase, Hibadh; 3-hydroxyisobutyrate dehydrogenase , Hmgcl; 3-hydroxy-3-methylglutaryl-Coenzyme A lyase, Hmgcs2; 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2, Lpl; lipoprotein lipase, Mut; methylmalonyl Coenzyme A mutase, Pcca, propionyl Coenzyme A carboxylase, Pfk; phosphofructokinase, Phgdhl1; phosphoglycerate dehydrogenase like 1, Scot-s; Succinyl-CoA:3-ketoacid-coenzyme A transferase 1.

acid levels were significantly lower in the 1% quercetin group (figure 4.5A). The plasma levels of ketone bodies and triglycerides decreased with increasing quercetin dose, but were not significantly different between control and quercetin groups (figure 4.5B & 4.5C).

DISCUSSION

In this study we established for the first time *in vivo* mechanisms of action of quercetin in lungs of rats chronically exposed to dietary quercetin. The effects of the long term nutritional polyphenolic intervention were explored on a whole genome level allowing for unbiased exploration. Quercetin coordinately upregulated genes involved in fatty acid catabolism pathways in lungs and lowered fatty acid levels in plasma. This indicates that chronic dietary intake of quercetin led to a modulation of fatty acid metabolism in rats.

Quercetin was shown to regulate genes in every step of the breakdown of triglycerides to short chain carbon molecules (figure 4.3). The first step in degradation of triglycerides is regulated by lipases that cleave fatty acids from glycerol for further processing in the cell. LPL is an extrahepatic lipase that is expressed in lungs [34], and is responsible for breakdown of plasma triglycerides from chylomicrons or lipoproteins. Entering the cell, fatty acids are converted to acyl CoA and either degraded by peroxisomal beta oxidation or transported into mitochondria, possibly by VDAC1 [35], for further degradation. VDAC1 is an outer mitochondrial membrane channel that permits passage of all metabolites, except for small membrane permeable compounds like oxygen, acetaldehyde and short chain fatty acids [36, 37]. After transit into the mitochondrial matrix, acyl CoAs are degraded by a large set of beta-oxidation enzymes to yield acetyl CoA. Acetyl CoA can be utilized in the TCA cycle for energy production or converted to water soluble transportable forms of acetyl CoA; acetoacetate and hydroxybutyrate by ketogenic enzymes HMGCS2, HMGL and HIBADH. Quercetin upregulated *Lpl*, *Vdac1*, peroxisomal beta-oxidation enzymes (*Acox1* and *Ech1*), mitochondrial beta oxidation enzymes (*Acaa2* and *Pcca*) and ketogenic enzymes (*Hmgcs2* and *Hibadh*). Peroxisomal *Ech1* together with *Hadh* are part of the bifunctional protein. Hadh was upregulated as well, but did not pass the significance filtering criteria (data not shown). In contrast to the upregulation of fatty acid catabolism pathways, adenine nucleotide translocator 2 (*Ant2*), together with one of the F0 subunits of ATP synthase, was downregulated by quercetin. ATP synthase and ANT2 are responsible for the last steps of the oxidative phosphorylation, generating ATP by utilizing the inner membrane proton

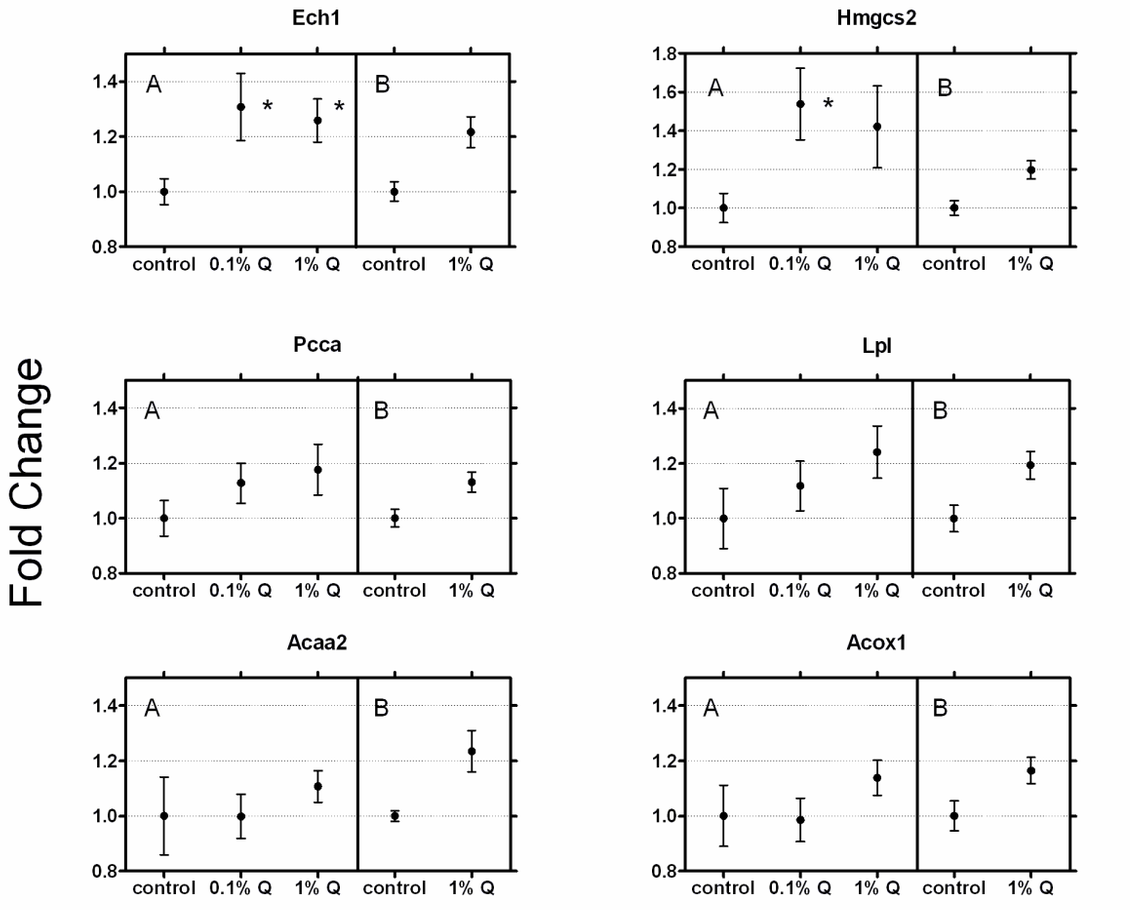

Figure 4.4
Expression of genes in lungs of rats treated with a control diet, 0.1% quercetin diet (0.1% Q) and 1% quercetin diet (1% Q) for 41 weeks. Panel A is the expression of genes analyzed with qRT-PCR, using *Hmbs* as reference gene (using *Rps26* as reference gene showed similar results (data not shown)). Panel B is the expression of genes analyzed with microarray. Data represent relative mean +/- SEM (n=6), control diet is set to 1.0. Significance is shown for qRT-PCR data only. Differences between expression levels in the control and 1% quercetin group in the microarray experiment were all significant (see supplemental table S2 for p-values). * $p<0.05$ as compared to control group.

gradient and transporting ATP out of the mitochondrial matrix in exchange for ADP [38]. The fatty acid catabolism pathways will mostly be affected by quercetin when rate limiting steps are modulated. Quercetin regulated the rate limiting steps in peroxisomal beta-oxidation (*Acox1* [39]) and ketogenesis (*Hmgcs2* [40]), however key regulators of mitochondrial beta oxidation, like *Cpt1* [41] and *Acad* [39], were not affected. *Vdac1* was upregulated by quercetin, but whether the upregulation of this highly abundant porin in the outer mitochondrial membrane is important in

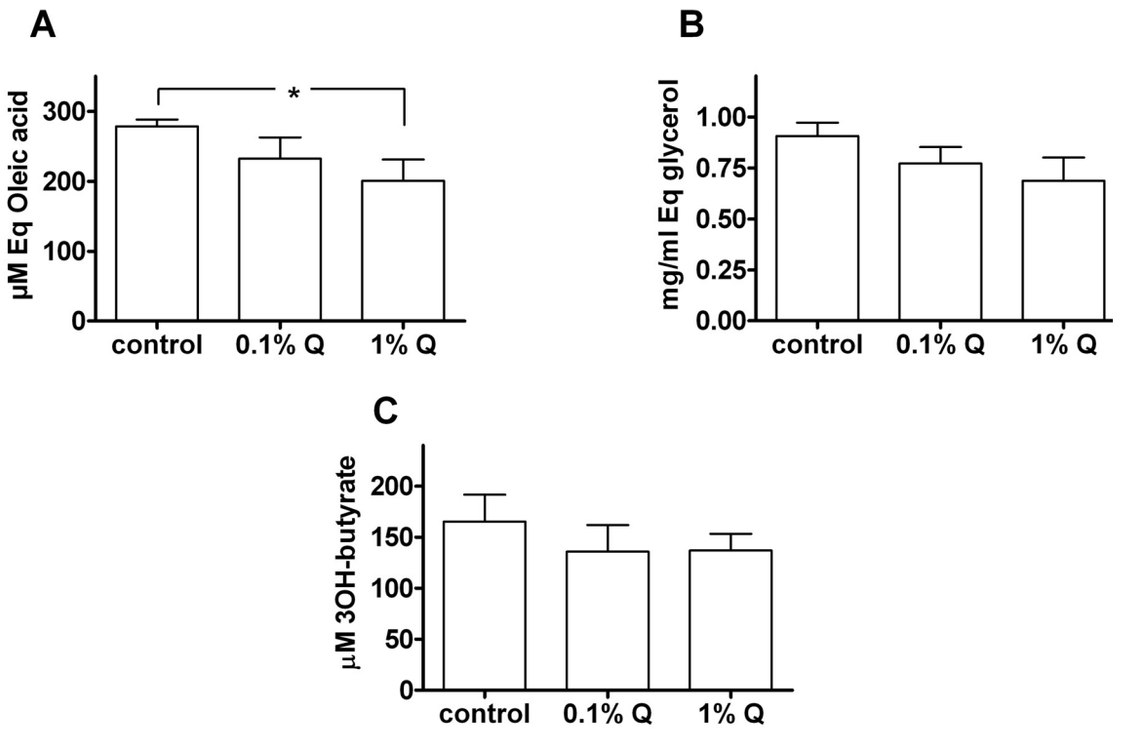

Figure 4.5
Plasma levels of fatty acid catabolism parameters in rats treated with a control diet, 0.1% quercetin diet (0.1% Q) or 1% quercetin diet (1% Q) for 41 weeks. Free fatty acids (A), triglycerides (B) and ketone bodies (C). Data represent mean +/- SEM (n=6). * $p<0.05$

controlling mitochondrial beta oxidation is largely unknown. *Vdac1* was shown to be upregulated by acetyl-L-carnitine in rat brain [42] and *Vdac1* gene expression was also shown to be important in regulation of ATP and ADP fluxes into and out of mitochondria [43], indicating that regulation of *Vdac1* on the gene expression level is of physiological relevance. Similarly, transcriptional regulation of *Lpl* by pharmacologic and physiological stimuli, resulted in enhanced LPL enzyme activities [44].

The downregulation of genes coding for ANT2 and ATP synthase in combination with the upregulation of substrate usage by quercetin may suggest an uncoupling of oxidative phosphorylation. Classical uncoupling in brown adipose tissue is typically regulated by uncoupling protein UCP1, leading to heat production instead of ATP [45, 46]. Uncoupling can also function to control the NAD+/NADH ratio, thereby regulating metabolic processes dependent on these cofactors like ketogenesis [47, 48]. However, UCP1 was not significantly modified by the quercetin intervention, nor were UCP2 and UCP3. Quercetin-mediated downregulation of ATP production and translocation in combination with an increase in ketone

body formation indicates that oxidation of fatty acids was not needed for energy production. Instead, this process seems to be taking care of an excess of fatty acids in the lungs, consequently producing ketone bodies that might be exhaled immediately via the pulmonary system and not being absorbed in plasma, explaining the fact that the level of plasma ketone bodies was not altered by quercetin. The increased expression of ketogenic enzymes can also be explained by the fact that glycolysis was decreased, as indicated by the downregulation of one of the key glycolytic enzymes, *Pfk*. Therefore, TCA cycle intermediates are not available from glycolysis and therefore the excess of acetyl CoA can only be converted into ketone bodies. This is supported by the fact that succinyl CoA seems to be available as an intermediate for the TCA cycle, because *Pcca*, the enzyme involved in the formation of succinyl CoA from odd-chain fatty acids, was upregulated and *Scot-S*, the enzyme that consumes succinyl CoA to produce, together with acetoacetate, acetoacetyl CoA, was downregulated. As a result, succinyl CoA probably complements for the decreased levels of intermediates from glycolysis.

The decrease in plasma free fatty acid levels in quercetin treated rats is in line with other studies showing that dietary quercetin can lower free fatty acid levels in rabbits [49]. The major site for metabolism of fatty acids is the liver. Therefore, the contribution of lung fatty acid catabolism to systemic fatty acid levels is probably low. However, the decrease in plasma fatty acid levels is consistent with the fact that quercetin modulates fatty acid metabolism in rats. Effects of quercetin on metabolism of energetic substrates were also reported by Mutch *et al.* [27]. Using affymetrix gene chips and cytochrome P450 reductase knockout mice, they showed that after a short, 4 hour, exposure to a 0.62% quercetin diet, amino acid metabolism, lipid metabolism and glutathione metabolism pathways were altered in liver and intestines. Moreover, genetically eliminating the expression of the phase I enzyme did not alter quercetin metabolism itself, but altered the tissue specific location of differentially regulated major metabolism pathways by quercetin [27].

Several other flavonoids have also been shown to interfere with the homeostasis of fat storage and fatty acid metabolism. The strongest evidence for modulation of lipid metabolism by a flavonoid was found for epigallocatechin galate (EGCg), a polyphenol found in high levels in green tea. Dietary supplementation of EGCg or extracts of green tea reduced the increase in body weight and adipose tissue mass induced by feeding a high fat diet in rodents (reviewed in [7, 50]). The anti-lipidemic effect of EGCg could be explained by a decrease in energy uptake in the intestines [51, 52]

and an increase in fatty acid oxidation rates [53, 54]. The physiological effects on energy expenditure and body weight by tea catechins, like EGCg, were accompanied by modulation of genes involved in fatty acid oxidation and fatty acid synthesis. Green tea extracts increased the expression of fatty acid oxidation genes, medium chain acyl-CoA dehydrogenase (*Mcad*) and acyl-CoA oxidase (*Acox1*), in liver and muscle in mice [54, 55]. In addition, tea catechins were shown to influence gene expression of important fatty acid synthesis genes; fatty acid synthase (*Fas*) and stearoyl-CoA dehydrogenase (*Scd*) were both downregulated by EGCg treatment in mice [52, 54]. Although in our study we did not challenge the animals with a high fat diet, we detected a similar upregulation of fatty acid oxidation genes *Acox1*, *Acaa2*, *Ech1* and *Pcca* by dietary quercetin in the lungs of rats.

Mechanistic insights on how quercetin can modulate fatty acid metabolism come from studies using models that show direct actions of quercetin on enzyme activities or indirect activation of regulating factors. Firstly, quercetin was shown to inhibit ATP synthase resulting in decreased mitochondrial and cellular ATP levels [56-59]. NAD^+/NADH ratios were also affected by quercetin in isolated perfused liver [60, 61]. Secondly, quercetin and other flavonoids are known to interact with several transcription factors and other regulatory proteins [62, 63]. Among these, PPAR nuclear transcription factors or sirtuins may be targets for quercetin action [21, 64-66] in lungs of rats mediating the observed effect of quercetin on fatty acid metabolism. Interpretation of studies illustrating *in vitro* activation of regulatory proteins by quercetin, however, is hampered by the fact that quercetin aglycone is normally not present in plasma and tissues of subjects [19, 26], unless local deconjugation of quercetin metabolites results in the formation of quercetin aglycone by enzymes with glucuronidase or sulfatase activity. Under certain physiological conditions, like inflammation and neoplastic growth, deconjugation of flavonoid glucuronides can occur, liberating the flavonoid aglycone [67, 68].

An additional biological response of rat lungs to quercetin supplementation seemed to be related to immune function. The highest fold changes of differential expression were found for genes coding for immunoglobulins. However, the variation between animals was large in both the control and quercetin groups. Cluster analysis of genes with highest fold change resulted in grouping of 20 immunoglobulin genes coordinately regulated in each animal. In 2 control animals and 5 quercetin animals immunoglobulin gene expression was relatively higher than in the remaining animals, demonstrating that immune response, possibly by an increase in lymphocyte infiltration in the lung, was enhanced in these

animals. This may be a bona-fide response, but the power of the present study is insufficient to conclude this. This shows the importance of using individual hybridization of samples on microarrays. It also shows that pooling of samples before hybridization or labeling would have resulted in identifying immune response as the most important process affected by quercetin treatment, and would render fatty acid catabolism unidentified.

Microarrays have evolved from a promising technique with a wide range of possibilities [69, 70] to a valuable and reproducible tool for assessing gene expression changes [71]. Application of microarrays to nutritional studies can give insights into how organisms react to dietary compounds [72]. A limitation of studying effects of dietary compounds on gene expression is that the changes induced by these compounds are most of the times small [73]. Unlike pharmaceutical interventions specifically directed at one high affinity molecular target, dietary intervention is less selective and generally results in small effects on gene expression. Using microarrays this problem can be overcome by studying a large number of genes at the same time. In this way, a combined effect of several small changes in the expression of genes belonging to a similar biological pathway can be easily detected. Other techniques, like RT-PCR and Northern Blotting, would fail to identify such effects, because of the relatively low number of genes tested at the same time. Pathway analysis software is in most cases indispensable in obtaining differentially modulated biological processes. A notable difficulty of assessing gene expression changes on a genome level is the problem of multiple testing. To obtain significant changes in gene expression the sample size should be sufficiently large, else correcting for multiple comparisons within the same experiment (e.g. 44000 comparisons on one microarray) does not yield significant data. In other words, the proportion of false positives in the dataset is large when thousands of genes are analyzed simultaneously. Methods for correcting for false discovery rate, like Benjamini-Hochberg, Holm and Bonferroni methods [74], are commonly used. Also Bayesian approaches are used that take into account the expression level of the genes for statistical analysis, assuming similar variances in genes with similar expression levels [75]. However, the costs for microarray experiments are most of the time still too high to allow for a high number of biological replicates. We prioritized the data on the basis of p-value and used the genes with the lowest p-values ($p<0.05$) for further analysis, without using statistical multiple correction techniques. When we combined the small effects of the most significantly changed genes into common biological pathways, we were able to identify effects of quercetin on fatty acid catabolism pathways. Individual gene expression changes were

confirmed with qRT-PCR and a decrease in plasma fatty acid levels indicated that quercetin altered fatty acid metabolism in rats. This biological validation of microarray data with independent techniques is the most powerful way to eliminate the possible misinterpretation of processes being false positively assigned as being regulated [76].

In conclusion, this study shows that quercetin induces fatty acid catabolism pathways in rat lung. In addition, quercetin showed an effect similar to uncoupling of oxidative phosphorylation combined with an upregulation of genes involved in the formation of ketone bodies. Quercetin is therefore newly identified as another flavonoid that can regulate fatty acid metabolism *in vivo*.

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CHAPTER 5

Quercetin aglycone and quercetin 3-O-glucuronide modulate energy utilization in mature 3T3-L1 adipocytes

ABSTRACT

Polyphenols are dietary compounds with potentially beneficial effects on human health. Previously, we demonstrated that chronic dietary quercetin, a polyphenol abundant in onions, apples and tea, alters fatty acid catabolism in rat lungs. To further explore the role of quercetin in modulation of energy providing metabolic pathways, we aimed to study the effects of quercetin on glucose and lipid utilization by mature 3T3-L1 adipocytes. For extrapolation of the observed *in vitro* effects to potential *in vivo* mechanisms, we also studied the effects of an important human metabolite of quercetin, quercetin 3-O-glucuronide. In addition to metabolic analyses, gene expression changes were analyzed. We demonstrated that quercetin aglycone and quercetin 3-O-glucuronide enhanced glucose levels, which could point to attenuated glucose utilization and/or uptake. Expression of *Pdk4*, a negative regulator of glycolysis, was significantly upregulated by both quercetin aglycone and quercetin 3-O-glucuronide. A strong downregulation of the expression of genes coding for proteins involved in lipid metabolism was seen for quercetin aglycone, but not for quercetin 3-O-glucuronide. Both quercetin aglycone and quercetin 3-O-glucuronide altered glycerol and NEFA levels as well, but the implications of these alterations are not clear. Nevertheless, this study is the first to demonstrate alterations in carbohydrate and lipid metabolism parameters induced by both quercetin aglycone and quercetin 3-O-glucuronide in mature 3T3-L1 adipocytes.

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INTRODUCTION

Polyphenols are dietary compounds from plants, abundant in vegetables, fruits, wine and tea [1]. A high dietary polyphenol intake has been associated with decreased levels of cardiovascular disease risk factors like LDL cholesterol, free fatty acids and triglycerides in blood [2]. Moreover, epidemiological studies suggest an inverse association between a high dietary polyphenol intake and development of cardiovascular diseases and possibly lung cancer, but not colon cancer [3].

