Bioavailability of genistein and its glycoside genistin

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Abstract

Genistein belongs to the class of isoflavones. The main sources of isoflavones in food are soybeans and soy-based products. Most isoflavones in plants are bound to sugars such as the glycosides genistin and daidzin. Understanding the various factors that influence absorption and metabolism of isoflavones and especially of their naturally occurring glycosides, is essential to fully elucidate and understand the physiological activity of these dietary ingredients in the human body. It is also essential to fully explore the possible beneficial health effects of the isoflavones, which are considered to be promising as healthy constituents in enriched (novel) foods or supplements. The aim of this thesis is to obtain data on the transport (uptake) and metabolism of the isoflavones genistein and daidzein and their glycosides genistin and diadzin. Special focus is directed to the possible role of SGLT1 in the mechanism involved in the transport and absorption of the isoflavonic glycoside genistin. Another objective is to explore, develop, validate and optimise *in vitro*, *in situ* and *in vivo* gastrointestinal model systems for studying the bioavailability and metabolism of genistein, daidzein and their glycosides.

This thesis reveals that in all intestinal models studied genistein is more bioavailable than its glycoside genistin. The major route of absorption of genistin appeared to be deglycosylation of genistin to genistein and the subsequent intestinal metabolism of genistein to genistein glucuronides and sulfates. Mechanistic studies show that the bioavailability of genistin could be improved by the naturally occurring compound phloridzin, present in apples, because phloridzin inhibits the efflux-transporters located at the brush border (apical) membrane of the enterocyte. On the basis the results described in this thesis, it is concluded that the best gastrointestinal model system appears to be freely moving unanaesthetized cannulated rats. However, animal experiments are expensive and time-consuming and should be reduced, refined or replaced whenever feasible also for ethical reasons. The Caco-2 cells provide a good alternative for studying the mechanism of isoflavones absorption, although the hydrolase activity in this cell line should be improved to mimic the human situation to a better extent.

All together the results presented in this thesis provides an insight in the mechanisms underlying the bioavailability of this important class of health beneficial soy based food ingredients and even point at inhibition of apical transporters as a way to improve the bioavailability of genistin and genistein.

Contents

Chapter 1

Introduction

Cancer is considered to be a world wide major disease. It is the second leading cause of death in the Western countries (Ames et al., 1995). Epidemiological studies have shown that not only genetic factors but also environmental factors, such as diet, play an important role in the disease aetiology (Boyle et al., 1995). It has been estimated that about one third of all human cancers are related to the modern diet (Doll and Peto, 1981; Peto, 2001), although dietary factors may also reduce cancer incidences.

Several epidemiological studies indicate that for example a high consumption of fruits and vegetables, particularly fresh fruits and raw and fresh green-yellow vegetables, is associated with a decreased cancer incidence (Steinmetz and Potter, 1991a and b; Block et al., 1992; Smith et al., 1995). In addition, laboratory studies indicate that fruits and vegetables in the animal diet reduce the incidence of adenomas in chemically-induced colon carcinogenesis models (Wattenberg, 1992; Alink et al., 1993; Dragsted et al., 1993). However, more recent studies also show the opposite effects (Rijnkels et al., 1998; Van Kranen et al., 1998; Tijhuis et al., 2005; Van Gils et al., 2005).

In addition to many nutritional compounds, fruits and vegetables contain several constituents named non-nutrients, as these compounds have been considered to be of no or unknown nutritional value (Wattenberg, 1992). Non-nutrients occur widely throughout the plant kingdom, including food crop plants. Several functions of the non-nutrients in plants have been proposed, including the protection of plants against damage by UV light, insects, fungi, viruses and bacteria. Some non-nutrients influence the quality, acceptability and stability of the food through their role as flavouring and colouring agents or even as protective agents by scavenging free radicals (anti-oxidants). Nowadays certain non-nutrients are regarded as essential components of the human diet because of their chemopreventive effects in humans (Block et al., 1992; Steinmetz et al., 1993; Dragsted et al., 1993; Ames et al., 1995; Hertog et al., 1995; Smith et al., 1995). One group of these non-nutrients are the flavonoids, which gained significant attention over the last decade from the scientific community due to their possible beneficial health effects.