Mechanistic studies *in vivo* point to a role for polyphenols in modulating energy providing metabolic pathways in mice and rats [4-7]. Polyphenols abundant in red wine and green tea; resveratrol and EGCg respectively, decreased adipose tissue mass in mice [4, 5]. Chronic dietary resveratrol prolonged lifespan of mice on a diet containing 60% fat and reduced lipid content in livers of mice [6]. We identified that quercetin, a polyphenol abundant in onions, apples and tea, could increase expression levels of genes coding for enzymes involved in fatty acid catabolism in rat lung after a chronic oral exposure [7]. Concomitantly, the plasma free fatty acid levels in rats decreased upon quercetin intervention [7]. As mentioned, the effects of dietary quercetin on fatty acid catabolism gene expression were observed in lungs of rats. It is not known whether dietary quercetin affects energy supply and utilization pathways in adipose tissue similar to EGCg and resveratrol.

One of the mechanisms explaining alterations in energy providing metabolic pathways was proposed to be through activation of SIRT1 by polyphenols. SIRT1 senses the energy state of the cell through its NAD+ dependent activity. Its homologs in yeast and *Drosophila* are involved in regulating the life-span prolonging effects induced by caloric restriction [8]. Quercetin, resveratrol and EGCg were shown to increase the activity of recombinant SIRT1 [9, 10]. Furthermore, resveratrol decreased intracellular triglyceride levels in differentiated mouse 3T3-L1 cells, an adipocyte cell culture model [11]. This reduction of triglyceride levels by resveratrol was mitigated when SIRT1 was knocked down and enhanced when SIRT1 was overexpressed in 3T3-L1 adipocytes [11]. In addition, the decrease in adipose tissue weight in high fat diet fed mice by resveratrol was mediated by activation of the SIRT1 target protein, PGC1α, a mediator of mitochondrial biogenesis, resulting in decreased lipid storage [5]. Furthermore, Baur *et al.* demonstrated that resveratrol enhanced PGC1α activity and lowered lipid levels in liver of mice on a high fat diet [6]. These findings are suggestive of a role for SIRT1 in the lipid lowering effect of

resveratrol [5, 6]. Whether quercetin and EGCg have similar effects on lipid storage *in vivo* and lipolysis *in vitro* is currently not known.

To address the question whether quercetin alters energy metabolism in adipocytes *in vitro*, we studied substrate utilization upon quercetin intervention by analyzing glucose, glycerol, NEFA and triglyceride levels in medium and/or cell lysates of mature 3T3-L1 adipocytes. Additionally, we analyzed concomitant alterations in gene expression of key enzymes involved in carbohydrate and lipid metabolism. Upon uptake, quercetin is metabolized in the intestine to glucuronide and sulfate conjugates. For extrapolation of the observed *in vitro* effects to potential *in vivo* mechanisms, we therefore included quercetin 3-O-glucuronide, an important human metabolite of quercetin [12-14], in our studies.

MATERIAL AND METHODS

Cell culture and exposure conditions

3T3-L1 mouse embryonic fibroblasts (ATCC) were cultured in DMEM/F12 (D6421, Sigma) supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin (PS) in a humidified 37 ^{o}C incubator with 5% CO_2. Confluent 3T3-L1 fibroblasts were differentiated in 6-well plates for three days in DMEM/F12 medium containing 10% FBS, 1% PS, and an adipogenic cocktail containing: 0.5 mM IBMX (Sigma), 1 μM dexamethasone (Sigma), 10 μM prostaglandin I_2 (Biomol) and 1 μM insulin (Sigma). After the three-day initiation of differentiation, 3T3-L1 cells were maintained in DMEM/F12 containing 10% FBS, 1% PS and 1 μM insulin. Maintenance medium was refreshed every two/three days and differentiation was monitored by the visual appearance of fat droplets in the cells. Twelve to fourteen days after the three day differentiation initiation period, 3T3-L1 cells were exposed to polyphenols for one or three days in exposure medium, which consisted of DMEM/F12 without phenol red containing 10% FBS, 1 μM insulin, 500 μM vitamin C (VWR), 60U/ml catalase (Sigma), 30U/ml superoxide dismutase (Sigma). Vitamin C, catalase and superoxide dismutase were added to prevent auto-oxidation of quercetin aglycone in cell culture medium [15, 16]. Resveratrol (Sigma), quercetin (Sigma) and quercetin 3-O-glucuronide (Apin Chemicals) were dissolved in ethanol at 10 mM and dissolved in exposure medium to a final concentration of 50 μM. Exposure medium of control adipocytes did not contain polyphenols and was supplemented with 0.5% EtOH. In the case of a three day exposure period, exposure medium was refreshed daily. Exposure medium was supplemented with fatty acids (linoleic acid, oleic

acid arachidonic acid - Albumin (Sigma)) to a final concentration of 40 μM total NEFA (non-esterified fatty acids), where indicated. After exposure, medium was collected and cells were washed with HBSS, trypsinized, constituted in 500 μl MQ and lysed by three consecutive freeze-thaw cycles in liquid nitrogen and a 37°C water bath. Cell lysates were stored at -80 °C and collected medium samples were stored at -20 °C until analysis was performed. For quercetin 3-O-glucuronide exposed adipocytes, only medium samples were collected. Cell viability was assessed using Cell Titer Blue assay kit (Promega) according to the manufacturer's protocol with slight adjustments. To exclude interference with the cell viability assay of compounds in exposure medium, exposure medium was discarded and subsequently diluted cell viability reagent was added.

Oil red O staining of adipocytes

Oil red O (ORO) staining was used to quantify intracellular neutral lipids in adipocytes grown in 6 well plates, using a method adapted from Koopman *et al.* [17, 18]. In short, 3T3-L1 adipocytes were washed with HBSS, fixed with 3.7% formaldehyde in culture medium without serum for 1 hour, rinsed three times with deionized water and quickly rinsed with 70% EtOH. Subsequently, cells were stained with Oil Red O solution for 30 minutes and washed six times with deionized water. After staining and washing, the neutral lipid-bound pigment was dissolved by adding DMSO per well and the absorbance at 540 nm was measured. Preadipocytes without lipid accumulation were used as controls for unspecific binding of Oil Red O stain.

Enzymatic analysis of glycerol, NEFA and triglycerides

Glycerol levels in cell lysates and medium were analyzed using a quantitative enzymatic assay (free glycerol determination reagent, Sigma). To prevent interference of vitamin C in medium samples, we added ascorbate oxidase (5U/ml, Sigma) to all samples before analysis. Triglyceride levels were analyzed using Triglyceride L-Type assay (WAKO chemicals) and NEFA levels were analyzed using the NEFA C assay kit (WAKO chemicals). Vitamin C did not interfere in NEFA and triglyceride assays because reagents already contained ascorbate oxidase. All assays were adjusted so that small sample volumes (5 -10 μl) could be used and analyzed with a microplate reader (BioTec Synergy HT). Polyphenols did not interfere in the enzymatic analyses, which was checked by standard addition of polyphenols to blank samples (data not shown). Intracellular levels of glycerol, triglycerides and NEFA were corrected for cellular protein content which was measured using the DC protein assay (Bio-Rad).

Table 5.1
Primers used for quantitative real time PCR. All primers were intron-spanning

Gene symbol	Gene Name	Sequence ID	Forward primer (5' - 3')	Reverse primer (5' - 3')
Fabp4	fatty acid binding protein 4	NM_024406	AATCACCGCAGACGACAGGAAG	TGCCCTTTCATAAACTCTTGTGGAAG
Dgat2	diacylglycerol O-acyltransferase 2	NM_026384	CTATTTGGCTACGTTGGCTGGTAAC	TTGGAGAGCAAGTAGTCTATGGTGTC
Hsl	hormone sensitive lipase	NM_010719	TCAGGGACAGAGGCAGAGGAC	TCCACTTAGTTCCAGGAAGGAGTTG
Scd1	stearoyl-Coenzyme A desaturase 1	NM_009127	TCATGGTCCTGCTGCACTTGG	CTGTGGCTCCAGAGGCGATG
Plin	perilipin	NM_175640	CCAGGCTGTCTCCTCTACCAAAG	TCGATGTCTCGGAATTCGCTCTC
Acox1	acyl-Coenzyme A oxidase 1	NM_015729	GCAGATAAACTCCCCAAGATTCAAGAC	TAAAGTCAAAGGCATCCACCAAAGC
Cpt2	carnitine palmitoyl transferase 2	NM_009949	GCTCCGAGGCATTTGTCAGG	TTGTGGTTTATCCGCTGGTATGC
Pck1	Phosphoenol pyruvate carboxykinase 1	NM_011044	GTTTGTAGGAGCAGCCATGAGATC	CCAGAGGAACTTGCCATCTTTGTC
Pdk4	pyruvate dehydrogenase kinase, isoenzyme 4	NM_013743	CTTACAATCAAGATTTCTGACCGAGGAG	ATAATGTTTGAAGGCTGACTTGTTAAAGAC
Canx	calnexin	NM_007597	GCAGCGACCTATGATTGACAACC	GCTCCAAACCAATAGCACTGAAAGG
Actb	beta actin	NM_007393	CGTTGACATCCGTAAAGACCTCTATG	TCATCGTACTCCTGCTTGCTGATC

Analysis of glucose levels in medium

Glucose levels in medium samples were analyzed using an automated enzymatic/coulometric based analysis method, primarily used to analyze glucose levels in whole blood (Freestyle, Abbott Diabetes Care). To confirm the suitability for analysis of glucose in tissue culture medium samples, we analyzed glucose calibration curves, prepared in medium without glucose, within each experiment. Calibration curves were linear up to 25 mM glucose and reproducible between days (data not shown).

Polyphenols did not interfere in the analysis of glucose levels in medium, which was assessed by standard addition of polyphenols to blank samples (data not shown).

RNA isolation and quantitative real time RT-PCR

After a one day exposure of 3T3-L1 adipocytes cultured in 6 wells plates to polyphenols, RNA was isolated from adipocytes cultured in individual wells (n=6, per treatment) using Trizol (Invitrogen) according to manufacturers protocol with an additional phenol/chloroform/isoamylalcohol (25:24:1, v:v:v) step followed by a second chloroform purification step. Isolated RNA samples were purified using RNeasy columns (Qiagen). Integrity and quality of RNA samples was excellent as was assessed by spectophotometric analysis using a Nanodrop (isogen Life Science) and automated electrophoresis (Experion, Bio-Rad). cDNA was synthesized from 1 μg total RNA for each sample using the iScript cDNA Synthesis kit (Bio-Rad). Primers were designed for Sybr Green probes with Beacon Designer 4.0 (Premier Biosoft International) (table 5.1). PCR amplification and detection was performed with the iQ SYBR Green Supermix and the MyIQ single-color real-time PCR detection system (Bio-Rad). A standard curve for all genes including reference genes was generated using serial dilutions of a pool prepared from all cDNA samples. The level of mRNA for each gene was normalized using calnexin and beta-actin as reference genes. PCR amplification and detection for all samples and genes was performed in duplicate or triplicate on 2 or 3 separate days.

RESULTS

Quercetin did not affect intracellular lipid levels in cultured adipocytes

Oil red O staining of intracellular neutral lipids demonstrated that after exposing cells for three days to quercetin, the total level of intracellular lipids did not decrease, as compared to adipocytes exposed to identical medium but without quercetin (control adipocytes) (figure 5.1). On the other hand, resveratrol lowered intracellular lipid levels as was previously observed by Picard *et al.* [11]. Although visual inspection of adipocytes after a three day polyphenol exposure did not show any morphological differences from control adipocytes, cell viability declined when adipocytes were exposed to 50 μM quercetin as compared to control adipocytes (data not shown). Consequently, Oil red O staining data have been corrected for cell viability decline. Nevertheless, one day quercetin exposed adipocytes

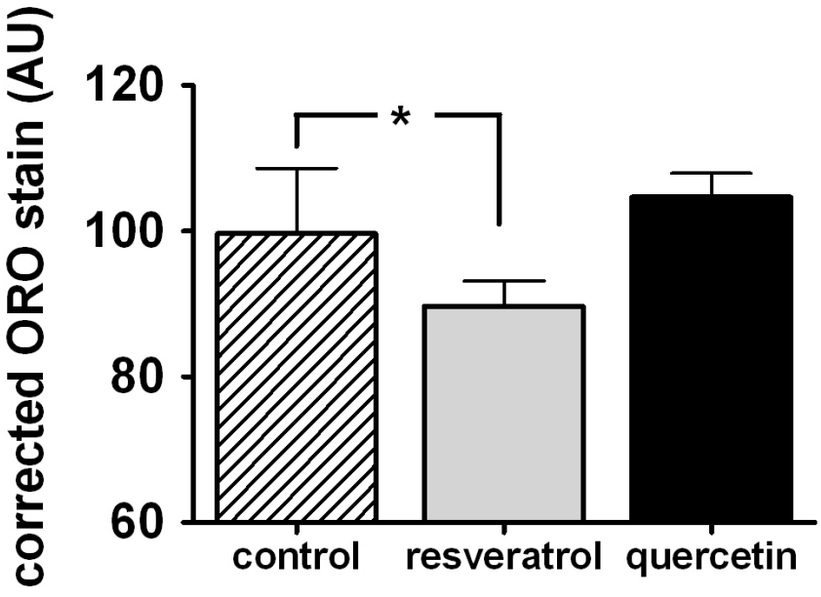

Figure 5.1
Intracellular neutral lipid levels after polyphenol exposure corrected for cell viability. Intracellular lipids were quantified with ORO stain after a three day exposure to 50 μM resveratrol or 50 μM quercetin (exposure medium was refreshed daily). * = $p<0.05$. Columns and error bars represent mean +/- SD (n=6).

were as viable as control adipocytes (data not shown). Exposure of adipocytes to resveratrol for either one day or three days did not affect cell viability (data not shown).

Quercetin enhanced glucose levels in adipocyte medium

Because of the cell viability decline, we assessed the effects of quercetin on energy providing metabolic pathways in all further experiments only after one day exposures to quercetin. First, we measured the effect of quercetin on extracellular levels of glucose added to the cells as energy source. Medium of quercetin exposed adipocytes contained higher levels of glucose after 24h incubation than medium of control adipocytes (figure 5.2A). This points to a lower utilization or uptake of glucose in quercetin exposed adipocytes. In contrast, resveratrol did not consistently affect glucose levels in medium of exposed adipocytes, even slightly decreased levels were observed occasionally in resveratrol exposed adipocytes (data not shown). Next, we supplemented exposure medium additionally with fatty acids to assess whether glucose utilization and/or uptake was differentially affected

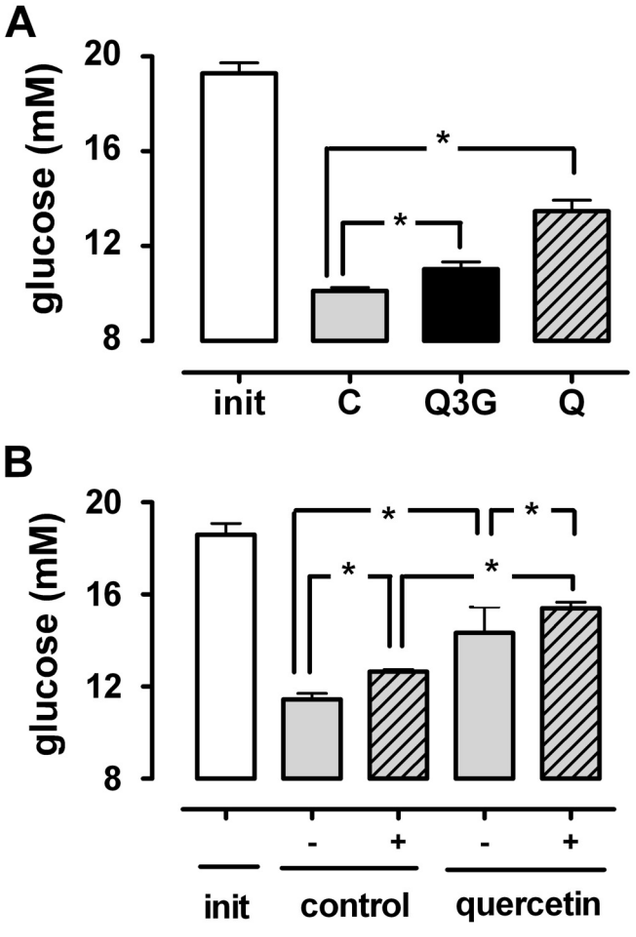

Figure 5.2
Effect of quercetin and quercetin 3-O-glucuronide on extracellular glucose levels.
A) Glucose levels in medium of adipocytes exposed to 50 μM quercetin or 50 μM quercetin 3-O-glucuronide for one day. B) Glucose levels in medium of adipocytes exposed to 50 μM quercetin for one day with (+) or without (-) supplementation of 40 μM fatty acids. C = control, Q = quercetin, Q3G = quercetin 3-O-glucuronide, init = initial glucose levels in medium before start of exposure. * = $p<0.05$. Columns and error bars represent mean +/- SD (n=6).

by quercetin when fatty acids in medium were abundant. Supply of fatty acids as additional energy source besides glucose resulted in less utilization and/or uptake of glucose in quercetin exposed cells as well as control cells (figure 5.2B). Consistent with our findings that quercetin lowered glucose utilization and/or uptake in the non-fatty acid supplemented adipocytes, quercetin had similar effects in the fatty acid supplemented adipocytes (figure 5.2B)

Quercetin altered levels of glycerol, NEFA and triglycerides

We next analyzed parameters of lipid metabolism in medium and cell lysates from control and quercetin exposed adipocytes. Intracellular triglyceride levels in cell lysates of adipocytes exposed to quercetin were not different from triglycerides in control adipocytes (figure 5.3) as was already demonstrated for the three-day exposure (figure 5.1). On the other hand, intracellular glycerol and NEFA concentrations were higher in cells exposed to quercetin than in control cells (figure 5.3). Medium concentrations of glycerol decreased upon quercetin intervention. NEFA levels could not be detected in medium using enzymatic assays before and after quercetin or control exposures (figure 5.3). One day resveratrol exposure did not result in consistent alterations of lipid metabolism parameters (data not shown).

With the increased supply of fatty acids in exposure medium the intracellular absolute levels of triglycerides, NEFA and glycerol were higher in quercetin exposed cells as well as control cells (figure 5.4). However, quercetin clearly depressed the increased cellular storage of triglycerides induced by additional supplementation of fatty acids as was shown by lower intracellular triglyceride levels in quercetin exposed adipocytes (figure 5.4) Glycerol levels did not differ between control and quercetin exposed cells (figure 5.4). NEFA levels decreased from the initial 40 μM to non-detectable levels, in quercetin exposed as well as control cells, indicating that adipocytes consumed the supplemented fatty acids (figure 5.4). Furthermore, in fatty acid supplemented adipocytes glycerol was detected only at very low levels in medium after exposure (figure 5.4). Consequently, we could not detect any differences in medium glycerol and NEFA levels between quercetin and control exposures.

Quercetin 3-O-glucuronide had similar effects on glucose, glycerol and NEFA levels as quercetin aglycone

We analyzed medium samples from adipocytes exposed to quercetin 3-O-glucuronide to assess the effect of a major *in vivo* metabolite of quercetin on levels of glucose, glycerol and NEFA. Similar to the effect of quercetin aglycone on medium levels of glucose and lipid metabolism parameters, quercetin 3-O-glucuronide significantly altered these parameters as well. Cell viability was not affected by the quercetin 3-O-glucuronide treatment (data not shown). As after quercetin aglycone exposure, glucose levels were significantly higher in medium of quercetin 3-O-glucuronide exposed adipocytes than in medium of non-exposed cells (figure 5.2A). Furthermore, medium glycerol levels decreased upon quercetin 3-O-glucuronide exposure

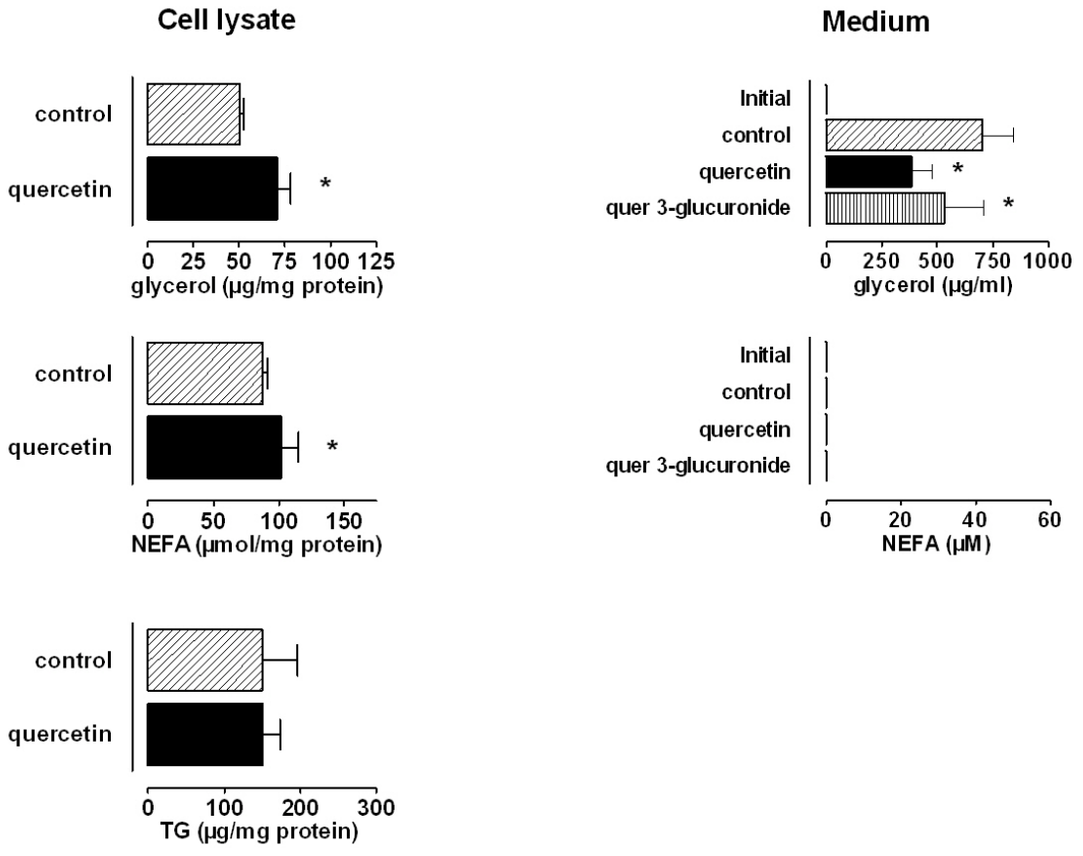

Figure 5.3
Effect of quercetin and quercetin 3-O-glucuronide on levels of lipid metabolism parameters without additional fatty acid supplementation. Triglyceride (TG), glycerol and NEFA (non-esterified fatty acids) levels in cell lysates and glycerol and NEFA levels in medium of adipocytes after exposure to 50 µM quercetin or 50 µM quercetin 3-O-glucuronide for one day. * = $p<0.05$ as compared to control treatment. Columns and error bars represent mean +/- SD (n=6).

(figure 5.3). The results obtained indicate that quercetin 3-O-glucuronide has similar effects as quercetin aglycone, but to a lower extent.

Quercetin aglycone mostly downregulated gene expression of enzymes involved in carbohydrate and lipid metabolism and upregulated *Pdk4*, whereas quercetin 3-O-glucuronide only upregulated *Pdk4*

In order to substantiate the observed effects of quercetin and quercetin 3-O-glucuronide, we analyzed expression of selected genes coding for proteins involved in carbohydrate and lipid metabolism pathways. Both

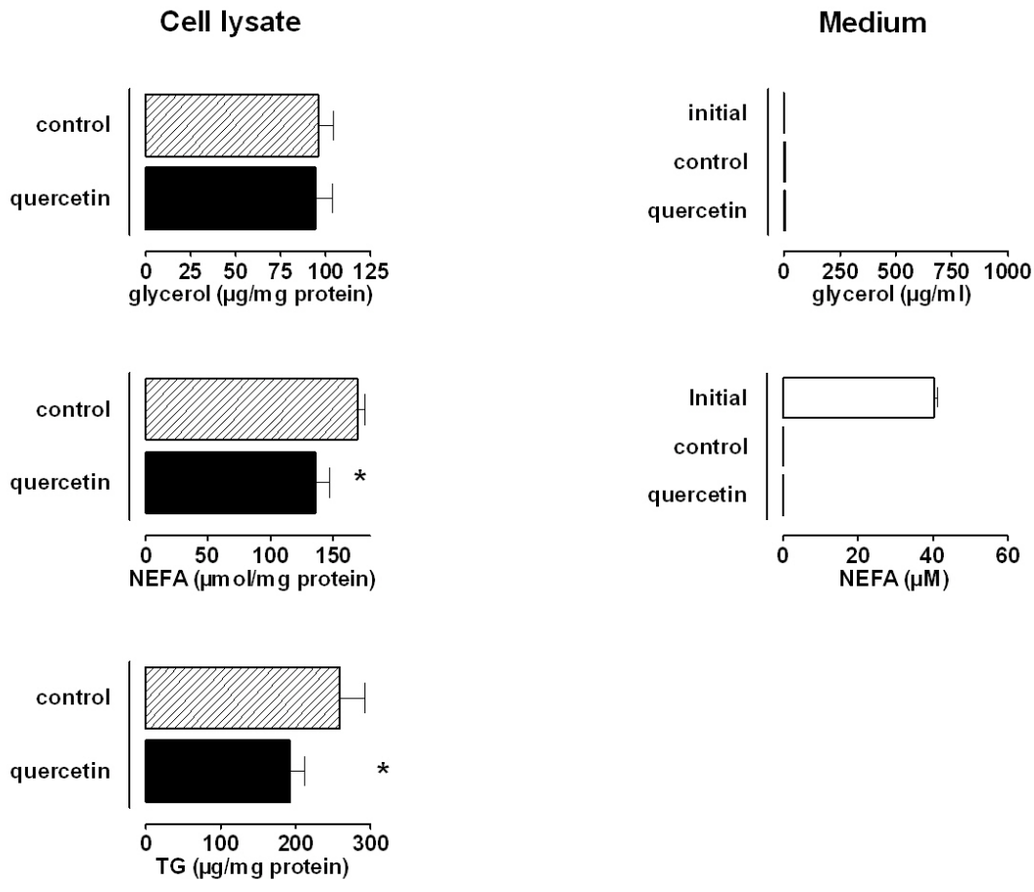

Figure 5.4
Effect of quercetin on levels of lipid metabolism parameters with additional fatty acid supplementation. Triglyceride (TG), glycerol and NEFA (non-esterified fatty acids) levels in cell lysates and glycerol and NEFA levels in medium of adipocytes after exposure to 50 µM quercetin for one day. * = $p<0.05$ as compared to control treatment. Columns and error bars represent mean +/- SD (n=6).

quercetin aglycone (2.4 +/- 0.6 fold, mean +/- SD) and quercetin 3-O-glucuronide (1.4 +/- 0.2 fold) significantly upregulated pyruvate dehydrogenase kinase 4 (*Pdk4*), a negative regulator of glycolysis (figure 5.5). This possibly indicates that glycolysis was decreased by quercetin aglycone and quercetin 3-O-glucuronide. Strikingly, quercetin aglycone strongly downregulated all other analyzed genes by 2 to 7-fold (figure 5.5A), whereas quercetin 3-O-glucuronide did not affect expression of any of these genes (figure 5.5B). We observed that genes coding for enzymes involved in beta-oxidation (*Cpt2*, *Acox1*) and lipid biosynthesis (*Dgat2* and *Scd1*) were downregulated by quercetin aglycone only. In addition, fatty acid binding protein 4 (*Fabp4*), hormone sensitive lipase (*Hsl*), perilipin (*Plin*) and

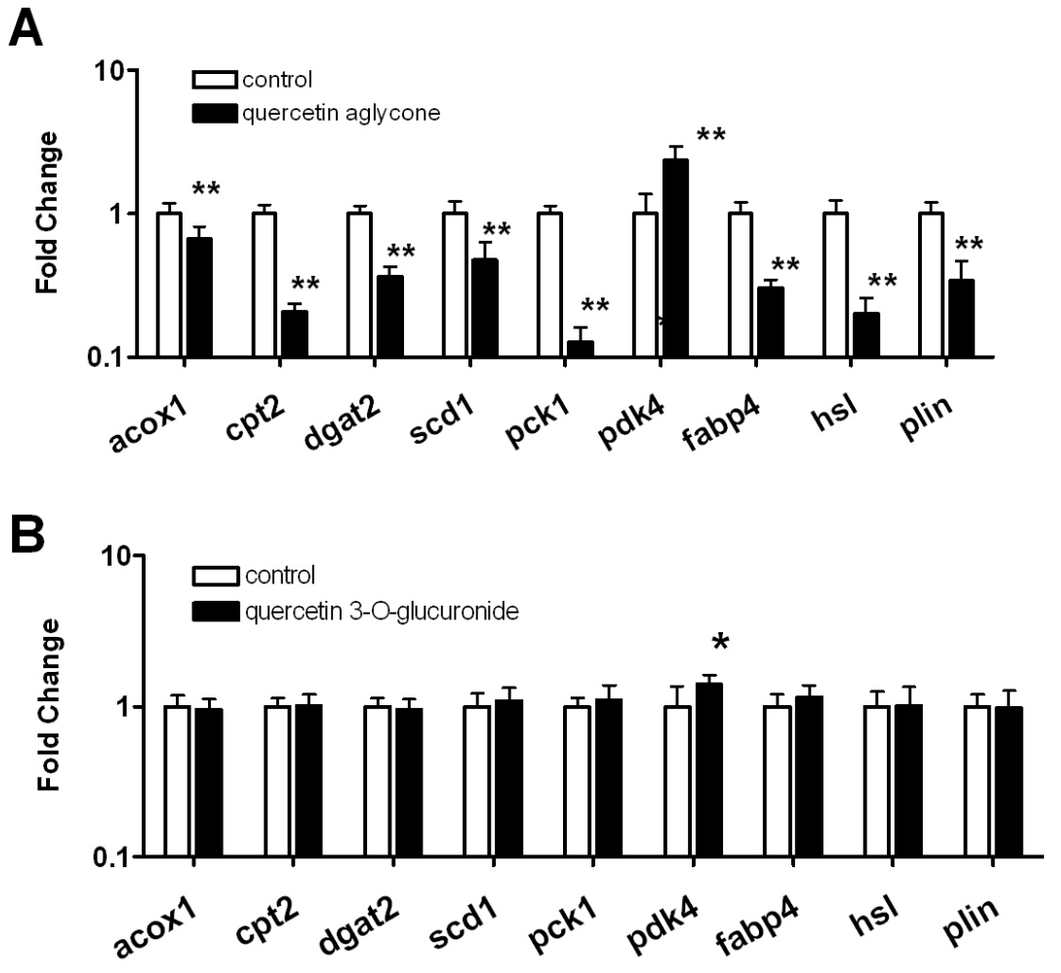

Figure 5.5
Gene expression changes in 3T3-L1 adipocytes induced by a one day exposure to 50 μM quercetin (A) or 50 μM quercetin 3-O-glucuronide. Gene expression of target genes was normalized using calnexin as reference gene. Similar results were obtained using beta-actin as reference gene. Average expression levels in control adipocytes were set to one. * *= $p<0.01$. * = $p<0.05$. Columns and error bars represent mean +/- SD (n=6).

phosphoenolpyruvate carboxykinase (*Pck1*) were all downregulated by quercetin aglycone (figure 5.5A), whereas quercetin 3-O-glucuronide exposed adipocyte gene expression levels were not different from expression levels in control adipocytes (figure 5.5B).

DISCUSSION

We found that quercetin aglycone and quercetin 3-O-glucuronide attenuated adipocyte glucose utilization or uptake. These changes were

supported by an upregulation of *Pdk4*, a negative regulator of glycolysis. In addition, levels of lipid metabolism intermediates, glycerol and NEFA, were altered significantly by exposure of adipocytes to quercetin aglycone or quercetin 3-O-glucuronide. Therefore, our study is suggestive for a shift in energy source from glucose to fatty acids. This study is the first to demonstrate alterations in carbohydrate and lipid metabolism parameters induced by quercetin 3-O-glucuronide.

Both quercetin aglycone and quercetin 3-O-glucuronide increased the levels of glucose in medium significantly. This effect can either be caused by a decrease in the rate of adipocyte glycolysis or a decrease in the uptake of glucose or both. The upregulation of *Pdk4* by quercetin aglycone as well as quercetin 3-O-glucuronide points to decreased glycolysis. PDK4 is an important regulator of pyruvate entry into the tricarboxylic acid (TCA) cycle, and is responsible for switching cellular energy sources from glucose to fatty acids [19]. PDK4 inactivates pyruvate dehydrogenase by phosphorylation which inhibits glycolysis [19] and has been demonstrated to be upregulated in adipose tissue in response to fasting [20]. Upregulation of *Pdk4* gene expression, therefore, suggests that adipocytes might shift their cellular energy source from glucose to fatty acids. Decreased glucose utilization and decreased tryglycerides and NEFAs in NEFA-supplemented adipocytes after quercetin aglycone exposure are supportive for this shift. However, results obtained in the non-NEFA-supplemented cells are not clear. In addition, a shift to fatty acid oxidation was not reflected by increased expression of beta oxidation genes *Cpt2* and *Acox1*. Further studies should be directed towards a more comprehensive identification of gene expression profiles in response to quercetin and quercetin 3-O-glucuronide treatment.

Quercetin has been shown to inhibit methylglucose uptake mediated by GLUT4 in rat adipocytes [21]. In addition, the widely expressed GLUT1 transporter [22] was inhibited by quercetin aglycone in cell culture models [23]. One of the predominant intestinal glucose transporters, GLUT2, was non-competitively inhibited by quercetin as well [24]. Interestingly, as the authors of the GLUT2 study pointed out [24], inhibition of GLUT transporters located in tissues other than gastro-intestinal tissues by quercetin aglycone is probably not physiologically relevant, because quercetin aglycone is rapidly conjugated *in vivo* to quercetin glucuronides and sulfates. Our result that quercetin 3-O-glucuronide also inhibited glucose utilization and/or uptake might suggest that quercetin 3-O-glucuronide could inhibit *in vivo* adipocyte GLUT transporters as well. This is further supported by the fact that we detected quercetin glucuronides

and/or sulfates in adipose tissue of rats chronically exposed to dietary quercetin, whereas quercetin aglycone was not detected [25].

Resveratrol has been shown to increase lipolysis in 3T3-L1 adipocytes in a SIRT1 dependent manner [11]. This *in vitro* result was consistent with *in vivo* findings demonstrating that dietary resveratrol lowered adipose tissue weight in mice and increased energy expenditure in mice fed a high fat diet [5, 6]. Because quercetin also altered fatty acid catabolism *in vivo* [7] and activated SIRT1 activity *in vitro* [9, 10], we hypothesized that quercetin might possibly alter lipolysis in 3T3-L1 cells as well. We observed, by using similar three day exposure conditions a decline in intracellular triglycerides induced by resveratrol, but we did not observe increased levels of glycerol in medium after a one day or three day exposure to resveratrol. Adipocytes exposed to quercetin for one day did also not show increased levels of glycerol in medium as compared to medium of control adipocytes. On the other hand, intracellular levels of glycerol and fatty acids did increase upon quercetin exposure, suggesting an overall higher rate of cellular lipolysis. This was however not reflected in decreased intracellular triglycerides nor in glycerol levels accumulated in medium over the exposure period of 24 hour. These data do not support an effect of resveratrol or quercetin on lipolysis. However, because glucose utilization and/or uptake was lowered in quercetin treated adipocytes, it is not clear how energy homeostasis could have been maintained. In a study by Kuppusamy *et al.* [26] quercetin aglycone was shown to increase glycerol release from primary rat adipocytes cells in culture. However, this effect was only observed when lipolysis was induced by epinephrine, a known hormonal stimulator of lipolysis [26].

Apart from quercetin and resveratrol, other polyphenols have been shown to interfere in adipocyte lipid metabolism as well. Genistein induced lipolysis, analyzed by glycerol released from exposed 3T3-L1 cells, independent of epinephrine [27], procyanidins increased glycerol levels in adipocyte medium [28], anthocyanidins increased glycerol release from adipocytes [29] and EGCg enhanced intracellular glycerol levels in 3T3-L1 adipocytes [30]. Several studies tried to elucidate possible mechanisms underlying the effect polyphenols on energy providing metabolic pathways. Modulation of PPARγ activity [31-33], activation of SIRT1 [5, 6, 11], inhibition of cAMP dependent phosphorylation of PKA [34, 35] and inhibition of PI3K [36, 37] have been proposed as possible molecular targets of polyphenols. This demonstrates that the effect of polyphenols on 3T3-L1 energy providing metabolic pathways is complex and might be regulated through several possible mechanisms.

In our study we identified a strong downregulation by quercetin aglycone of most of the analyzed genes selected for their role in carbohydrate and lipid metabolism. Gene expression of proteins involved in fatty acid beta oxidation (*Cpt2*, *Acox1*), lipid biosynthesis (*Scd1*, *Dgat2*) and gluconeogenesis (*Pck1*) were all strongly downregulated. This suggests an overall repression of adipocyte genes involved in carbohydrate and lipid metabolism. Given that the analyzed genes in our study are target genes of nuclear receptors (NRs) the observed effects of quercetin aglycone might be regulated through mechanisms involving NRs. A similar transcriptional response of adipocytes was induced by RIP140 (receptor interacting protein 140). This co-repressor of a number of NRs (*in vitro*), like PPARs, ERs and RXRs [38], globally suppressed the expression of genes coding for carbohydrate and lipid metabolism proteins. This was demonstrated by studying gene expression changes upon re-expression of the *Rip140* gene in *Rip140*-null adipocytes [39, 40]. Overexpressed genes in *Rip140*-null adipocytes, e.g. fatty acid synthase (*Fas*), acyl-CoA oxidase 1 (*Acox1*), acetyl-CoA synthetase (*Acs*), carnitine palmitoyl transferase 1b (*Cpt1b*), lactate dehydrogenase (*Ldh*) and pyruvate dehydrogenase kinase 4 (*Pdk4*), were strongly repressed when the *Rip140* gene was re-expressed [39, 40]. Interestingly, the polyphenol activated protein SIRT1 promotes fat mobilization in 3T3-L1 adipocytes by docking with NR co-repressors NCoR and SMRT [11]. Quercetin aglycone can activate recombinant SIRT1 [9, 10], but whether quercetin aglycone can activate SIRT1 in 3T3-L1 adipocytes is not known. Therefore, studying the effect of quercetin aglycone on SIRT1 activity in 3T3-L1 adipocytes would probably give clues on the involvement of SIRT1 and NRs in the quercetin mediated effect on carbohydrate and lipid metabolism parameters.

In conclusion, we demonstrated that quercetin aglycone and quercetin 3-O-glucuronide attenuated glucose use or glucose uptake in 3T3-L1 adipocytes in culture. Although quercetin aglycone and quercetin 3-O-glucuronide altered lipid metabolism parameters in cell lysates and medium of exposed adipocytes differently than resveratrol, the implications of these alterations are not clear. Further studies should be performed to address the effect of quercetin and quercetin glucuronides on lipid metabolism. Nevertheless, our results indicate that quercetin 3-O-glucuronide is a potential bioactive compound with similar effects on glucose and lipid metabolism parameters as quercetin aglycone. Given that quercetin aglycone is generally not found *in vivo*, we speculate that observed *in vivo* effects of dietary quercetin on fatty acid catabolism in rats [7] could possibly be induced by quercetin glucuronides.

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CHAPTER 6

SIRT1 stimulation by polyphenols is affected by their stability and metabolism

ABSTRACT

SIRT1 is the human ortholog of the yeast sir2 protein; one of the most important regulators of lifespan extension by caloric restriction in several organisms. Dietary polyphenols, abundant in vegetables, fruits, cereals, wine and tea, were reported to stimulate the deacetylase activity of recombinant SIRT1 protein and could therefore be potential regulators of aging associated processes. However, inconsistent data between effects of polyphenols on the recombinant SIRT1 and on *in vivo* SIRT1, led us to investigate the influence of 1) stability of polyphenols under experimental conditions and 2) metabolism of polyphenols in human HT29 cells, on stimulation of SIRT1. With an improved SIRT1 deacetylation assay we found three new polyphenolic stimulators. Epigallocatechin galate (EGCg, 1.76 fold), epicatechin galate (ECg, 1.85 fold), and myricetin (3.19 fold) stimulated SIRT1 under stabilizing conditions, whereas without stabilization, these polyphenols strongly inhibited SIRT1, probably due to H_2O_2 formation. Using metabolically active HT29 cells we were able to show that quercetin (a stimulator of recombinant SIRT1) could not stimulate intracellular SIRT1. The major quercetin metabolite in humans, quercetin 3-O-glucuronide, slightly inhibited the recombinant SIRT1 activity which explains the lack of stimulatory action of quercetin in HT29 cells. This study shows that the stimulation of SIRT1 is strongly affected by polyphenol stability and metabolism, therefore extrapolation of *in vitro* SIRT1 stimulation results to physiological effects should be done with caution.

Keywords: SIRT1, sir2, polyphenols, quercetin, resveratrol, EGCg

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INTRODUCTION

SIRT1 (Silent Information Regulator Two ortholog 1) is the human ortholog of the yeast sir2 protein. It belongs to a class of proteins called sirtuins that possess a NAD+-dependent deacetylase activity. Sir2 most likely is one of the key proteins in mediating the caloric restriction dependent lifespan extension in *S. cerevisiae*, *C. elegans* and *D. melanogaster* [1]. Lifespan extension by caloric restriction is not seen in yeast, *C. elegans* and drosophila mutants that do not express the *sir2*-gene [2-4]. Because *sir2* is conserved from prokaryotes to mammals, the human SIRT1 protein could also be involved in regulating life extending processes in humans. Recent discoveries have shown that SIRT1 regulates several stress related processes [5-10], fatty acid metabolism and adipogenesis [11], axonal neurodegeneration [12] and muscle cell differentiation [13]. SIRT1 controls these processes by NAD+-dependent deacetylation of acetylated lysine groups of several transcription factors and other proteins. Histones [14, 15], p53 [9, 16], FOXO transcription factors [5, 6, 17], ku70 [7], TAFI168 [18], myoD [13], p300 [19] and PGC1α [20, 21] are all substrates for SIRT1 deacetylation.

An interesting finding was that the deacetylation activity of SIRT1 can be stimulated by several polyphenolic compounds [22]. Polyphenols are a wide group of dietary compounds from plants, occurring in high amounts in fruits, vegetables, cereals, wine and tea. Epidemiological studies suggest that a diet rich in polyphenols may protect against cardiovascular diseases [23, 24], and mechanistic studies in cells and animals have shown that polyphenols have a wide range of properties that also may play a role in the prevention of other diseases, such as cancer [25, 26] and neurodisfunctions [27, 28]. Polyphenols are strong antioxidants, but are also known to interfere in signal transduction pathways [29-31], inflammation [32-34], and can interact with a number of proteins involved in cell proliferation [33, 35, 36]. The stimulation of SIRT1 could possibly be an additional process that may explain the mechanisms by which dietary polyphenols exert their beneficial effect in humans.