Flavonoids are ubiquitously present in foods of plant origin. Epidemiological studies indicate that a high dietary intake of flavonoids reduces the risk of chronic diseases, including coronary heart defects and cancers (reviewed by Barnes et al., 1996;

Adlercreutz and Mazur, 1997; Setchell and Cassidy, 1999; Clarkson, 2000). It has been estimated that on the average the human intake of flavonoids is a few hundreds of milligrams per day (Hollman and Katan, 1999). Over 6000 different naturally occurring flavonoids have been described and the list is still growing (Harborne and Williams, 2000). Flavonoids are polyphenolic substances with a low molecular weight based on a flavan moiety (i.e. 2-phenyl-benzo-γ-pyrane). This basic structure of flavonoids consists of two benzene rings A and B linked through a heterocyclic oxygen containing pyrane ring C (Kühnau, 1976) (Figure 1.1).

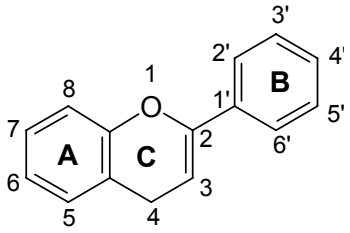


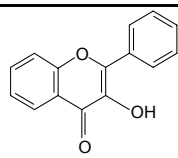
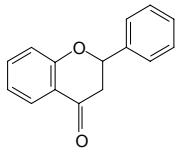
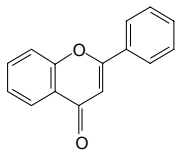
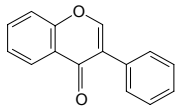
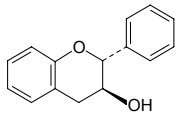
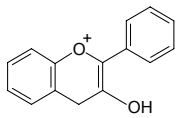
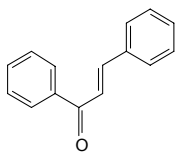
Figure 1.1*: The basic structure of flavonoids.*

The flavonoids can be classified into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanides and chalcones as the major classes (Table 1.1). The classification of these compounds is based on the various chemical modifications of the heterocyclic C ring. The structure of flavonoids varies widely within these major classes and substitutions include hydrogenation, hydroxylation, methoxylation, malonylation, sulfation and glucuronidation. In plants, flavonoids are predominantly bound to sugar derivatives (glycosylated) and called glycosides, whereas the flavonoids without a sugar are called aglycones.

Isoflavones

Isoflavones are a large and very distinctive subclass of the flavonoids. The isoflavones differ for instance structurally from the other classes of the flavonoids by having the phenyl ring (B-ring) attached at the 3- rather than at the 2-position of the heterocyclic ring C (Table 1.1). They are more restricted in the plant kingdom than other flavonoids, since they are found regularly in especially one subfamily of Papilionoideae of the Leguminosae. Although many varieties of vegetables, grains and legumes contain small amounts of isoflavones, by far the largest quantities are found in soybeans. Soy contains approximately1 mg of genistein and daidzein/g of dry beans (Reinli and Block, 1996). Genistein, daidzein and glycitein are the

***Table 1.1**: The flavonoid classes with their chemical structure, the major source of the flavonoids, the common as well as the chemical name of some predominant aglycones.*