Howitz *et al.* showed that the deacetylation activity of SIRT1 could be enhanced by the following polyphenols: resveratrol (up to 13-fold), butein (8.5 fold), picaetannol (7.9 fold), isoliquiritigenin (7.6 fold), fisetin (6.6 fold) and quercetin (4.6 fold). Stimulation of deacetylation activity by resveratrol resulted in regulation of several SIRT1 mediated physiological processes. Resveratrol treatment in experimental model systems expressing SIRT1 or its homologous genes increased the lifespan of *S. cereviseae*, *C. elegans* and *D.*

melanogaster [22, 37], suggesting that resveratrol could mimic the effects of caloric restriction in model organisms. In mammalian cell culture models resveratrol reduced fat storage and triglyceride release in differentiated 3T3-L1 cells [11], increased p53-mediated cell survival in HEK293 cells [22] and inhibited NFκB-dependent transcription in NSCLC cells [10]. Regulation of these effects by resveratrol was abolished in the analogous SIRT1 knockdown model. Although other polyphenols (quercetin and piceatannol) were shown to have a marked effect on SIRT1 activity, they did not have any effect on lifespan in yeast. Only resveratrol and fisetin were shown to have a physiological effect that was mediated by sir2 [22, 37].

We hypothesize that stability of polyphenols under experimental conditions and metabolism of polyphenols in cells eliminate the stimulatory action of polyphenols on SIRT1 activity. Firstly, it is well known that after ingestion, polyphenols are metabolized by phase II enzymes in the intestine and liver. As a result, all tissues, except those of the gastro-intestinal tract, are only exposed to glucuronidated and sulfated metabolites of polyphenols. This has a profound effect on their bioactivity [38]. Secondly, most polyphenols are readily oxidized in aqueous media with a pH higher than 7, resulting in the formation of polyphenolic oxidation products and H_2O_2, which can lead to misinterpretation of experimental *in vitro* results [39].

Previous studies that determined sir2 deacetylase activity used radioactive methods with ^{14}C-NAD$^+$, ^{14}C-acetylated p53 or ^{3}H-acetylated histone groups as radioactive substrate [8, 40-42], HPLC methods to analyze substrate conversion/product formation [43-45] or spectrophotometric plate reader methods with a synthetic substrate containing a specifically cleaved fluorochrome [46-48]. These methods are either difficult to perform or not very specific. Spectrophotometric analysis of fluorochromes is hampered by autofluorescence of polyphenols and inherent background of fluorescent synthetic substrates. Therefore, we developed a more specific HPLC method. With this method we investigated whether stability and metabolism affect the polyphenolic stimulation of SIRT1. We used recombinant SIRT1 as well as metabolically active HT29 colon carcinoma cells, because colonic cells are exposed to unconjugated polyphenols from the diet via the lumen of the gastro-intestinal tract. Several polyphenols (figure 6.1), including resveratrol, catechins, quercetin and its major metabolite in humans, quercetin 3-O-glucuronide, were tested.

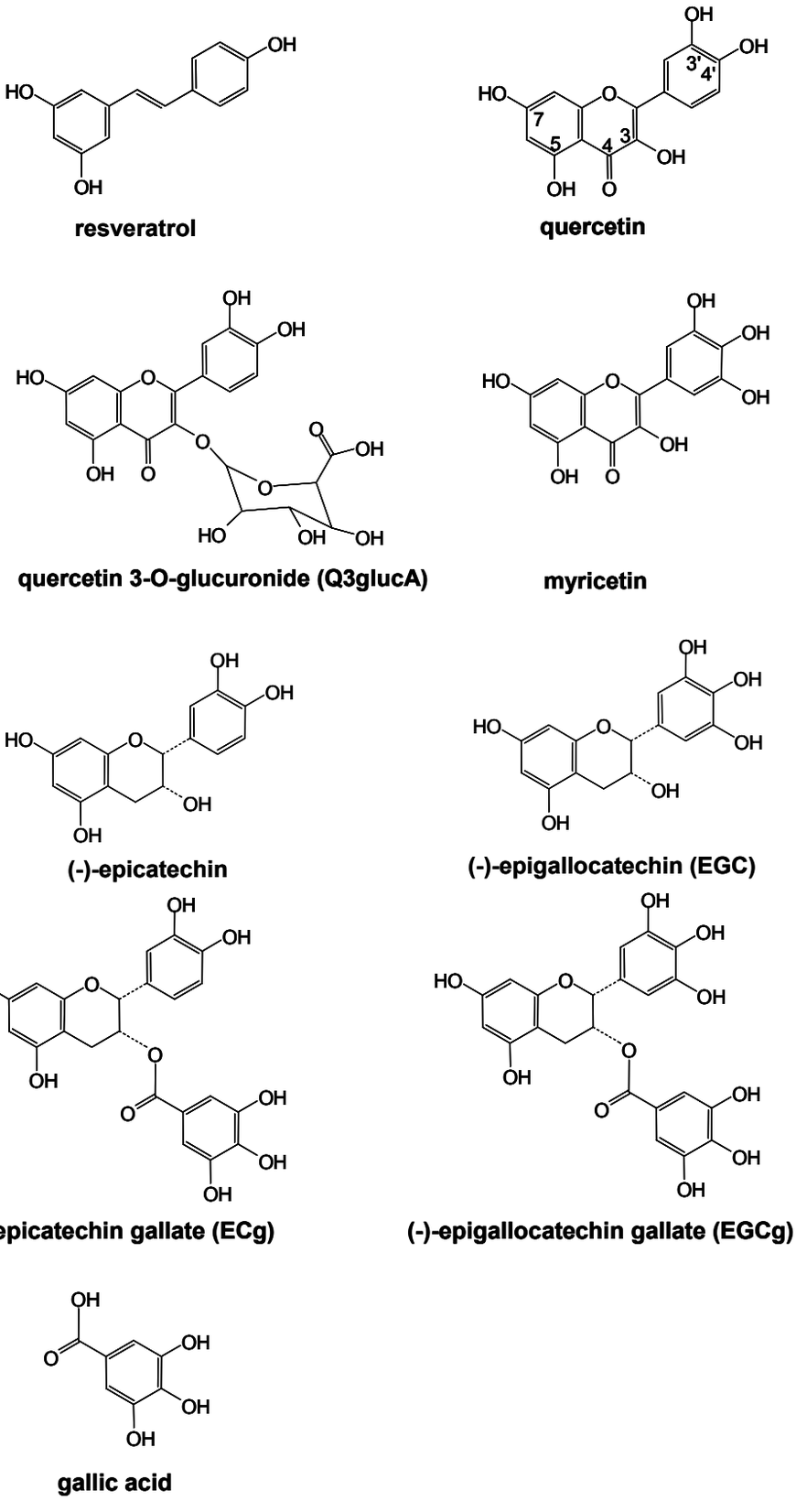

Figure 6.1
Molecular structures of analyzed polyphenols

EXPERIMENTAL PROCEDURES

Materials

Quercetin, resveratrol, myricetin, nicotinamide, catalase, 7-amino-4-methylcoumarin (AMC), (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECg), (-)-epigallocatechin gallate (EGCg) and gallic acid were purchased from Sigma-Aldrich. Isorhamnetin was purchased from Roth, tamarixetin from Extrasynthese and Quercetin 3-O-β-glucuronide from Apin Chemicals. Human recombinant SIRT1, Fluor de Lys-SIRT1 deacetylase substrate, Fluor de Lys Developer II 5x concentrate, NAD+ and Fluor de Lys deacetylated standard were purchased from Biomol. All chemicals used were of analytical grade.

Cell culture

HT29 colon carcinoma cells (ATCC) were cultured in DMEM (D5648, Sigma-Aldrich) with 5% fetal bovine serum, 25 mM HEPES, penicillin, streptomycin and non-essential amino acids in a humidified 37 ^{o}C incubator with 5% CO_2. Cells were subcultured once a week and medium was refreshed once a week. For SIRT1 incubation experiments, ~$5 \cdot 10^3$ cells/well were plated in a 96-wells tissue culture plate one day before the start of the incubation. For quercetin uptake experiments cells were plated at ~$3 \cdot 10^4$ cells/well in a 6-wells tissue culture plate.

SIRT1 activity: overview

The Biomol SIRT1-assay is based on the deacetylation by SIRT1 of a synthetic substrate (Fluor de Lys-SIRT1 substrate), consisting of four amino acids with one acetylated lysine group (Arg-His-Lys-Lys(Ac)) and a fluorochrome (7-amino-4-methylcoumarin, AMC). After deacetylation, the fluorochrome is specifically released only from the deacetylated substrate by adding Developer II. To analyze the effects of polyphenols on deacetylation by the recombinant SIRT1 protein and intracellular deacetylation, we adjusted the Biomol method. We substituted the original detection with a fluorescence plate reader, with a more specific HPLC-fluorescence method to quantify AMC release.

SIRT1 activity: recombinant SIRT1

The assay was performed in a total volume of 50 μl in assay buffer (25 mM Tris/HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mg/ml BSA) with 0.5 – 1U of SIRT1 protein (depending on different activities of different protein batches). The concentration of the synthetic substrate was 25 μM and the NAD+ concentration was 500 μM. Final concentrations of

all polyphenols were 100 μM (levels of organic solvent (DMSO or methanol) did not exceed 1%). Assays were performed with or without 1 mM ascorbic acid, 100U/ml catalase or a combination of both. Before incubation, polyphenols were mixed with SIRT1 and were preincubated at 37 °C for 10 minutes. Substrate solution was also prewarmed for 10 minutes at 37 °C. Incubation was started by combining the substrate with the SIRT1 and polyphenol solution. Control samples were incubated with 1% solvent and without polyphenols. Blank samples (without SIRT1 protein and polyphenols and with 1% solvent) were taken along as well. Three replicates of each incubation were performed in each experiment. After 15 minutes the incubation was stopped by addition of 50 μl 0.5x developer II concentrate with 2 mM of the SIRT1 inhibitor nicotinamide in assay buffer. The samples were allowed to develop for 15 minutes at room temperature with additional shaking (~500 rpm). Samples were further processed for HPLC analysis by adding 200 μl acetonitrile and 100 μl 20%-H_3PO_4 after which the samples were centrifuged for 10 minutes at 10000rpm. The supernatant was injected on the HPLC system.

SIRT1 activity: intracellular SIRT1

For analysis of intracellular SIRT1 activity, HT29 cells were incubated with 25 μM substrate, 1 μM trichostatin A (TSA, to inhibit class I and II histone deacetylases) and 1 mM ascorbic acid in DMEM with 25 mM HEPES, 1% non-essential amino acids and 1% penicillin/streptomycin but without FBS. Expression of SIRT1 in HT29 cells was confirmed by PCR (data not shown). Polyphenols were incubated at a concentration of 100 μM. Incubations were performed for 4 hours in a 37 °C tissue culture incubator. After incubation, the conversion of substrate to deacetylated substrate was analyzed in the exposure medium. Intracellular levels of deacetylated substrate were analyzed as well (cell processing was similar to the intracellular quercetin analyses, see below), and were about 40 times lower than medium concentrations. Apparently the deacetylated substrate is efficiently transported out of HT29 colon cells. Therefore medium concentrations were taken as a marker for intracellular substrate conversion. Control incubations were performed without cells and 8 to 32 replicates of each incubation were performed in each experiment. The same developer reaction to release the fluorochrome was performed as in the recombinant SIRT1 assay with slight modifications. Twenty-five μl of medium sample was incubated in a 96 well pcr plate (preplugged tubes plate, Bioplastics) with 25 μl 0.5x developer II concentrate with 2 mM nicotinamide in assay buffer for 15 minutes on a well plate shaker at 600

rpm at room temperature. With the addition of 100 µl acetonitrile and 50 µl 20%-H_3PO_4 the developer reaction was stopped and the protein was precipitated by centrifugation at 1780×g. The supernatant was transferred to a 96-well Collection Plate (Captiva, Varian) and injected on the HPLC system. Total protein levels of all wells were analyzed with the Biorad protein assay reagent, based on the Lowry method. No differences in protein levels between control and polyphenol incubations were seen after 4 hours of exposure.

Analysis of quercetin uptake and metabolism

HT29 cells were exposed to 10 µM quercetin in a 6-well tissue culture plate in the presence of 1 mM ascorbic acid in DMEM with 25 mM HEPES, 1% penicillin/streptomycin and 1% non-essential amino acids and 5% fetal bovine serum and incubated at 37 ^{o}C. A time course of exposure was performed up to exposures of 24 hours. After exposure, cells were washed with HBSS, trypsinized and the cell pellet was dissolved in 100 µl miliQ water. The cells were lysed with four freeze-thaw cycles in liquid nitrogen and a 37 ^{o}C water bath. The method for further processing of samples for HPLC injection was based on the analysis of quercetin in plasma [49, 50].

HPLC analysis of AMC

The HPLC system consisted of 2 Merck Hitachi LaChrom Elite L2130 pumps (Hitachi) and a LaChrom Elite L2200 injector, with an insert for injection of 96-well plates. Separation was achieved by injecting 20 µl sample onto an Inertsil column (150 mm x 4.6 mm, 5 µm, Alltech), protected by a NewGuard RP18 guard column (15.0 mm x 3.2 mm, 5 µm, Perkin Elmer) with a flow of 1 ml/min. The solvent for elution was 30% acetonitrile in 10 mmol/L phosphate buffer, pH 6.9. Total run time was 7.5 minutes. Peaks were detected with a fluorescence detector (FP-920, Jasco) set at 347 nm excitation and 440 nm emission wave lengths. Samples were compared to a standard curve of AMC in 50% acetonitrile and 5%-H_3PO_4 and corrected by subtracting the AMC levels in blank samples. Fold stimulation by polyphenols was calculated by comparing the AMC levels in the polyphenol treated samples with the control samples.

HPLC analysis of quercetin

The same HPLC system was used for quercetin analysis as for AMC analysis. Separation was achieved by injecting 20 µl sample onto a Chromolith RP-18e column (100.0 x 4.6 mm, Merck), protected by a NewGuard RP18 guard column (15.0 mm x 3.2 mm, 5 um, Perkin Elmer). Columns were housed in a column heater (ESA) set at 30°C. The solvents

for elution were 5% acetonitrile in 25 mmol/l citrate buffer, pH 3.7 (solvent A) and 70% acetonitrile in 25 mmol/l citrate buffer, pH 3.7 (solvent B). The elution program at a flow rate of 2.5 ml/min was as follows: 0–12 min, linear gradient from 0 to 43% B; 12-13 min, from 43 to 100%; 13-14.5 min, isocratic at 100% B; 14.5-15.5 min, linear return to 0% B. Total run time was 17 min. Peaks were detected with a coulometric array detector (Coularray detector model 6210; ESA) set at 75, 250, and 500 mV (Pd as reference). Quercetin was quantified at the lowest potential (75 mV).

Statistical tests

Power calculations were performed to calculate the number of samples needed to detect a significant difference in SIRT1 deacetylation activity between quercetin treated cells and control cells. The expected intracellular fold stimulation of SIRT1 by quercetin (1.19) was calculated by using the intracellular fold stimulation of SIRT1 by resveratrol (1.65), and the ratio of the fold changes found in the recombinant assay. A significant change (β= 0.8, α= 0.05) between control samples and quercetin treated cells could have been found with a sample size of n=32. P values were calculated with a t-test.

RESULTS

Specific HPLC method for analyzing SIRT1 activity

Polyphenols and synthetic fluorescent substrates can potentially interfere with the analysis of a fluorochrome in a deacetylation assay. We therefore developed a method that analyzed the deacetylase activity of SIRT1 more specifically than other methods that are based on the release of a fluorochrome. In the published Biomol assay [22], we added a protein precipitation step and analyzed the samples by HPLC with fluorescence detection. With this method we could separate resveratrol and the fluorescent substrates from the fluorochrome (AMC). Resveratrol clearly had a higher retention on the column than AMC (figure 6.2A and 6.2B), therefore interference by resveratrol was eliminated. The identity of the first peaks in the chromatograms (figure 6.2A and 6.2B) was confirmed by injecting substrate or deacetylated substrate standards without any reaction. The other polyphenols also did not interfere with AMC analysis (data not shown). Recovery of spiked AMC in the recombinant and intracellular assay was 98%-100%. This method allowed us to analyze the influence of polyphenols on the deacetylation activity of recombinant SIRT1 and intracellular SIRT1, without interference by autofluorescence from the

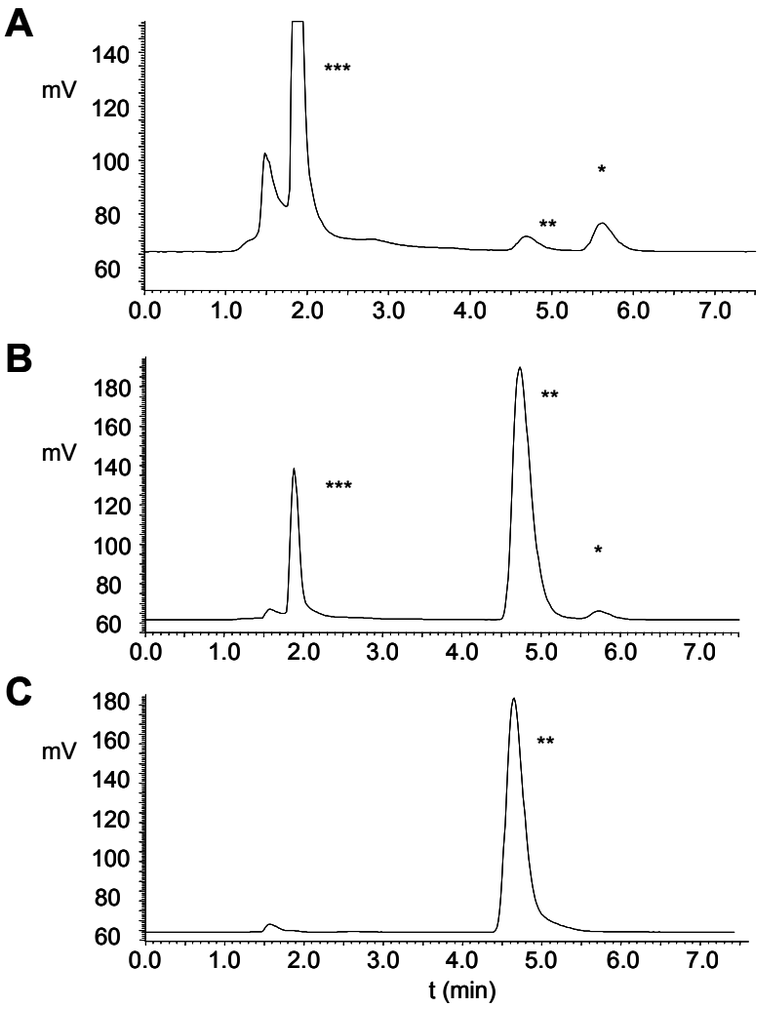

Figure 6.2
HPLC chromatograms of a typical recombinant SIRT1 protein assay. Blank sample 15 minutes after start of incubation with substrates and resveratrol but without SIRT1 (A), resveratrol stimulated SIRT1 sample 15 minutes after start of incubation with SIRT1, substrates and resveratrol (B) and standard of 62.5 nM AMC (C). The samples were taken from a reaction where SIRT1 (1U) was incubated with 100 μM resveratrol with 25 μM substrate and 500 μM NAD+. * = resveratrol, ** = AMC and *** = substrate and deacetylated substrate peaks.

polyphenols and without high background fluorescence from the synthetic substrate.