Flavonoids	Basic chemical structure	Major source	Predominant aglycones	Chemical name
Flavonols		tea, onions, apples	quercetin kaempferol myricetin	3,5,7,3',4'-pentahydroxy flavone 3,5,7,4'-tetrahydroxy flavone 3,5,7,3',4',5'-hexahydroxy flavone
Flavones		herbal, celery, green pepper, and camille tea	apigenin luteolin	5,2',5'-trihydroxy flavone 5,7,3',4'-tetrahydroxy flavone
Flavanones		citrus fruits	hesperetin naringenin	5,7,3'-trihydroxy-4'-methoxyflavanone 5,7,4'-trihydroxyisoflavanone
Isoflavones		legumes (soybeans)	genistein daidzein glycitein	5,7,4'-trihydroxy isoflavone 7,4'-dihydroxy isoflavone 7,4'-dihydroxy-6-methoxy isoflavone
Catechins (flavane)		tea, chocolate	catechin epicatechin gallocatechin epigallocatechin	3,5,7,3',4'-pentahydroxyflavan 3,5,7,3',4'-pentahydroxyflavan 3,5,7,3',4',5'-hexahydroxyflavan 3,5,7,3',4',5'-hexahydroxyflavan
Anthocyanides		fruits such as berries and cherries; wine	cyanidin	3,5,7,3',4'-pentahydroxy anthocyanide
Chalcones		apples	phloretin	5,7,9,4'-tetrahydroxy chalcone

predominant isoflavones. Their 7-*O*-glycosides are called genistin, daidzin and glycitin, respectively, and the conjugates of these glycosides found in soy are 6'-*O*-acetylglycosides and 6'-*O*-malonylglycosides containing either an acetyl or a malonyl ß-glucoside in addition to the 7-*O*-glycoside moiety.

History of health claims of isoflavones

The interest in isoflavones originates from the discovery in 1940 that isoflavones in subterranean clover were the cause of infertility effects observed in Western Australian sheep (Bennetts et al., 1946). Formononetin, the 4'-methoxy derivate of daidzein, is the major isoflavone in clover. It can be converted to daidzein and subsequently metabolised to equol (Batterman et al., 1965). These compounds have an estrogenic activity, and equol and daidzein possess a greater estrogenic activity than formononetin. Since then, studies on the estrogenic activity and metabolism of the isoflavones in sheep and other animals have been reported (Bennetts et al., 1946; Braden et al., 1967; Schutt et al., 1970). In general, there was little interest in these phytoestrogens, until the discovery in 1984 of amounts of equol in human urine by Axelson et al. (1984) and Setchell et al. (1984). In addition, when soy based foods were consumed, levels of isoflavones in female urine and blood were shown to far exceed the concentration of endogenous estrogens in humans. Furthermore, a diet rich in soybean has been associated with a lower incidence of hormonally dependent cancers in Asian countries (Barnes et al., 1996; Adlercreutz and Mazur, 1997). The chemopreventive effects of soybeans and soy-containing foods may be related to their isoflavonic content. Asian people consume approximately 50 mg of isoflavones per day, whereas the average daily intake of isoflavones in Western countries has been estimated to be less than 1 mg per day (Kimira et al., 1998; Chen et al., 1999). In 1994, the Japanese Ministry of Health and Welfare approved soy based protein products as food of special health use (FOSHU). In 1999, the Food and Drug Administration (FDA) in the United States approved a cardiovascular health claim that can be used on labels of soy-based foods containing at least 6.25 g of soy protein/ serving. These health claim-approvals stimulated the food industry to develop more soy-enriched foods (examples of so-called functional foods) and soy supplements.

Mechanism of activity of isoflavones

Many studies have confirmed the health promoting activity of isoflavones in preventing heart diseases (FSA, 2003; Kurzer, 2003). The preventive cardiovascular effects of genistein are unclear, but may be due to the inhibition of lipoprotein oxidation (antioxidant activity), lowering of serum cholesterol, inhibition of tyrosine kinase and/or improvement of vascular reactivity (Fotsis et al., 1993; Potter, 1995).