Polyphenols influence recombinant SIRT1 activity directly and indirectly

To determine the effects of polyphenols on SIRT1 deacetylase activity, we used the recombinant SIRT1 protein. Because several of the tested polyphenols are known to be unstable in aqueous solution we conducted

experiments with and without addition of vitamin C or catalase. Addition of vitamin C did not change the effect on SIRT1 activity significantly for resveratrol, quercetin, EC and ECg (table 6.1). However, a striking finding was that EGCg, EGC, gallic acid and myricetin inhibited the activity of SIRT1 when the compounds were incubated without any stabilizing agents (table 6.1). For example, the substrate deacetylation was approximately 25 times lower in the presence of EGCg than in the control reaction. Addition of 100U/ml catalase negated the inhibitory effect of EGCg, whereas addition of 1 mM vitamin C resulted in stimulation of SIRT1 (figure 6.3A). This indicates that the inhibition probably is an indirect effect caused by oxidation products of EGCg or by H_2O_2. To investigate whether formation of oxidation products of EGCg or H_2O_2 formation was responsible for the inhibition of SIRT1 activity, we preincubated EGCg for half an hour without vitamin C, catalase, SIRT1 or substrate. SIRT1 and substrate were incubated afterwards with the preincubated solution with and without addition of stabilizing agents. Stabillizing agents again eliminated the inhibitory effects of polyphenols on SIRT1 activity (figure 6.3B). This suggests that H_2O_2 formation is the major process that caused the activity

Table 6.1
Modulation of SIRT1 activity by polyphenols (100 μM) with and without addition of 1 mM vitamin C. + = stimulation, o = no effect, - = inhibition

	+ vitamin C				**- vitamin C**			
	Fold change	**SD**	**n**	**Effect**	**Fold change**	**SD**	**n**	**Effect**
Polyphenols where addition of vitamin C does not change the SIRT1 effect								
resveratrol	4.66	0.60	5	+	5.18	0.70	12	+
quercetin	2.15	0.62	11	+	2.54	0.11	3	+
ECg	1.85	0.35	6	+	1.91	0.26	6	+
EC	0.99	0.06	3	o	1.09	0.25	6	o
Polyphenols where addition of vitamin C does change the SIRT1 effect								
EGCg	1.90	0.40	6	+	0.04	0.02	12	-
EGC	0.91	0.20	5	o	0.41	0.11	6	-
myricetin	3.19	0.61	9	+	0.09	0.03	3	-
gallic acid	1.02	0.07	6	o	0.44	0.06	6	-

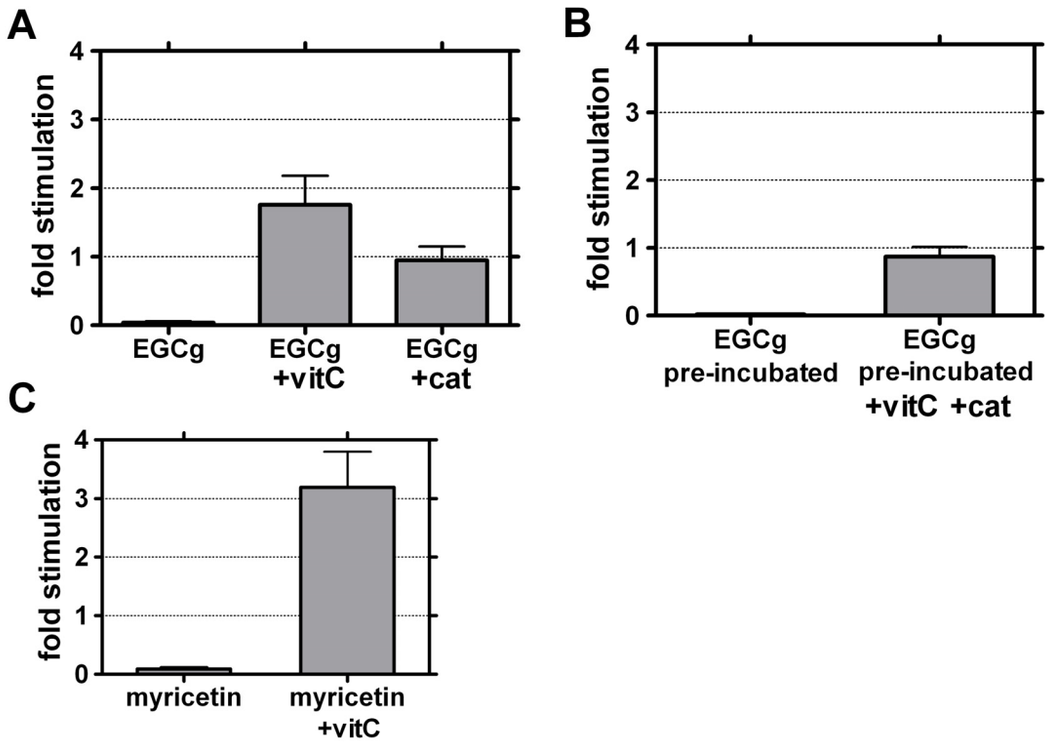

Figure 6.3
Fold stimulation of the activity of the recombinant SIRT1 protein incubated under several conditions with polyphenols. SIRT1 incubated with and without stabilizing agents and EGCg (A), preincubated EGCg (B) and myricetin (C). All reactions were performed for 15 minutes with 0.5-1U SIRT1, 25 μM substrate, 500 μM NAD+, 100 μM polyphenol and with or without 1 mM vitamin C (+vitC) or 100 U/ml catalase (+cat). EGCg pre-incubated = preincubation (30 min) with 100 μM EGCg without addition of stabilizing agents, substrates and SIRT1 protein.

of the protein to decrease. H_2O_2 formation during EGCg incubations did not influence the developer reaction or AMC stability (data not shown). Myricetin, gallic acid and EGC also inhibited SIRT1 activity when no vitamin C was added. With addition of vitamin C, myricetin stimulated SIRT1 3.19 +/- 0.6 fold (average +/- SD, figure 6.3C). All further incubations were done with the addition of 1mM vitamin C.

Several tested polyphenols influenced the deacetylation activity of the recombinant SIRT1 protein. Resveratrol was the best stimulator of SIRT1 activity; it increased the deacetylation activity 5.03 +/- 0.7 fold (figure 6.4). The two flavonols, quercetin and myricetin, stimulated SIRT1 activity by 2.15 +/- 0.62 and 3.19 +/- 0.61 fold respectively. With the proper incubation conditions, catechins conjugated to a gallic acid group (EGCg

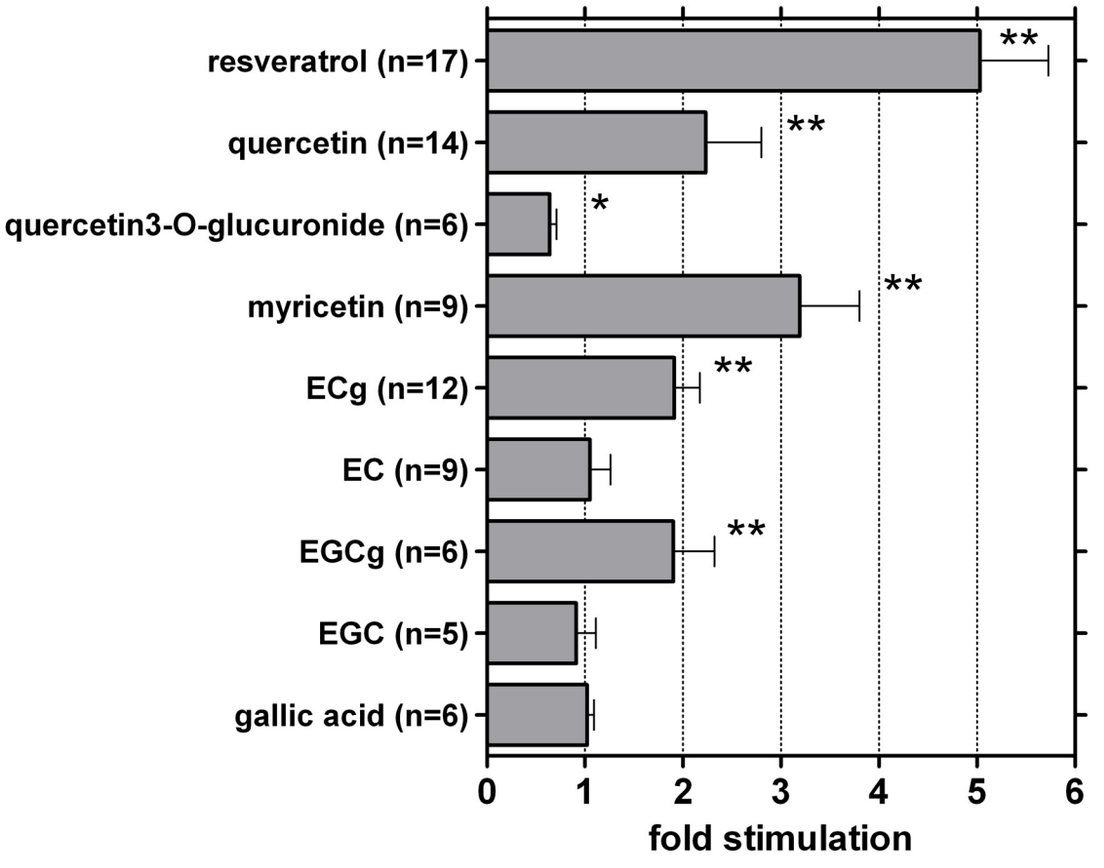

Figure 6.4
Fold stimulation of the recombinant SIRT1 deacetylase activity by polyphenols. For the polyphenols where addition of stabilizing agents did not result in a significant change in SIRT1 stimulation, both results from experiments without and with stabilizing agents were used. All reactions were performed for 15 minutes with 0.5-1U SIRT1, 25 μM substrate, 500 μM NAD+, 100 μM polyphenol and 1 mM vitamin C. n = number of replicates. * = $p<0.05$, **=$p<0.01$ meaning a significant difference from control incubations without polyphenols. protein.

and ECg) stimulated SIRT1 activity significantly (EGCg: 1.76 +/- 0.42 fold, ECg: 1.85 +/- 0.35 fold). Epicatechin (EC) and epigallocatechin (EGC) had no effect on SIRT1 activity. Also gallic acid itself had no effect on SIRT1 activity. Because most polyphenols are rapidly metabolized in epithelial cells before entering the blood, it is of relevance to test metabolites of these compounds to better simulate the effects of polyphenols in an *in vivo* system. In contrast to the stimulating effect of quercetin, one of the metabolites of quercetin (quercetin 3-O-β-glucuronide) did not show a stimulatory effect. In fact, a slight, but significant, inhibition of SIRT1 activity was seen for quercetin 3-O-β-glucuronide (figure 6.4).

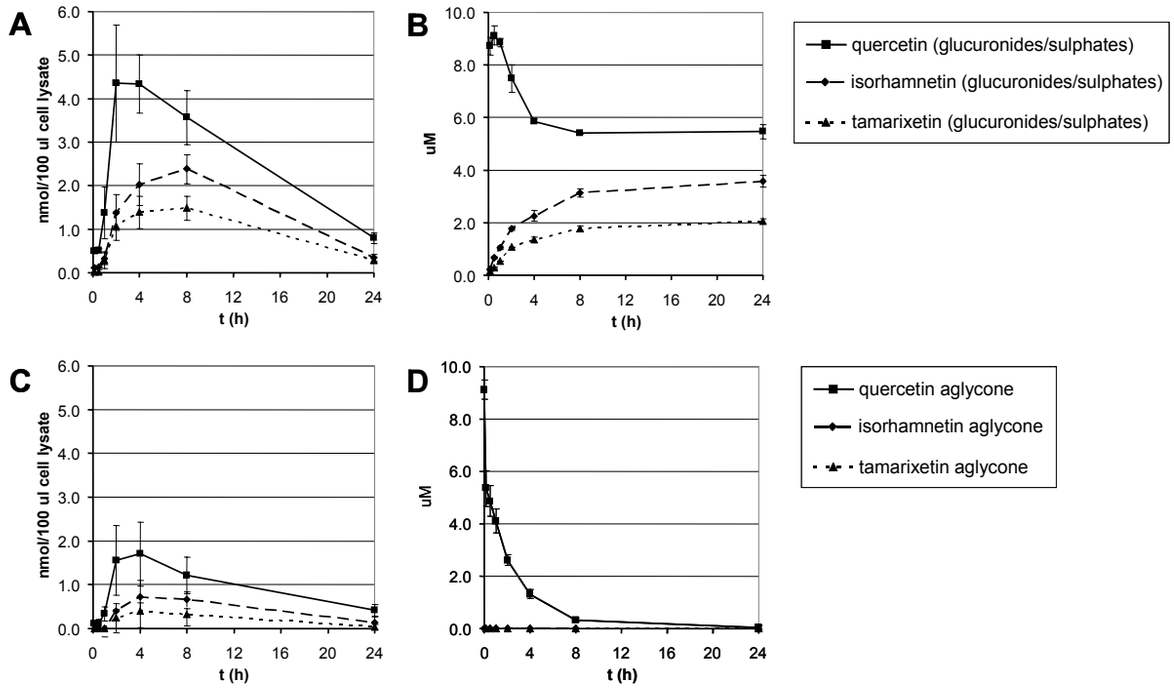

Figure 6.5
Quercetin uptake in HT29 colon carcinoma cells. Quercetin uptake was analyzed in incubation medium (B, D) and cell lysate (A, C) after exposure up to 24 hours to 10 μM quercetin with 1 mM vitamin C. Panels A and B show levels of quercetin, isorhamnetin (3'-OCH3-quercetin) and tamarixetin (4'-OCH3-quercetin) aglycone and glucuronides/sulfates in cell lysate and medium. Samples are enzymatically hydrolyzed with glucronidase/sulfatase before extraction. Panels C and D show levels of quercetin, isorhamnetin and tamarixetin aglycones in cell lysate and medium. Samples are not hydrolyzed before extraction.

Polyphenol effects on SIRT1 activity in HT29 cells

The stimulatory effects of polyphenols on the isolated SIRT1 protein are only relevant if these effects can be reproduced in an *in vivo* situation. Therefore we chose to analyze the effects of one of the stimulators (quercetin) in a cellular system (HT29 colon carcinoma cell line). Before determining the effects on SIRT1, we first analyzed the uptake kinetics and intracellular levels of quercetin. For this, HT29 cells were incubated with 10 μM quercetin and 1 mM vitamin C for up to 24 hour. We analyzed quercetin and quercetin metabolites in cellular extracts and incubation medium with and without enzymatic hydrolysis of the samples with a *Helix pomatia* enzyme mixture containing both sulfatase and glucuronidase activity. In this way, we could distinguish between aglycone forms and conjugated forms of quercetin, isorhamnetin (3'-O-methoxy quercetin) and tamarixetin (4'-O-methoxy quercetin). Quercetin was quickly taken up by

HT29 cells and immediately metabolized to methoxylated (isorhamnetin and tamarixetin) and glucuronidated/sulfated conjugates (figure 6.5A). The intracellular concentrations of total quercetin metabolites reached a maximum at 2 to 4 hours after start of exposure. After 24 hours only low levels of conjugated quercetin were left inside the cell (figure 6.5A). The levels of intracellular quercetin aglycone were much lower than conjugated quercetin and also peaked at 4 hours after start of exposure (figure 6.5C). The concentrations of free quercetin aglycone in the incubation medium quickly decreased (figure 6.5D) and the levels of quercetin metabolites in the medium increased till a steady-state was reached approximately 8 hours after start of exposure (figure 6.5B).

Because the maximum intracellular levels of quercetin aglycone and quercetin conjugates were reached 2 to 4 hours after start of exposure we chose to incubate HT29 cells for 4 hours with polyphenol and SIRT1 substrate. Resveratrol was taken as a positive control. Incubations of HT29 cells with resveratrol showed that the deacetylation activity was increased 1.65 fold in cells exposed to resveratrol (figure 6.6), indicating that the stimulation of deacetylation activity by resveratrol could also be reproduced in HT29 cells. In contrast, quercetin did not show any effect on the deacetylation activity of SIRT1 in HT29 cells (figure 6.6).

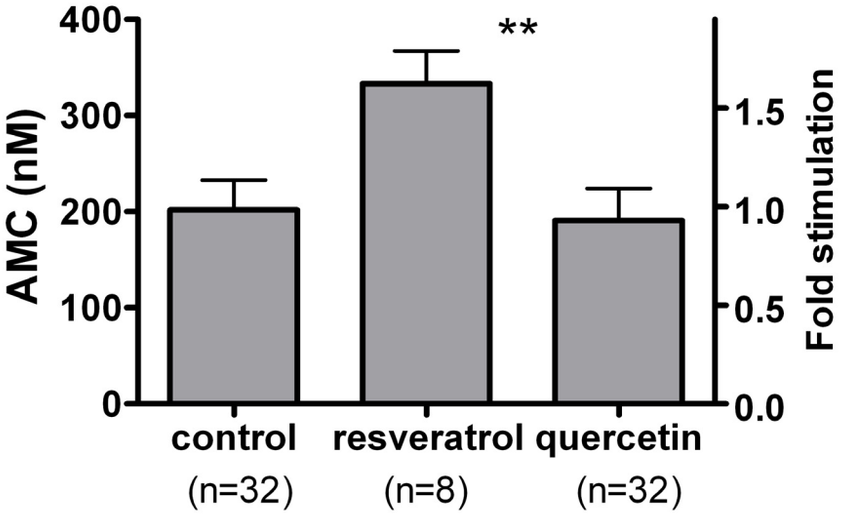

Figure 6.6
AMC levels and fold stimulation after exposure of HT29 cells for 4 hours to 25 μM substrate, 1 μM TSA and 100 μM polyphenol. AMC levels represent the deacetylase activity of intracellular SIRT1 protein. n = number of replicates. ** = $p<0.01$.

DISCUSSION

The inconsistent finding that polyphenols could stimulate the deacetylase activity of SIRT1 on the protein level, but not in a cellular system, prompted us to compare the effects of polyphenols on recombinant SIRT1 with the effects on SIRT1 deacetylase activity in metabolically active HT29 colon carcinoma cells. We show that intracellular activity of SIRT1 in HT29 cells is only stimulated by resveratrol and not by quercetin. This is probably due to rapid metabolism of quercetin because quercetin 3-O-glucuronide did not stimulate recombinant SIRT1. In this paper, we also show for the first time that the catechins EGCg and ECg and the flavonol myricetin can stimulate the activity of the recombinant SIRT1 protein, but only under the proper incubation conditions.

The stimulating effect of polyphenols on SIRT1 has been shown in Hela cells [22] and in drosophila S2 cells [37]. However, large differences were seen between polyphenolic effects on intracellular SIRT1 and on the isolated SIRT1 protein. For example, butein showed a 8.5 fold stimulation on the isolated protein, but did not have any effect in Hela cells [22]. On the other hand butein showed a stimulating effect both on the drosophila sir2 protein and in drosophila S2 cells [37]. Intracellullar effects of quercetin on SIRT1 were not found, whereas effects on isolated sir2 proteins were high; 4.6 fold on human SIRT1 [22] and 1.25 fold on C. elegans sir2.1 [37]. We found a 2.15 fold stimulation of recombinant SIRT1 deacetylase activity by quercetin (figure 6.4). However, no effect was seen on intracellular SIRT1 activity in quercetin exposed HT29 cells. We used colon cells because these cells are readily exposed to polyphenols via the diet. Quercetin was conjugated in HT29 to glucuronides and immediately transported out of the cell, as was shown by high concentrations of quercetin glucuronides in the medium. Additional metabolites of quercetin found in the medium of quercetin exposed HT29 cells were methoxylated quercetin (isorhamnetin and tamarixetin), these metabolites were also conjugated to glucuronides. Van der Woude *et al.* [51] showed that the major metabolites formed by HT29 cells were quercetin 3-O-glucuronide and quercetin 4'-O-glucuronide. Quercetin 3-O-glucuronide is also one of the major quercetin metabolites in human plasma [52] and is formed in the intestine and liver by UDP glucuronosyl transferases [53]. In contrast to the stimulating effect of quercetin on SIRT1, quercetin 3-O-glucuronide did not have a stimulating effect on the deacetylase activity of SIRT1, even a slight inhibition of activity was seen. This difference between the effects of

a major quercetin metabolite and quercetin itself may explain the lack of stimulating effect of quercetin in a cellular system.

Resveratrol was able to stimulate the activity of SIRT1 in HT29 cells by 1.65 fold. This relatively low stimulation of intracellular SIRT1 activity as compared to the effects of resveratrol on the recombinant protein, points to an effective metabolism or low uptake of resveratrol in HT29 cells. Uptake and metabolism studies of resveratrol in hepatocytes and Caco-2 cells [54, 55] showed that resveratrol was conjugated to sulfates and glucuronides and effectively transported out of the cell. However, some accumulation of resveratrol in Caco-2 cells was also found [55]. A higher potency and higher accumulation than quercetin probably explains why resveratrol is able to stimulate intracellular SIRT1, whereas quercetin is not.

Apart from the metabolism of polyphenols, stability of polyphenols in incubation medium can influence the effects of polyphenols on SIRT1 activity. Polyphenols are antioxidants and are readily oxidized in aqueous media [39]. Several polyphenols oxidize and form hydrogen peroxide. EGCg and gallic acid (200 μM) produce up to ~650 μM H_2O_2 when incubated for 1 hour in HEPES buffered DMEM medium. When these compounds are incubated in combination with an excess vitamin C, the H_2O_2 production is decreased to ~100 μM [56]. In our experiments EGC and gallic acid inhibited the activity of SIRT1 slightly when no vitamin C was added, whereas after stabilization with vitamin C, EGC and gallic acid did not have an effect (table 6.1). The two other compounds with three hydroxyl groups in the B-ring, EGCg and myricetin, almost completely inhibited the activity of SIRT1 without vitamin C. When stabilized with vitamin C, EGCg and myricetin stimulated the activity of the recombinant SIRT1 protein (figure 6.3). Wee *et al.* [56] showed that vitamin C could prevent the breakdown of gallic acid in culture medium, whereas catalase could not. We found similar results with EGCg (figure 6.3A); EGCg in combination with catalase did not stimulate recombinant SIRT1, whereas EGCg stabilized with vitamin C stimulated SIRT1. Howitz *et al.* [22] found a slight inhibition of SIRT1 activity by myricetin (0.89 fold) and a more pronounced inbition by EGC (0.41 fold) and EGCg (0.32 fold), indicating that Howitz did not use stabilizing conditions when incubating polyphenols with SIRT1.

Both ECg and EGCg stimulated the activity of recombinant SIRT1, while EC and EGC were not able to stimulate SIRT1. Thus the attached gallic acid group seems to be necessary for a stimulating activity of catechins. An advantage of this gallolyation of the polyphenolic backbone is that these polyphenols are hardly conjugated to glucuronides and sulfates in the

human body. Therefore, EGCg is present in human plasma for 77-90% in its free form [57], whereas other polyphenols, like resveratrol, myricetin and quercetin, are conjugated to glucuronides and sulfates. Tissues, other than gastro-intestinal tract tissues, are also mainly exposed to conjugates of resveratrol [58] and quercetin [59], while EGCg was found in tissues in its unconjugated form [60]. This may have a significant impact on the bioactivity of EGCg in the human body.

Recent studies have shown that the activation of recombinant SIRT1 by resveratrol only occurs when a fluorochrome is attached to the synthetic substrate, whereas without the fluorochrome resveratrol did not stimulate SIRT1 [41, 61]. Nevertheless, resveratrol has been shown to be a stimulator in sirtuin dependent processes extensively [10, 11, 22, 37]. Secondly, Sir2 proteins do not seem to discriminate their substrates on the basis of sequence, but by conformation [62]. The conformation of the fluorescently tagged substrate may resemble the conformation of an *in vivo* substrate better than substrates without a fluorochrome. This suggests, in combination with the fact that only SIRT1 and not the other human sirtuins deacetylate p53-based substrates *in vitro* [63], that the Biomol substrate may be well suited for analysis of SIRT1 activity. Resveratrol increases the affinity of the substrate for the protein probably by inducing a conformational change in the SIRT1 protein [41]. Whether other polyphenols, like EGCg and quercetin, act in the same way as resveratrol is not yet established, but is likely.

With our improved method for determining polyphenolic modulation of recombinant and intracellular SIRT1 deacetylase activity, we were able to identify three new polyphenolic stimulators of SIRT1 deacetyase activity, ECGg, ECg and myricetin. Furthermore, we found that probably the metabolism of polyphenols is responsible for diminished or absent SIRT1 modulating activity in a human cellular system as compared to the effect of the polyphenol on the isolated protein. Therefore, extrapolation of *in vitro* SIRT1 stimulation results to physiological effects should be done with caution.

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ABBREVIATIONS

sir2, silent information regulator 2, SIRT1, silent information regulator two ortholog 1, EGCg, (-)-epigallocatechin gallate; AMC, 7-amino-4-methylcoumarin; EC, (-)-epicatechin; EGC, (-)-epigallocatechin; ECg, (-)-epicatechin gallate; NAD, nicotinamide adenine dinucleotide; TSA, trichostatin A.

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CHAPTER 7

General Discussion

IDENTIFICATION OF FUNCTIONAL EFFECTS OF POLYPHENOLS

Epidemiological studies suggest that selected dietary polyphenols can be protective in development of cardiovascular heart diseases in humans and mechanistic studies demonstrate that polyphenols possess beneficial properties in *in vitro* and animal model systems. For these reasons polyphenols gained a lot of attention and are currently sold as food supplements containing high amounts of polyphenols (up to 50 fold of the average dietary intake). In contrast to the extensive testing that drug therapies undergo before they are allowed to be used in the clinic, food supplements do not need to undergo these extensive trials. Therefore, adequate data for safety assessment are often lacking. Furthermore, limited knowledge mostly from *in vitro* studies on possible mechanisms of action of polyphenols is often used to claim multiple beneficial effects of polyphenols on human health. This extrapolation is often misleading and claims cannot be substantiated unless additional *in vivo* research is conducted. Furthermore, epidemiological studies can only hint on possible interactions between dietary intakes of nutritional compounds and disease risk, whereas mechanisms of action are not considered. Therefore, to explain data from epidemiological studies, mechanistic *in vivo* and relevant *in vitro* studies should be performed as well.

Our approach to identify *in vivo* functional effects of polyphenols was based on elucidation of tissue targets and unbiased exploration of alterations in rat physiology by using transcriptomics techniques. The study of tissue bioavailability of polyphenols is of particular importance, because polyphenols are extensively metabolized and evidence for polyphenol availability to tissue targets is limited. Based on our finding that the highest quercetin metabolite concentrations were found in the lungs (chapter 4) and the fact that dietary intake of quercetin is likely to result in exposure of the gastro-intestinal tract, we selected lungs and colon as target organs for elucidation of mechanism of action of quercetin. A chronic high quercetin diet was used to mimic the continuous dietary intake of quercetin in humans (chapter 3 & 4). Furthermore, individual animals were used to analyze large scale gene expression to take biological significance of the quercetin intervention into account instead of technical significance, which is the case when samples are combined for microarray cost reduction.

The functional effects observed in the *in vivo* studies were further explored by using cell culture models: HT29 colon carcinoma cells (chapter 6) and 3T3-L1 adipocytes (chapter 5). Appearance of the aglycone in *in vivo*

target tissues might be possible (explained below). However, phase II metabolites are much more abundant in *in vivo* target tissues (chapter 2). Therefore, in our *in vitro* studies we included an important human metabolite of quercetin, quercetin 3-O-glucuronide, to obtain relevant effects of a possible *in vivo* bioactive form of quercetin. Using this approach we were able to identify relevant *in vivo* mechanisms of action of polyphenols. In this chapter, we will discuss the experimental findings, propose potential mechanisms underlying the effects of polyphenols on energy metabolism and suggest future studies that will strengthen the hypotheses generated in this thesis.

QUERCETIN ALTERS *IN VIVO* ENERGY METABOLISM

To assess the effects of a chronic quercetin diet, we fed rats a high quercetin diet (~500 mg/kg BW/day) for 41 weeks. We selected lungs as target organ, because lungs were identified as the organ containing the highest levels of quercetin metabolites after chronic dietary quercetin supplementation (chapter 2). In addition, epidemiological studies indicated that a high flavonol diet is possibly protective against lung cancer [1]. By using whole genome oligo microarrays we were able to identify physiological processes that were affected by the chronic quercetin treatment. Among the genes that were most significantly differentially expressed between rats fed a quercetin and control diet, a majority coded for proteins involved in the catabolism of fatty acids. We verified and confirmed individual differentially expressed genes in rat lung by quantitative real time RT-PCR and found in addition that plasma levels of non-esterified free fatty acids (NEFA) were lower in rats treated with the high quercetin diet as compared to NEFA plasma levels in control rats (chapter 4).

Further support for a role of quercetin in altering processes related to energy metabolism comes from a similar study with mice fed dietary quercetin (0.62% quercetin aglycone). In contrast to our chronic 41 week exposure, this study only used a single oral dose of quercetin and analyzed large scale gene expression differences between quercetin and control mice in liver and intestine 24 hours after supplementation. Several physiological processes that were significantly affected by the quercetin intervention were related to energy metabolism, in particular lipid metabolism and amino acid metabolism [2]. Additional indications for the energy metabolism altering effect of quercetin come from animal experiments using a high fat (HF) diet intervention in combination with polyphenols. Dietary

supplementation of quercetin 3-O-rhamnoglucoside (50 mg/kg BW/day) for 4 weeks attenuated body weight gain induced by a HF diet. Moreover, liver weight and plasma triglyceride levels were lower in HF diet fed mice supplemented with quercetin 3-O-rhamnoglucoside as compared to mice fed a HF diet without quercetin 3-O-rhamnoglucoside supplementation [3]. Lowering of plasma triglyceride levels was also seen in mice fed a HF diet supplemented orally with 1% quercetin for 2 weeks as compared to HF diet controls [4]. Although, body weight, liver weight and food intake did not differ between quercetin and control HF groups, the observed lowering of plasma triglycerides was accompanied by a decrease in expression and activity of lipogenic enzymes: fatty acid synthase, ATP citrate lyase and malic enzyme in liver after quercetin supplementation [4].

In the same animal experiment as our 41 weeks quercetin supplementation study, we investigated the effect of dietary quercetin on colon tumor formation induced by a colon carcinogen, azoxymethane (chapter 3). We identified a dose dependent decrease in the development of tumors in the colon with increasing dosages of dietary quercetin. Although, very diverse mechanisms can underlie the inhibition of carcinogenesis by quercetin, it is tempting to speculate on the role of energy metabolism in cancer cells. This speculation is supported by unpublished results that provide evidence for an alteration of metabolic pathways in the colon by the dietary quercetin supplementation (Dihal *et al.* unpublished results). RNA and proteins were isolated from colon scrapings of rats that received the 1% quercetin diet or control diet for 11 weeks. Both transcriptomic and proteomic data point to an alteration in metabolic pathways; e.g. glycolysis enzymes were downregulated and beta oxidation gene expression was upregulated.

For long it has been known that cancer cells have increased rates of glycolysis and an impairment of mitochondrial respiration (first described by Warburg [5]). Normal cells convert glucose to pyruvate, which can be utilized in mitochondria by the tricarboxylic acid (TCA) cycle for generation of NADH and concomitant aerobic ATP production by oxidative phosphorylation. The predominant pathway for energy production in cancer cells is the anaerobic production of ATP by shuttling glucose through the glycolysis pathway to be converted to lactate in the cytoplasm [6]. Downregulation of enzymes involved in the glycolytic pathway by quercetin in the colon of rats exposed for 11 weeks to dietary quercetin is suggestive of a shift towards a cellular phenotype resembling that of normal cells instead of cancerous cells.

In summary, alteration of *in vivo* cellular physiology by dietary quercetin is reflected by changes in levels of lipid metabolism parameters and changes in gene expression profiles of metabolic pathways that regulate the consumption and production of energy rich substrates. Relevant molecular mechanisms underlying the observed *in vivo* alteration of energy metabolism by quercetin are difficult to identify, because of the discrepancy between *in vitro* mechanistic studies and *in vivo* dietary supplementation studies. Mechanisms of action generated from *in vitro* studies are based most of the time on effects of quercetin aglycone. However, lack of evidence for the presence of quercetin aglycone at the molecular target after *in vivo* dietary supplementation yields these *in vitro* studies possibly not physiologically relevant. Because the evidence for *in vivo* alterations in energy metabolism by quercetin is substantial, the question remains: How can dietary quercetin exert its *in vivo* effect? We aimed to answer this question by studying *in vitro* effects of both quercetin aglycone and quercetin metabolites. This rationale and possible mechanisms of action of quercetin will be further outlined in the next sections.

NATURE OF THE BIOACTIVE COMPOUND: POLYPHENOL AGLYCONE OR POLYPHENOL METABOLITE?

Experimental findings

In general, an orally absorbed dose of polyphenols is metabolized relatively fast. Therefore, most polyphenols, with the exception of certain catechins and isoflavones [7], occur predominantly in plasma and tissues as polyphenol conjugates containing methoxy, sulfate and/or glucuronide groups. We analyzed the levels of quercetin metabolites in tissues after chronic dietary exposure of rats to quercetin for 11 weeks (chapter 2). The degree of conjugation of quercetin in tissues was assessed by analyzing tissue samples before and after enzymatic hydrolysis with exogenously added glucuronidase and sulfatase during extraction. In general, quercetin was detected as quercetin conjugates but we identified quercetin aglycones in tissue extracts of lung, kidney and liver as well. These aglycones, however, could possibly be formed during extraction, because of high endogenous tissue levels of proteins with glucuronidase activity. Standard addition of quercetin 3-O-glucuronide to tissue samples before extraction resulted in deconjugation of spiked quercetin 3-O-glucuronide during extraction, as was shown by the formation of quercetin aglycone. We thus identified high glucuronidase activity in lungs, liver and kidney, but low glucuronidase

activity in brain, muscle and bone (chapter 2). These results have two implications for the identification of aglycones in tissues. First, detection of aglycones in tissue samples can possibly be a result of deconjugation of polyphenol glucuronides by glucuronidase during extraction, and second, high levels of endogenous glucuronidase levels in tissues may enable aglycone formation at the target site *in vivo*.

In tissue distribution studies of polyphenols by Abd El Mohsen *et al.* [8-10], naringenin, resveratrol and pelargonidin were identified as aglycones in tissues after an oral dosing of radiolabeled compound. Deconjugation of glucuronides during extraction was checked for naringenin only, using purified naringenin glucuronide standards. No deconjugation of the standard was found in control tissue samples [9]. This suggests that naringenin was present as aglycone in tissues and that endogenous glucuronidase levels were low or even absent. The authors speculated that the high single dose of polyphenol saturated the capacity of the UDP-glucuronosyl transferase activity of the rats to conjugate the polyphenols, giving rise to unconjugated polyphenols in tissues [9]. In a 6 week quercetin supplementation study in rats, Graf *et al.* [11] detected quercetin aglycones in tissues, but did not report a control experiment with standard addition of quercetin glucuronides to eliminate the possibility of glucuronide deconjugation during extraction. Therefore, these samples possibly contained high levels of endogenous enzymes with glucuronidase activity as was seen in our 11 week tissue distribution study in rats.

We also analyzed the *in vitro* cellular uptake of quercetin in colonic cells (chapter 6) and endothelial cells (unpublished results). In colonic cells, quercetin was rapidly methoxylated and glucuronidated intracellularly and transported out of the cell. Intracellular levels of both conjugated quercetin and quercetin aglycone peaked between 2 – 4 hours after start of exposure (chapter 6). Intracellular methoxylation occurred in endothelial cells as well, because we identified methoxylated quercetin in the medium after exposure to quercetin. However, endothelial cells did not glucuronidate quercetin (unpublished results). This implies that quercetin aglycone enters both endothelial and colonic cells in culture, but differences in cell type cause a difference in metabolism of the polyphenol. Both direct and indirect evidence for the uptake of polyphenol glucuronides has been reported [12-15]. Direct evidence for intracellular (or cell associated) levels of quercetin 3-O-glucuronide came from a study using differentiated PC12 neuronal cells [12]. Shirai *et al.* demonstrated low levels (1.5 pmol/million cells) of quercetin 3-O-glucuronide in extracts of PC12 cells exposed for 4 hours to 10 μM quercetin 3-O-glucuronide [12]. Indirect evidence showed that

methoxylated quercetin 3-O-glucuronide was detected in medium after exposure of mouse fibroblasts to quercetin 3-O-glucuronide, indicating that quercetin 3-O-glucuronide was metabolized intracellularly [13]. Hepatocytes exposed to quercetin 7-O-glucuronide converted quercetin 7-O-glucuronide intracellulary to quercetin 3'-sulfate [15]. In both experiments exposure medium did not contain detectable levels of quercetin aglycone, demonstrating that the glucuronide was taken up, metabolized and transported out of the cell [13, 15]. In addition, exposure of endothelial cells to hesperetin glucuronides and naringenin glucuronides resulted in (intra)cellular accumulation of the glucuronides of hesperetin and naringenin [16].

Mechanisms of action

The findings that polyphenol metabolites can be taken up by cells and that polyphenol aglycones can be either taken up by cells or generated intracellularly by deconjugating enzymes, provide three possible mechanisms by which polyphenols can exert their intracellular molecular effect (figure 7.1). In the first mechanism intracellular polyphenol glucuronide is the active compound (figure 7.1A). The polyphenol glucuronide has to be transported over the plasma membrane, and intracellular polyphenol glucuronide may alter cellular physiology (figure 7.1A). Second, the transported glucuronide may be deconjugated by intracellular enzymes with glucuronidase activity. In this way, polyphenol aglycone is released and may have intracellular effects (figure 7.1B). Third, the polyphenol glucuronide is extracellularly deconjugated by glucuronidases and quercetin aglycone is subsequently taken up into the cell, where it may alter cellular physiology (figure 7.1C). Additionally, intracellulary or extracellularly released aglycone can be metabolized intracellularly to polyphenol glucuronides that can exert their action in the cell. Furthermore, polyphenols can interact with extracellularly located targets or with targets in the cell membrane to trigger an intracellular response. For these interactions cellular uptake of the polyphenol is not essential. Therefore, these interactions will not be described in this section.

For all three mechanisms evidence is accumulating. Polyphenol conjugates have been shown to be bioactive compounds [17]. Therefore, if polyphenol glucuronides are able to be taken up by cells, mechanism A provides a process that could be feasible *in vivo*. In this mechanism the polyphenol metabolite would be the active compound. The physiological effect of the polyphenol metabolite can be different from their corresponding aglycones. We demonstrated that quercetin 3-O-glucuronide inhibited the activity of

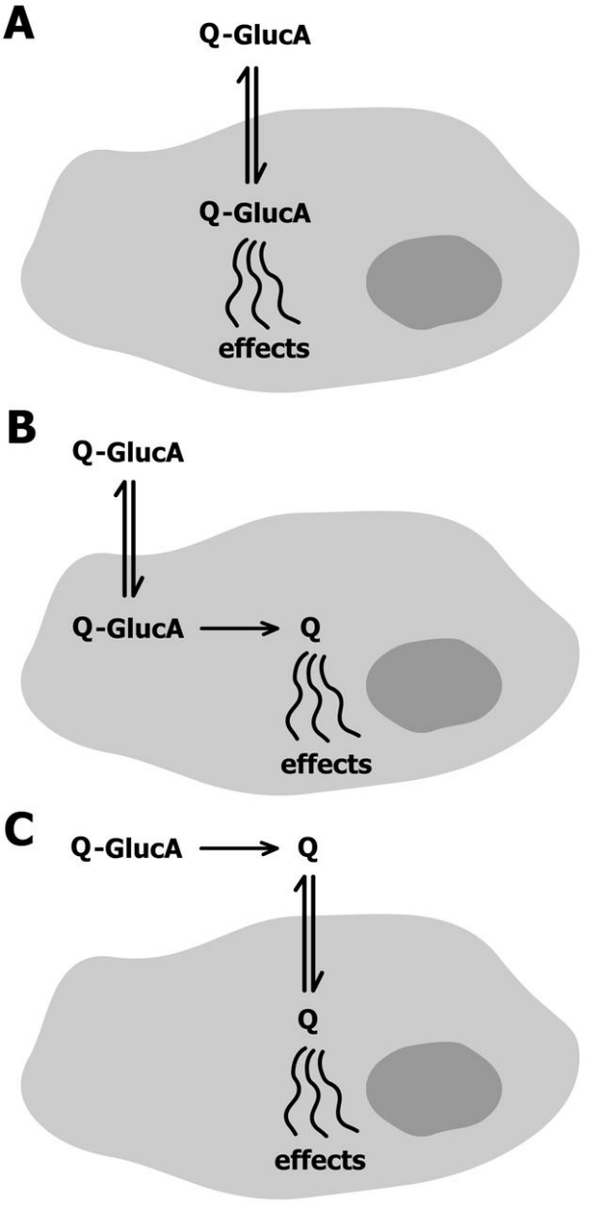

Figure 7.1
Mechanisms for the cellular availability of polyphenols to exert their intracellular molecular effect. Quercetin is used a model compound, but similar mechanisms hold true for other polyphenols that are conjugated to glucuronides or sulfates. Q-GlucA = Quercetin glucuronide and Q = quercetin aglycone. See text for explanations of mechanisms A, B and C.

recombinant SIRT1, whereas quercetin aglycone stimulated SIRT1 activity (chapter 6). In addition, we analyzed the effect of quercetin 3-O-glucuronide on mitochondrial respiration of cultured human lung cells (BEAS2B). Quercetin aglycone or quercetin 3-O-glucuronide (final concentration 100 μM) were injected into a closed chamber containing

continuously stirred lung cells and equipped with a polarographic oxygen sensor [18]. Quercetin aglycone instantaneously inhibited the rate of oxygen consumption in human lung cells, whereas quercetin 3-O-glucuronide did not affect the rate of oxygen consumption. This indicated that either quercetin 3-O-glucuronide was not taken up by cells or quercetin 3-O-glucuronide was ineffective in inhibiting mitochondrial respiration (unpublished results).

Efflux of polyphenol glucuronides out of liver and intestinal cells has been demonstrated. However, influx of polyphenol glucuronides has been hardly studied, probably due to the fact that polyphenol glucuronide standards are not commonly available. Efflux of polyphenol glucuronides has been demonstrated in intestinal cells to occur through MRP and BCRP transport proteins [19-21]. Glucose transporters from the SGLT and GLUT families have been suggested as influx transporters for polyphenol glucuronides [19, 22]. Influx of non polyphenolic glucuronides has been studied more extensively. Influx of estradiol 17-glucuronide into hepatic microsomes was studied in detail using radiolabeled estradiol 17-glucuronide. This study provided evidence for an ATP independent transport mechanism for estradiol 17-glucuronide influx into microsomes [23]. In addition, it was demonstrated that bilirubin monoglucuronide can be internalized into hepatic microsomes with concomitant glucuronidation to bilirubin diglucuronide [24].

The high activity of glucuronidases found in selected tissues of rats after feeding a chronic quercetin diet (chapter 2) implies that mechanisms B and C are possible *in vivo*. Mechanism B is dependent on intracellular deconjugating activity, whereas mechanism C relies on extracellular deconjugating activity. Mechanism C has been demonstrated for genistein glucuronides and luteolin glucuronide in conditions of neoplasm and inflammation, respectively. *In vivo* extracellular glucuronidases in necrotic areas of tumor lesions were suggested to be the cause of high levels of genistein aglycones at tumor sites in mice [25]. In addition, luteolin aglycone was formed when glucuronidases were released from immune cells [26]. Therefore, mechanism C may be particularly important in certain pathological conditions. That *in vivo* glucuronidase activity can activate glucuronides to corresponding aglycones has been demonstrated in cancer therapy development. Differences between levels of glucuronidase activity in normal and tumor tissue were used for development of a strategy to selectively deliver chemotherapeutic agents to tumor sites [27, 28]. Doxorubicin is used in the clinic as antitumor agent for the treatment of breast carcinomas and other carcinomas, but cardiotoxicity of doxorubicin

restricts the use of high dosages of doxorubicin [29, 30]. A glucuronide of doxorubicin was used as prodrug to selectively target doxorubicin to sites with high glucuronidase activity (i.e., necrotic areas of tumor lesions, where enzymes with glucuronidase activity are released into the extracellular environment due to cell necrosis) where the prodrug was activated by deglucuronidation to the corresponding aglycone [31, 32].

Mechanism B is less established in the field of polyphenol research than mechanism C. Only one study showed evidence for this mechanism of cellular absorption of the glucuronide and subsequent intracellular deconjugation [15]. Quercetin 7-O-glucuronide was taken up in cultured hepatocytes and further metabolized by deconjugation and sulfatation, yielding quercetin 3'-sulfate. Whether quercetin aglycone accumulated intracellularly is not known, because it was immediately conjugated to quercetin 3'-sulfate. Nonetheless, this study demonstrated that glucuronides can be intracellularly deconjugated in cultured liver cells.