Epidemiological studies also suggest that isoflavones decrease the risk of developing hormonally dependent cancers (i.e. prostate and breast cancer) (reviewed by Bingham et al., 1998; Branca and Lorenzetti, 2005). This is explained by their ability to bind to

the estrogen receptor (Willard and Frawley, 1998; Hargreaves et al., 1999), thereby changing the metabolism of hormones and/or inhibiting cell proliferation. The reason why isoflavones bind to estrogen receptors and exert their typical (anti) estrogenic effects lays in the fact that they are diphenolic compounds with structural similarities to endogenous estrogens. The hydroxyl groups on the 7 and 4'-position of isoflavones seem to be important for this estrogenic activity. Isoflavones are therefore also called phytoestrogens, natural compounds that possess estrogenic activity. Furthermore, isoflavones inhibit steroid biosynthesis enzymes, such as 17 ß-hydroxysteroid oxidoreductase (Mäkälä et al., 1995) and aromatase (Kao et al., 1998). This might result in a reduced or modified production and bioavailability of endogenous estrogens (Mazur and Adlercreutz, 1998).

In addition, genistein has also been shown to interfere directly with the transforming growth factor-ß (TGFß) signaling pathway (Kim et al., 1998). These growth factors regulate cell proliferation and thus, genistein is considered to be an antiproliferative agent. Enzymes involved in cell proliferation include tyrosine protein kinases (Akiyama et al., 1987) and type II DNA topoisomerase (Kaufmann, 1998). Topoisomerases are DNA-associated enzymes that cleave and then reseal either one (topoisomerase I) or two (topoisomerase II) strands of DNA during cellular processes such as replication or transcription, thereby allowing these strands to pass through one another. Interaction with these enzymes can lead to accumulated DNA breaks and mutations, without the need for a covalent interaction with DNA itself (Ferguson, 1994 and 2001). If this would predominantly occur in malignant cells, it would add to the anti-proliferative effects of isoflavones. Topoisomerase II inhibition is one of a number of postulated effects of genistein, including also antiproliferative activity, apoptosis induction and effects on differentiation. Record and co-workers (1997) reported that genistein failed to induce chromosome damage (measured as micronuclei) in mice *in vivo*, despite doing so *in vitro*. Furthermore, a daily intake of 45 mg genistein caused the modulation of the menstrual cycle of premenopausal women (Ross et al., 1994).

Finally, intervention studies showed that isoflavones possess significant bone sparing effects among postmenopausal women (Kurzer, 2003). *In vitro* and animal studies suggest that isoflavones may exert beneficial effects on bone mineral density and bone turnover (reviewed by Setchell et al., 2003c). The exact mechanisms are presently elusive and speculative, but an isoflavone rich diet appears to offer a high potential for the prevention of osteoporosis (Setchell et al., 2003c).

Metabolism of isoflavones

The metabolism of isoflavones may play an important role in the proposed health effects and the mechanism of activity of isoflavones. Numerous metabolism studies of daidzein and genistein in different kind of animals and humans have been reported. It is well established that intestinal cells are the major site of metabolism for genistein and daidzein and not the liver (Setchell, 1998). Genistein and daidzein are predominantly conjugated in the intestine with glucuronic acid and to a lesser extent with sulfate (Adlercreutz et al., 1995). The sulfate and glucuronide conjugates may also be important as carriers of aglycones to target tissues such as breast and prostate. In target tissues these conjugates may be biologically active or hydrolysed to generate the aglycon (Wong et al., 1997). Intestinal bacteria appear to contribute as well to the conversion of formononetin, biochanin A (the 4'-methoxy derivate of genistein), daidzein and genistein.

Figure 1.2 shows a possible pathway for conversion of formononetin and daidzein to equol and *O*-desmethylangolensin (ODMA) and of biochanin A and genistein to 4-ethylphenol. Diadzein can be converted by the gut microflora to dihydrodaidzein, which can be further metabolised to both equol and ODMA (Figure 1.2). Sheep metabolise formononetin into approximately 70% equol and approximately 5-20% ODMA (Braden et al., 1967; Lindner, 1975). In human urine equol, dihydrodaidzein, tetrahydrodaidzein, ODMA and 2'-dehydro-ODMA have been identified (Setchell et al., 2001). Equol is an interesting metabolite, because in humans, only one third of the population is capable of producing it in relatively high amounts (Joannou et al., 1995; Rowland et al., 2000). It has been suggested that equol-producers may have a particular health benefit from soy (Setchell et al., 2002a).