Further evidence for mechanism B comes from studies with hormone glucuronides. Endogenous hormones, like estrogens, androgens and thyroid hormones, are glucuronidated by UDP-glucuronosyltransferases [33]. The thyroid gland secretes the hormone thyroxine (T4), which can be deiodinated in tissues to triiodothyronine (T3). Both compounds are glucuronidated and sulfated in tissues, giving glucuronidated thyroxine (T4G) and triiodothyronine (T3G) and sulfated thyroxine and triiodothyronine. Interestingly, a mechanism for fine-tuning of local thyroid hormone levels and action was discovered in cardiac cells [34]. Cardiac fibroblasts produced T4G and T3G after *in vitro* exposure to T4 and T3, whereas cardiomyocytes were unable to glucuronidate T4 and T3. In contrast, cardiac fibroblast did not take up T4G and T3G, whereas cardiomyocytes effectively accumulated T4G and T3G intracellularly. More strikingly, intracellular glucuronidase activity in cardiomyocytes deglucuronidated the imported thyroid hormone glucuronides. Intracellularly released thyroid aglycones concomitantly stimulated the differentiation of the cardiomyocytes [34]. The authors suggested that glucuronidation represents a mechanism to uncouple local thyroid hormone action in the heart from that in other peripheral tissues and in the systemic circulation [34]. It would be interesting to study fine-tuning reactions in this model with cardiomyocytes or other cell types that would prefer polyphenol glucuronide uptake instead of polyphenol aglycone. Local deconjugation could be a mechanism for polyphenols to exert there effects at selected target sites as well. Therefore, glucuronidation of polyphenols could not

merely be a process for efficient elimination of polyphenols, but also a mechanism of distributing polyphenols to tissue targets.

A similar local fine-tuning mechanism for polyphenol action occurs in plants. Polyphenols are synthesized in plants from malonyl CoA and phenylalanine, and further conjugated to glycosides by glycosyltransferases probably located in the cytoplasm of the plant cell [35]. Polyphenol glycosides are stored in vacuoles and form a conjugate pool for responding adequately to physiological alterations [36]. The response is partly regulated by specific enzymes with β-glycosidase activity. Upon stimulation of the plant cell by certain environmental inducers or changing levels of polyphenol synthesis intermediates, the plant cell is supposed to release the conjugate pool by exocytosis of the vacuole to the cell wall [36-38]. β-Glycosidases located in the cell wall of the plant cell deconjugate the stored polyphenol glycosides and polyphenol aglycones are released into the extracellular environment [38]. In the case of plant root cells, aglycones are excreted to induce gene expression changes in nitrogen fixing bacteria in the soil [39]. This storage and release of glycosides is a common mechanism for plants to interact with their environment or regulate hormone levels [40, 41]. Whether a fine-tuning mechanism of polyphenol action also occurs in mammals warrants further investigation.

Taken together, both polyphenol aglycones and metabolites can possibly be bioactive compounds *in vivo*. Mechanism A (direct intracellular effect of polyphenol conjugates) is likely to occur, because of the *in vivo* abundance of polyphenol metabolites over polyphenol aglycones and demonstrated intracellular availability of polyphenol glucuronides. On the other hand, mechanisms B and C (intra- and extracellular release of polyphenol aglycone, respectively) are very attractive and could partially justify *in vitro* experiments with polyphenol aglycones. However, until the direct deconjugation of polyphenol conjugates with a causative effect of the released polyphenol aglycone has been demonstrated *in vivo*, this is still a hypothesis which needs to be further studied. Nevertheless, the high quercetin 3-O-glucuronide deconjugating capacity detected in lung, liver and kidney of rats after chronic feeding of a high quercetin dose (chapter 2), entails that mechanisms B and C would be plausible mechanisms of action of polyphenols *in vivo*.

MOLECULAR TARGETS OF POLYPHENOLS RELATED TO ENERGY METABOLISM

Polyphenols have been shown to interact with several mammalian proteins. Both the type of interaction and nature of the target protein are very

diverse. Polyphenols can for example function as ligands for specific receptors, activate enzymes or inhibit them by competitively or non-competitively altering substrate availability to the active site and/or by increasing gene transcription by activating transcription factors. All of these interactions can possibly trigger a change in cellular homeostasis, but whether these interactions significantly alter cellular physiology *in vivo* is most of the time not fully understood.

We observed that quercetin induced upregulation of *in vivo* fatty acid catabolism in rats after chronic dietary supplementation (chapter 4). Moreover, quercetin and quercetin 3-O-glucuronide induced changes in *in vitro* differentiated adipocytes indicative for a shift energy supply from glucose- to fatty acid-utilizing pathways (chapter 5). These experimental observations were made by analyzing levels of carbohydrate and lipid metabolism parameters and expression levels of genes coding for enzymes involved in carbohydrate and lipid metabolism. Although metabolite and gene expression profiles provide us with snapshots of the cellular state after quercetin induced alteration in cellular physiology, mechanisms by which quercetin triggers alterations in energy metabolism related pathways were not directly elucidated. However, metabolite and gene expression profiles of cellular state after quercetin intervention can give clues about what molecular targets will be affected by quercetin.

Several studies using similar profiling techniques have identified carbohydrate and lipid metabolism pathways as significantly altered by polyphenols as well. Proteomic profiling of HT29 colon cells after a 24h quercetin exposure, showed that quercetin altered a number of different processes [42]. Among these processes several proteins involved in energy metabolism were modulated by quercetin. The fatty acid catabolism enzyme, acyl CoA dehydrogenase, was upregulated by quercetin, whereas OXPHOS protein succinate dehydrogenase was downregulated [42]. Interestingly, in line with our results from lungs of quercetin exposed rats, the authors suggested that "quercetin causes severe alterations of metabolism that could lead to a reduced oxidative phosphorylation of substrates and an enhanced energy generation via fatty acid oxidation" [42]. Furthermore, both resveratrol and genistein opposed the effects on energy metabolism related pathways induced by feeding mice a high fat diet in liver [43, 44]. *In vitro* exposure of human adipocytes to anthocyanins resulted in an upregulation of fatty acid catabolism genes [45]. Taken together, it seems that polyphenols can trigger several cellular responses related to energy metabolism. In the following section, we will describe the mechanistic *in vitro* studies that aimed to explain the polyphenolic induced alterations in

cellular energy metabolism. These experiments can help us to trace the observed *in vivo* results back to cellular interactions of polyphenols.

SIRT1

Several polyphenols, such as resveratrol, EGCg, myricetin and quercetin were shown to induce the activity of the recombinant SIRT1 protein (chapter 6 and [46]). SIRT1 is a NAD+ dependent deacetylase involved in regulating processes related to energy metabolism, like adipogenesis and insulin secretion [47]. Resveratrol, a polyphenol abundant in red wine, has been shown to lower the Km for NAD+ and acetylated SIRT1 target peptides, possibly by increasing the binding efficiency of substrates through a resveratrol induced conformational change in SIRT1 structure [48]. Extensive *in vitro* data demonstrate that several resveratrol mediated alterations of biological function require an active SIRT1 protein. For example: Resveratrol induced lipolysis in differentiated 3T3-L1 adipocytes only when SIRT1 protein was expressed [49]. PGC1α, a regulator of mitochondrial biogenesis, was activated by resveratrol in cultured cells, as was demonstrated by decreased acetylation status of PGC1α and increased expression of PGC1α target genes, whereas PGC1α was not activated when SIRT1 was knocked down by RNAi [50]. Furthermore, in cells isolated from SIRT1 knockout mice resveratrol did not activate PGC1α, whereas in cells isolated from wild-type mice resveratrol did activate PGC1α [50]. Thus, evidence is accumulating that resveratrol can alter *in vivo* energy metabolism pathways through interacting with SIRT1. On the other hand, the repression of insulin induced activation of MAPK and AKT by resveratrol in HEK293 cells was not dependent on SIRT1, because RNAi of SIRT1 in these cells did not alter the resveratrol mediated effect [51]. This indicates that resveratrol possibly can interact with several targets that will alter cellular physiology.

GLUT

The ability of quercetin to activate SIRT1 renders SIRT1 a possible molecular target by which quercetin could alter energy metabolism *in vivo*. However, other SIRT1-independent mechanisms might be involved as well. Quercetin has been shown to inhibit glucose transport proteins GLUT2, expressed in the intestines, and GLUT4, expressed in muscle and adipose tissue [52-54]. Simultaneously administering a single oral dose of glucose (2g/kg BW) and quercetin (3-65 mg/kg BW) to rats resulted in lower plasma glucose levels as compared to plasma glucose levels after oral glucose only [54]. The authors speculated that the observed lowering of

plasma glucose levels by quercetin could be explained by decreased glucose uptake in the intestines, through the inhibition of GLUT2 by quercetin.

PI 3-kinase

Quercetin has been extensively used *in vitro* as an aspecific inhibitor of phosphatidylinositol 3-kinase (PI 3-kinase, IC50 = 3.8 μM [55]). Furthermore, a more specific inhibitor LY294002 (IC50 = 1.4 μM) was designed based on the molecular structure of quercetin [55, 56]. The PI 3-kinase protein family is a large family of proteins involved in a number of cellular signaling pathways, like insulin signaling [57, 58]. Therefore, quercetin has been suggested to act as modulator of signaling pathways [59]. Interestingly, PI 3-kinase inhibition by LY292004 was recently shown to induce the cytoplasmic localization of SIRT1 in myoblasts, which was previously thought to be localized only in the nucleus [60]. Quercetin might affect SIRT1 localisation by inhibiting PI 3-kinase as well, which means that it would indirectly regulate SIRT1. However, *in vivo* evidence for the inhibitory effect of quercetin on PI 3-kinase is lacking.

PPAR

Peroxisome proliferators-activated receptors (PPARs) are nuclear receptors that regulate transcription of a large set of genes that are mostly involved in energy metabolism. Three different PPARs can be distinguished: PPARα, PPARβ/δ and PPARγ [61-63]. Thuillier et al. [64] studied the effects of quercetin on PPAR mediated keratinocyte differentiation. Quercetin was shown to bind to all thee PPARs, with the highest affinity for PPARα (Ki = 9μM), increase the activity of a PPAR-responsive element and inhibit keratinocyte differentiation [64]. The authors concluded that the observed effect on keratinocyte differentiation was induced by interaction of quercetin with PPARs and subsequent alteration of PPAR-mediated gene expression [64]. Other polyphenols have been demonstrated to interact with PPARs as well [65]. Resveratrol selectively activated both PPARα and PPARγ *in vitro* in cultured and primary cells [66]. Moreover, resveratrol (20 mg/kg) attenuated brain infarct in a mouse stroke model when given orally for three consecutive days. Attenuation of brain infarct by resveratrol was dose dependent and similar to the effect of common PPARα ligands fenofibrate (30 mg/kg for 3 days) and Wy-14643 (30 mg/kg for 10 days) [66]. Furthermore, the resveratrol mediated effect on brain infarct was not seen in PPARα knockout mice [66]. Isoflavones, genistein and daidzein, were also shown to activate PPARs *in vitro* [67, 68]. Therefore, PPAR nuclear receptors could possibly play a role in the alteration of energy metabolism mediated by quercetin and other polyphenols.

ENERGY METABOLISM AS POSSIBLE POLYPHENOL TARGET PROCESS

The observed *in vivo* effects of quercetin and related polyphenols on energy metabolism in combination with *in vitro* cellular interactions of polyphenols with important energy metabolism transcription factors and proteins, point to a role for polyphenols as modulators of energy metabolism. Although direct *in vivo* evidence is still lacking, polyphenols might be able to either directly interact with cellular energy sensing proteins or indirectly trigger energy sensors to alter cellular energy metabolism. Direct interactions with energy sensors, like the demonstrated *in vitro* SIRT1/polyphenol interaction, are plausible *in vivo* mechanisms, but indirect alterations are also possible. Polyphenols can for example affect intracellular responses through there anti-oxidative or pro-oxidative properties [69]. Depletion of intracellular GSH, scavenging of ROS or alteration of mitochondrial membrane potential [70-72] could possibly affect energy sensing as well.

Further support for a role of polyphenols in modulating energy metabolism is illustrated when we revisit polyphenol functions in plants (as described in chapter 1). One of the major functions of polyphenols in plants is establishing a symbiotic interaction with rhizobia for energy assimilation. This interaction is initiated through a specific interaction of a polyphenol with a transcription factor *nodD* [73]. The polyphenol induces a structural change in the configuration of *nodD,* which results in increased DNA binding affinity of the transcription factor and consequently increased expression of *nodD* target genes [74]. The *nodD* transcription factor contains a LysR type transcriptional regulator (LTTR). LTTR containing proteins are one of the largest families of transcriptional regulators in prokaryotes. Interestingly, many LTTRs are functionally involved in basic energetic metabolism, such as amino acid biosynthesis, CO_2 fixation, nitrogen assimilation and catabolism of aromatic compounds [74]. Consequently, polyphenols can function as regulators of cellular energetic processes in prokaryotes. Similar functional interactions related to cellular energy metabolism, like the interaction of polyphenols with SIRT1, GLUTs, PI3K and PPARs, have been established *in vitro* in mammals. Moreover, *in vivo* alterations of energy metabolism by polyphenol intervention have been demonstrated in mammals. Thus, taken into account that mammals have relied on plant food sources abundant in polyphenols throughout evolution, it is tempting to propose that polyphenols have evolutionary evolved as modulators of cellular energetic processes, possibly through cellular interactions with different proteins.

FUTURE PERSPECTIVES FOR IDENTIFYING FUNCTIONAL EFFECTS OF POLYPHENOLS

Although the experiments described in this thesis demonstrate that quercetin and other polyphenols may play a role in modulating *in vivo* energy metabolism, several questions relating to the mechanisms of action of polyphenols remain unanswered. One of the foremost questions is the nature of the *in vivo* bioactive compound, whether it is the polyphenol aglycone, the polyphenol metabolite(s) or both. To address this research question, experiments should be performed to elucidate the glucuronidase activity at target tissues. One of the possible experiments to analyze deglucuronidating activity in tissues is to use perfusion models of (isolated) organs, such as lungs or liver. After perfusing tissues with polyphenol glucuronides, analysis of polyphenol metabolites and particularly formation of aglycones would give the first direct evidence for polyphenol deconjugating capacity of tissues. Applying different kinds of stress to the animal or isolated perfused organ, like inflammation or different oxygen tensions, would answer the question whether polyphenol metabolites are deconjugated more rapidly under specific pathologic conditions. Similar perfusion experiments were already performed in studies on the absorption of polyphenol glycosides from the intestines [75, 76]. These experiments demonstrated that polyphenol glycosides can be selectively deconjugated by glycosidase enzymes in the intestinal wall, accounting for the observed absorption differences for polyphenol glycosides [76]. Limited availability of polyphenol metabolites as pure chemical compounds did not allow for these experiments to be performed with polyphenol glucuronides. However, currently several research groups are able to synthesize polyphenol metabolites in quantities sufficient for performing *in vitro* and *in vivo* experiments [17]. We were able to perform a limited number of *in vitro* exposure experiments with quercetin 3-O-glucuronide to address whether quercetin metabolites possess similar molecular effects as quercetin aglycone. These experiments demonstrated that quercetin 3-O-glucuronide can be bioactive in cell culture models and elicit both similar (chapter 5) and opposite (chapter 6) effects as quercetin aglycone. Additional *in vitro* studies using polyphenol metabolites are essential for obtaining information on the possibility that polyphenol metabolites are bioactive compounds *in vivo*.

Mammalian molecular targets of polyphenols have been identified, but most of these investigations were restricted to *in vitro* experiments. Identification of the *in vivo* relevance of these molecular targets is essential for elucidating mechanisms of action of polyphenols. Metabolomics, proteomics and transcriptomics techniques can provide *in vivo* data on

cellular physiology after dietary intervention with polyphenols. These methods would give possible directions for the search for new *in vivo* molecular targets of polyphenols. In addition, the use of transgenic animals to address the relevance of possible *in vivo* targets can be of major importance. Even though physiological effects of resveratrol in mice fed a high fat diet resemble that of the *in vitro* activation of the SIRT1 protein, direct *in vivo* SIRT1 stimulating effects of resveratrol are not yet demonstrated. It would be intriguing to study the effects of several different SIRT1 stimulating polyphenols, like resveratrol, quercetin and EGCg, in SIRT1 transgenic mice. In combination with variations in mouse diet composition, this would generate key answers on the role of SIRT1 in the energy metabolism modulating effects of polyphenols.

Although our mechanistic studies point to possible alterations in rat physiology that might be beneficial for humans as well, like lowering of plasma free fatty acids (chapter 4) and attenuation of colon tumor incidence (chapter 3), we used high dosages of polyphenols. Up to now, these high dosages were necessary to answer the proposed research question. For example, current analytical techniques to accurately measure the levels of polyphenols in tissues are limited by detection levels in the nanomolar range. Using the relatively high dietary dosages (0.1% and 1% quercetin: ~50 and 500 mg/kg BW per day, resulting in nanomolar tissue concentrations) we were able to analyze the tissue distribution of quercetin accurately. When using lower dosages of quercetin, the analytical techniques we used would be insufficient to detect levels of quercetin metabolites in tissue which probably are in the picomolar range. Therefore, to address the question whether normal dietary intake levels of polyphenols (0.3-0.6 g/kg BW per day for flavonols [77]) give similar tissue distribution profiles and similar effects as the high dosages we used in our studies, more sensitive analytical methods for the identification of polyphenol metabolites in tissues should be developed.

CONCLUSIONS

Our approach to elucidate functional effects of polyphenols provided novel insights: First, lungs were newly identified as target tissues of dietary quercetin. Second, an effect on energy metabolism was identified; quercetin was shown to upregulate fatty acid catabolism in rats. Third, a possible role in cancer protection was identified: tumor incidence in a rat model for colon carcinogenesis was dose-dependently attenuated by chronic dietary quercetin. Fourth, the effect on energy metabolism was extended *in vitro*: carbohydrate and lipid metabolism parameters were found to be altered in

cultured adipocytes *in vitro* by both quercetin aglycone and a major human quercetin metabolite, quercetin 3-O-glucuronide. Finally, polyphenolic stimulation of *in vitro* SIRT1 activity was demonstrated to be affected by metabolism and stability of polyphenols and novel polyphenolic SIRT1 stimulators (myricetin, EGCg and ECg) were identified.

The experiments described in this thesis point to a possible beneficial effect of dietary polyphenols. However, as long as the molecular mechanisms in humans are unknown and the risk of increasing dietary intakes of polyphenols via food supplements is not thoroughly investigated, there is no scientific justification for supplementing the diet with large amounts of polyphenols. Nevertheless, our approach successfully identified modulation of energy metabolism by polyphenols as an important process involved in mediating the possible health effects associated with dietary polyphenol intake. Therefore, future research should be directed towards further identifying the molecular mechanisms underlying the effects of polyphenols on energy metabolism. Understanding the molecular mechanisms will help to interpret and substantiate the epidemiological data, and is necessary for targeted *in vivo* studies in humans. These studies would generate key answers on the potential effects of dietary polyphenols in humans and the possible application of polyphenols as healthy functional dietary component.

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SUMMARY

A diet rich in fruits and vegetables contains high levels of polyphenols (up to 1 gram per day). Epidemiological studies suggest that a high dietary intake of selected polyphenols can be protective against development of cardiovascular heart diseases in humans. In addition, mechanistic studies demonstrate that polyphenols possess beneficial properties in *in vitro* and animal model systems. Due to the possible beneficial health effects of polyphenols in the human diet, polyphenols are currently being sold extensively as food supplements. However, the basis for most of the health claims attributed to polyphenols in food supplements is often very small. Results from mechanistic *in vitro* studies are frequently directly extrapolated to possible beneficial effects in humans. Reliable *in vivo* studies and relevant *in vitro* studies on physiological effects of polyphenols are needed to conclusively obtain mechanisms that underlie associations between health effects and dietary polyphenol intake observed in epidemiological studies. Our objective was to elucidate relevant mechanisms of action of selected polyphenols. This was done by studying the tissue distribution and *in vivo* physiological effects of quercetin (a polyphenol abundant in the human diet), followed by *in vitro* elucidation of possible biological mechanisms explaining the observed *in vivo* effects.

An extensive number of studies have been performed to determine the absorption and disposition of polyphenols in animals and humans. These studies demonstrated that polyphenols are extensively metabolized to phase II conjugates and eliminated through urine and/or faeces. However, most of these studies used single doses and relatively little is known about the availability of polyphenols to tissues. Therefore, we developed a method to quantitatively analyze quercetin metabolite levels in tissues of rats after chronic exposure to a 0.1% and 1% quercetin diet (~50 and 500 mg/kg BW/per day) for 11 weeks. Our study demonstrated that quercetin metabolites were widely distributed to rat tissues (chapter 2). All collected tissues contained detectable levels of quercetin metabolites. The highest levels were found in lungs and the lowest in brain, white fat and spleen. The tissue distribution pattern was similar between rats fed a 1% and 0.1% quercetin diet, but levels in tissues of the 0.1% diet group were approximately 4 times lower (chapter 2).

Based on the findings that quercetin metabolites were the highest in lungs of rats we chose lung tissue as target tissue to elucidate possible

mechanisms of action of quercetin. In addition, lung tissue seems to be an important target based on epidemiological studies indicating that a high flavonol diet is protective against lung cancer. We used whole genome microarrays to elucidate gene expression changes in lung tissue induced by a chronic 41 week quercetin diet (10 g quercetin/kg diet, ~500 mg/kg BW/day) (chapter 4). This transcriptomic study provided us with evidence for a quercetin mediated alteration in expression profiles of fatty acid catabolism genes. This effect was corroborated by the finding that plasma free fatty acid levels were lowered after the chronic quercetin intervention (chapter 4).

Apart from lung, the gastro-intestinal tract is an additional tissue target of dietary. Because of the potential of quercetin to reduce the risk of developing cancer we studied the effect of dietary quercetin (0, 0.1, 1 or 10 g quercetin aglycone/kg diet) on the incidence of colon cancer induced by azoxymethane (AOM) (chapter 3). In addition, we studied the difference between the effect on tumor incidence of quercetin aglycone and quercetin 3-rutinoside (rutin), an abundant quercetin glycoside in the human diet. Interestingly, the chronic quercetin aglycone diet dose-dependently decreased the tumor incidence in colons of rats. On the other hand, the rutin diet (40 g/kg diet) did not alter tumor incidence as compared the control group. This indicated that the form in which quercetin is ingested via the diet determines its beneficial effect, possibly due to a lower bioavailability of quercetin 3-rutinoside as compared to quercetin aglycone.

The established *in vivo* effects of a chronic quercetin diet, described in the previous studies, were further explored in *in vitro* systems. We tried to mimic *in vivo* conditions by using a major *in vivo* metabolite of quercetin in humans, quercetin 3-O-glucuronide (chapter 5 and 6). We studied the effects of polyphenols on activation of SIRT1 (chapter 6). This NAD+ dependent deacetylase is an important protein regulating several different biological processes, like regulation of energy metabolism and stress responses. Experiments published by Howitz et al. {Nature (2003) 425:191} demonstrated that SIRT1 was activated by quercetin only when using the isolated SIRT1 protein. No activation of SIRT1 was observed when an *in vitro* cell system was used. This discrepancy prompted us to analyze the effect of stability and metabolism of polyphenols on their SIRT1 activating effect. Using an optimized method to analyze SIRT1 activation by polyphenols, we were able to demonstrate that polyphenols degrade under experimental aqueous conditions and only activated SIRT1 when they were stabilized with vitamin C. This resulted in the identification of three additional polyphenolic SIRT1 activators, myricetin, EGCg and ECg. We

also observed that quercetin 3-O-glucuronide inhibited the activity of SIRT1, whereas quercetin aglycone activated SIRT1. Exposing HT29 colon carcinoma cells to quercetin aglycone did not result in activation of SIRT1, probably because of the observed rapid metabolism of quercetin aglycone to quercetin glucuronides in HT29 cells (chapter 6).

Fatty acid catabolism pathways in rats were demonstrated to be altered by the chronic quercetin diet (chapter 4). Therefore, we used a 3T3-L1 adipocyte *in vitro* model to explore the role of quercetin and quercetin 3-O-glucuronide in altering metabolic pathways for energy supply. We chose this adipocyte model because of the previously identified alteration of lipolysis by resveratrol in a SIRT1 dependent manner {Picard et al. Nature (2004) 429:771}. We demonstrated that exposure of adipocytes for 24h to quercetin aglycone and quercetin 3-O-glucuronide resulted in reduced consumption of glucose, which could point to attenuated glucose utilization and/or uptake. Expression of Pdk4, a negative regulator of glycolysis, was significantly upregulated by both quercetin aglycone and quercetin 3-O-glucuronide. A strong downregulation of the expression of genes coding for proteins involved in lipid metabolism was seen for quercetin aglycone, but not for quercetin 3-O-glucuronide. Both quercetin aglycone and quercetin 3-O-glucuronide altered glycerol and free fatty acid levels as well, but the implications of these alterations are not clear. We demonstrated for the first time that quercetin 3-O-glucuronide affected energy providing pathways in adipocytes. Further studies should be performed to elucidate whether quercetin 3-O-glucuronide acts *in vivo* as energy metabolism modulator as well.

Finally, in chapter 7 we propose mechanisms by which polyphenols can alter *in vivo* biological processes. We review literature on the effects of quercetin on *in vivo* energy metabolism and discuss possible molecular targets of polyphenols that might play a role in the observed effects of polyphenols on energy metabolism. In addition, mechanisms of the *in vivo* availability of polyphenols to intracellular molecular targets are discussed.

To conclude, our approach to identify functional effects of polyphenols focused on identifying tissue targets, analyzing *in vivo* alterations of rat physiology and elucidating biological mechanisms *in vitro* using selected *in vivo* polyphenol metabolites. In addition, we used chronic quercetin diets to mimic the chronic dietary intake of quercetin in humans. Using this approach, we revealed lungs as novel tissue target of quercetin and demonstrated that dietary quercetin alters fatty acid catabolism pathways in rats. In addition, dietary quercetin lowered tumor incidence in the colon of rats in a model of colon carcinogenesis. Furthermore, a major *in vivo*

metabolite of quercetin, quercetin 3-O-glucuronide, opposed the effect of quercetin aglycone on SIRT1 activation *in vitro*, whereas quercetin 3-O-glucuronide attenuated glucose utilization in cultured adipocytes in a similar fashion as quercetin aglycone. Although we used high dietary dosages of quercetin and further studies should elucidate physiological effects of a normal dietary intake of polyphenols, the experiments described in this thesis point to a possible beneficial effect of dietary polyphenols. However, as long as the molecular mechanisms in humans are unknown and the risk of increasing dietary intakes of polyphenols via food supplements is not thoroughly investigated, there is no scientific justification for supplementing the diet with large amounts of polyphenols. Nevertheless, our approach successfully identified modulation of energy metabolism by polyphenols as an important process involved in mediating the possible health effects associated with dietary polyphenol intake.

SAMENVATTING

Een dieet rijk aan groente en fruit produkten bevat veel polyfenolen (tot 1 gram per dag). Epidemiologische studies suggereren dat een hoge inname van sommige van deze polyfenolen de kans op het krijgen van hart- en vaatziekten verlaagt in mensen. Ook mechanistische studies demonstreren dat polyfenolen goede eigenschappen hebben in *in vitro* en dier studies. Naar aanleiding van de mogelijke gezondheidsbevorderende effecten van polyfenolen, worden polyfenolen tegenwoordig als voedingssupplementen verkocht die grote hoeveelheden polyfenolen bevatten. Maar de basis voor de gezondheidsclaims die worden toegeschreven aan polyfenolen is vaak klein. Resultaten van mechanistische *in vitro* studies worden vaak direct geëxtrapoleerd naar mogelijke gezondheidsbevorderende effecten in mensen. Betrouwbare *in vivo* studies en relevante *in vitro* studies naar de fysiologische effecten van polyfenolen zijn nodig om definitief inzicht te krijgen in de onderliggende mechanismen die de epidemiologisch gevonden associaties tussen gezondheidseffecten en een hoge polyfenolen inname via de voeding verklaren. Ons doel was om relevante onderliggende mechanismen te ontdekken van geselecteerde polyfenolen. Dit hebben we gedaan door de weefselverdeling en *in vivo* fysiologische effecten van quercetine (een polyfenol die veel voorkomt in de menselijke voeding) te bestuderen, gevolgd door *in vitro* onderzoek naar de mogelijke biologische werkingsmechanismen die de gevonden *in vivo* effecten verklaren.

Een groot aantal studies is uitgevoerd om de absorptie en uitscheiding van poyfenolen in mensen en dieren te bepalen. Deze studies hebben laten zien dat polyfenolen worden gemetaboliseerd tot phase II conjugaten en geëlimineerd worden via de urine en/of ontlasting. Maar veel van deze studies hebben enkelvoudige doseringen gebruikt en relatief weinig is bekend over de beschikbaarheid van polyfenolen in weefsels. Wij hebben daarom een methode ontwikkeld om kwantitatief quercetine metabolieten in weefsels te analyseren van ratten die 11 weken chronisch bloot zijn gesteld aan een 0.1% of 1% quercetine dieet (~50 and 500 mg/kg lichaamsgewicht/per dag). Onze studie liet zien dat alle verzamelde weefsels quercetine metabolieten bevatten (hoofdstuk 2). De hoogste concentraties werden gevonden in de longen en de laagste in de hersenen, wit vet en milt. Het weefselverdelingspatroon was vergelijkbaar in de ratten die een 1% en 0.1% quercetine dieet hadden gevolgd, maar de concentraties

in de weefsels van de 0.1% dieet groep waren ongeveer 4 keer lager (hoofdstuk 2).

Op basis van het feit dat de concentraties van quercetine metabolieten het hoogst waren in de longen van ratten, hebben we gekozen voor longweefsel als target weefsel voor de opheldering van mogelijke "mechanisms of action" van quercetine. Bovendien, lijkt longweefsel een belangrijk doelorgaan te zijn, omdat uit epidemiologisch onderzoek is gebleken dat een hoog flavonolen dieet beschermend is tegen longkanker. We hebben gebruik gemaakt van "whole genome" microarrays om genexpressie verschillen in long weefsel op te pikken die geïnduceerd zijn door een chronisch 41 weken lang quercetine dieet (10g quercetine/kg dieet, 500 mg/kg lichaamsgewicht per dag) (hoofdstuk 4). In deze transcriptomics studie hebben we bewijs gevonden voor een door quercetine geïnduceerde verandering van expressie profielen van vetzuurkatabolisme genen. Dit effect werd bevestigd door de ontdekking dat concentraties van vrije vetzuren in plasma na het chronische quercetine dieet verlaagd waren (hoofdstuk 4).

Naast de longen, is het maag-darm stelsel een additioneel doelorgaan van quercetine vanuit de voeding. Doordat quercetine mogelijk de kans op de ontwikkeling van kanker verlaagd, hebben we het effect van quercetine vanuit de voeding (0, 0.1, 1 or 10 g quercetine aglycon/kg dieet) op de incidentie van dikkedarmkanker onderzocht (hoofdstuk 3). Dikkedarmkanker werd geïnduceerd door middel van azoxymethaan (AOM). Bovendien hebben we het verschil tussen het effect van quercetine aglycon en quercetine 3-rutinoside (rutine), een veelvuldig voorkomend quercetine glycoside in de humane voeding, op tumor incidentie bestudeerd. Interessant was dat het chronische quercetine aglycon dieet de tumor incidentie op een dosis afhankelijke manier in ratten verlaagde. Aan de andere kant had het rutine dieet (40 g/kg dieet) geen effect op de vorming van tumoren in vergelijking met de controle groep. Dit betekent dat de formulering waarin quercetine via de voeding wordt ingenomen bepaalt wat het effect van quercetine is. Dit komt mogelijk door de lage biobeschikbaarheid van quercetine 3-rutinoside in vergelijking tot die van het quercetine aglycon (hoofdstuk 3).

De gevonden *in vivo* effecten van een chronisch quercetine dieet, beschreven in voorgaande hoofdstukken, werden verder onderzocht met behulp van *in vitro* systemen. We hebben geprobeerd om de *in vivo* condities na te bootsen door gebruik te maken van een belangrijke *in vivo* metaboliet van quercetine in mensen, quercetine 3-O-glucuronide (hoofdstuk 5 en 6). We hebben de effecten van polyfenolen op SIRT1 activatie onderzocht

(hoofdstuk 6). Dit NAD+ afhankelijke deacetylase is een belangrijk eiwit dat verschillende biologische processen, zoals energie metabolisme en stress response reguleert. Experimenten gepubliceerd door Howitz *et al.* {Nature (2003) 425:191} hebben laten zien dat SIRT1 werd geactiveerd door quercetine alleen als het geïsoleerde eiwit werd gebruikt. Activatie van SIRT1 werd niet waargenomen wanneer een *in vitro* cel systeem werd gebruikt. Deze discrepantie was aanleiding voor ons om het effect van de stabiliteit en het metabolisme van polyfenolen op de SIRT1 activerende eigenschappen van polyfenolen te onderzoeken. Door gebruik te maken van een geöptimaliseerde methode om SIRT1 activatie door polyfenolen te meten, hebben we laten zien dat sommige polyfenolen onder experimentele waterige omstandigheden degraderen. Alleen als deze polyfenolen gestabiliseerd werden met vitamine C hadden ze een SIRT1 activerende werking. Hierdoor hebben we drie additionele SIRT1 activerende polyfenolen (myricetin, EGCg en ECg) ontdekt. We hebben ook waargenomen dat quercetine 3-O-glucuronide de activiteit van SIRT1 remde, in tegenstelling tot quercetine aglycon die SIRT1 activeerde. Blootstelling van HT29 colon carcinoma cellen aan quercetine aglycon resulteerde niet in een activatie van SIRT1, mogelijk door de waargenomen snelle omzetting van quercetine aglycon naar quercetine glucuroniden in HT29 cellen (hoofdstuk 6).

We hebben laten zien dat een chronisch quercetine dieet vetzuur katabolisme pathways in ratten verandert (hoofdstuk 4). Daarom hebben we een 3T3-L1 vetcel *in vitro* model gebruikt om de rol van quercetine en quercetine 3-O-glucuronide in de verandering van metabolische pathways voor energie gebruik verder te onderzoeken. We hebben dit vetcelmodel gekozen doordat al eerder is laten zien dat resveratrol de lipolyse op een SIRT1 afhankelijke manier verandert {Picard et al. Nature (2004) 429:771}. Wij laten zien dat blootstelling van vetcellen voor 24 uur aan quercetine aglycon of quercetine 3-O-glucuronide heeft geresulteerd in een verminderde consumptie van glucose. Dit kan wijzen op een lager glucose verbuik en/of lagere opname. Expressie van *Pdk4*, een negatieve regulator van de glycolyse, was significant opgereguleerd door quercetine en quercetine 3-O-glucuronide. Een sterke downregulatie door quercetine aglycon van de expressie van genen die coderen voor eiwitten betrokken bij vetmetabolisme werd waargenomen, maar niet voor quercetine 3-O-glucuronide. Quercetine aglycon en quercetine 3-O-glucuronide veranderden allebei ook de glycerol en vetzuur niveau's, maar de implicaties van deze veranderingen zijn niet duidelijk. We hebben voor het eerst laten zien dat quercetine 3-O-glucuronide het energiemetabolisme verandert in

vetcellen. Verdere studies moeten worden uitgevoerd om op te helderen of quercetine 3-O-glucuronide ook het energiemetabolisme *in vivo* verandert.

Ten slotte, stellen we in hoofdstuk 7 mechanismes voor die mogelijk een verklaring geven voor de door polyfenolen geïnduceerde veranderingen van biologische processen. We geven een overzicht van de literatuur over de effecten van quercetine op *in vivo* energiemetabolisme en stellen mogelijke targets van polyfenolen voor die misschien een rol spelen in de gevonden effecten van polyfenolen op het energiemetabolisme. Bovendien, beschrijven we de mechanismes van de *in vivo* beschikbaarheid van polyfenolen naar intracellulaire moleculaire targets.

Concluderend, onze aanpak om functionele effecten van polyfenolen te ontdekken was gericht op het identificeren van doelweefsels, analyse van *in vivo* veranderingen van rat fysiologie en ophelderen van biologische "mechanisms of action" met geselecteerde metabolieten van polyfenolen. Bovendien hebben we een chronisch dieet gebruikt dat de chronische inname van polyfenolen in mensen nabootst. Met deze aanpak hebben we de longen ontdekt als nieuw doelorgaan van quercetine en we hebben laten zien dat quercetine uit de voeding vetzuurkatabolisme pathways in ratten verandert. Ook hebben we laten zien dat quercetine uit de voeding de tumor incidentie in de colon van ratten in een rat colon tumor model verlaagt. Verder hebben we laten zien dat een belangrijke *in vivo* metaboliet van quercetine, quercetin 3-O-glucuronide, het tegenstelde effect op de activatie van SIRT1 *in vitro* had dan dat van quercetine aglycon, maar een vergelijkbaar effect als quercetine aglycon wat betreft de verlaging van glucose verbruik in gekweekte vetcellen. Ook al hebben we hoge doseringen van quercetine gebruikt en verdere studies moeten uitwijzen wat de fysiologische effecten zijn van een normale inname aan polyfenolen vanuit de voeding, de experimenten beschreven in dit proefschrift wijzen naar een mogelijk gezondheidsbevorderende effect van polyfenolen uit de voeding. Maar zolang de moleculaire mechanismes in mensen niet bekend zijn en het risico van het verhogen van de inname van polyfenolen via voedingssupplementen niet grondig onderzocht is, is er geen wetenschappelijke rechtvaardiging voor het supplementeren van het dieet met grote hoeveelheden polyfenolen. Onze aanpak heeft desondanks toch geresulteerd in de ontdekking dat de verandering van het energiemetabolisme door polyfenolen een belangrijk proces is dat mogelijk betrokken is bij het tot stand komen van mogelijke gezondheidseffecten van polyfenolen vanuit de voeding.

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Vincent
5 april 2007, Boston, USA

Dit proefschrift werd mede geschreven met behulp van:

Bill Evans – The Bill Evans Album
Stevie Wonder – Songs in the key of life
John Legend – Once Again
Paul Desmond – Easy Leaving
Kenny Barron – Live at Bradley's
D'Angelo - Voodoo
Miles Davis – The Sorcerer
Wayne Shorter – Adam's apple
Herbie Hancock – Maiden Voyage
Corine Bailey Rae – Corine Bailey Rae
Donny Hathaway – Live
Clifford Brown – Clifford Brown and Max Roach
Soulive – Soulive (Live)
Stevie Wonder – Innervisions
Kenny Barron – What if?
Branford Marsalis – Contemporary jazz
John Coltrane – A love Supreme
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Talisker 10 years

"Try to find the best teachers, listen to the finest playing, and try to emulate that. Be true to the music"
Wynton Marsalis

ABOUT THE AUTHOR

CV

Vincent Cornelis Johannis de Boer was born on September 11th 1977 in Edam, The Netherlands. After completing pre-university education (VWO, Don Bosco College, Volendam) in 1995, he studied Chemistry and Pharmaceutical Sciences at the Free University in Amsterdam (Vrije Universiteit, Amsterdam). He obtained his MSc degree in 2002 at the section of Analytical Chemistry & Applied Spectroscopy. During his MSc study, he worked on the analysis of isoflavones in plants at the Department of Ecology and Ecotoxicology (Biology faculty, VU Amsterdam) and wrote his dissertation on the identification of proteins and peptides with ion mobility spectrometry/mass spectrometry under supervision of Dr. Henk Lingeman. At the Department of Oncology (VU medical center, Amsterdam) he worked on a research project on drug gradients in tumor models supervised by Prof. Dr. Jan Lankelma. In 2001 he was employed as research associate working on the risk assessment of endocrine disruptors in surface waters at the Water Board of Noord-Holland, Edam. From October 2002 until January 2007 he was appointed as PhD student at the Wageningen University (section of Toxicology). He performed his research on the project entitled "Benefit-risk evaluation of flavonoids in foods and their use as functional food ingredients", as described in this thesis, at the RIKILT – Institute of Food Safety in Wageningen. From April 2007, Vincent de Boer is employed as postdoctoral fellow in the lab of Dr. Marcia Haigis at the Department of Pathology at Harvard Medical School (Boston, USA). His research will be focused on identifying molecular mechanisms regulated by SIRT4, a mitochondrial protein involved in energy metabolism and aging related biological processes.

Publications

de Boer VCJ, van Schothorst EM, Dihal AA, van der Woude H, Arts ICW, Rietjens IMCM, Hollman PCH, Keijer J
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Submitted

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Impact of multiple genetic polymorphisms on effects of a four-week blueberry juice intervention on ex vivo induced lymphocytic DNA damage in human volunteers.
Submitted

Dihal AA, van der Woude H, Hendriksen PJM, Charif H, Dekker HJ, IJsselstijn LJ, **de Boer VCJ**, Alink GM, Burgers PC, Rietjens IMCM, Luider TM, Woutersen RA, Stierum R
Transcriptomic and proteomic profiling of quercetin exposed colon mucosa from F344 rats
In preperation

Bieger J, Cermak R, **de Boer VCJ**, Hollman PCH, Kamphues J, Wolffram S
Tissue distribution of the flavonol quercetin and its methylated metabolites in the pig after long-term feeding of a quercetin-containing diet
In prepration

Training and supervision plan

VLAG graduate school

Discipline specific activities

Courses

- Ecophysiology of the GI-tract, VLAG, Wageningen, 2003
- NUGO: Hands on course Advanced MicroArray Data Analysis, Maastricht, 2005
- Introduction to Bioinformatics, UVA, Amsterdam, 2003
- Masterclasses Nutrigenomics, Wageningen, 2003 and 2005
- Human and Animal Physiology, WUR, Wageningen, 2005
- FEBS Advance course. Frontiers in Molecular Biochemistry of Mitochondria, Warsaw, 2006

Meetings

- Nutrigenomics congres, Wageningen, 2003
- Conference on Polyphenols, Vichy, France 2003
- International conference on polyphenols, Helsinki, Finland, 2004
- NuGO Week 2004, Wageningen
- NuGO Week 2005, Lucca, Italy
- 2nd International Conference on Polyphenols and Health, Davis, USA, 2005
- Networks in BioInformatics, AMC, A'dam, 2005
- BioASP Pathway meeting, AMC, A'dam, 2004
- International society Free Radical Research 13th biennial Congress, Davos, Switserland, 2006
- Molecular genetics of ageing, Cold Sping Harbor, USA, 2006
- NWO Nutrition, Papendal, The Netherlands 2002-2006
- ZonMW research meetings 2002 - 2006
- Discipline specific activities

General courses

- NWO Talent Days, The Hague, 2004, 2005, 2006
- Presentation skills, WUR, Wageningen, 2005
- Undergraduate student supervision, WUR, Wageningen, 2004
- Intermediate English, WUR, Wageningen, 2003

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