The metabolic pathways suggested for genistein are presented in figure 1.3.It is proposed that genistein is first transformed by gut bacteria to dihydrogenistein, followed by a cleavage of the C-ring to form 6'-hydroxy-ODMA, which can be further degraded by the colonic microflora to 4-hydroxyphenyl-2-propionic acid and trihydroxybenzene. Decarboxylation of 4-hydroxyphenyl-2-propionic acid could lead to the metabolic product 4-ethylphenol.

Oxidative metabolism of the soy isoflavones daidzein and genistein catalyzed by cytochrome P450 enzymes *in vivo* and by liver microsomes *in vitro* showed several hydroxylated metabolites (i.e. 3'-OH-daidzein, 6-OH-daidzein, 8-OH-daidzein and 3'-OH-genistein) (Kulling et al., 2001; Heinonen et al., 2004). 3'-OH-Genistein

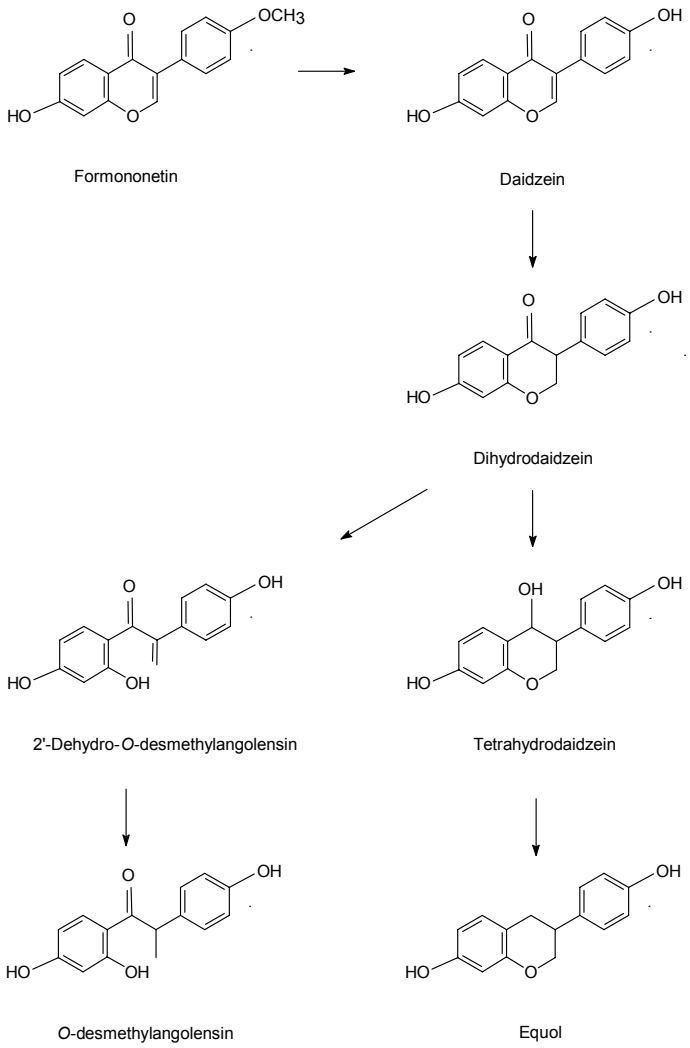


***Figure 1.2**: Potential metabolic pathways of formononetin or daidzein to equol and O-Desmethylangolensin.*

(3',4',5,7-tetrahydroxy-isoflavone) is also known as orobol, which is at least as active as genistein in several *in vitro* tests (Yamashita et al., 1990; Tomonaga et al., 1992).

It can be assumed that genistein and daidzein undergo a further biotransformation by catechol-*O*-methyltransferase (COMT), because the aromatic hydroxylation products of genistein and daidzein contain a catechol or pyrogallol moiety (Kulling et al., 2001). COMT converts catechol like compounds into their less active methoxy-derivates. This may be important because the catechol metabolites of estrogens are

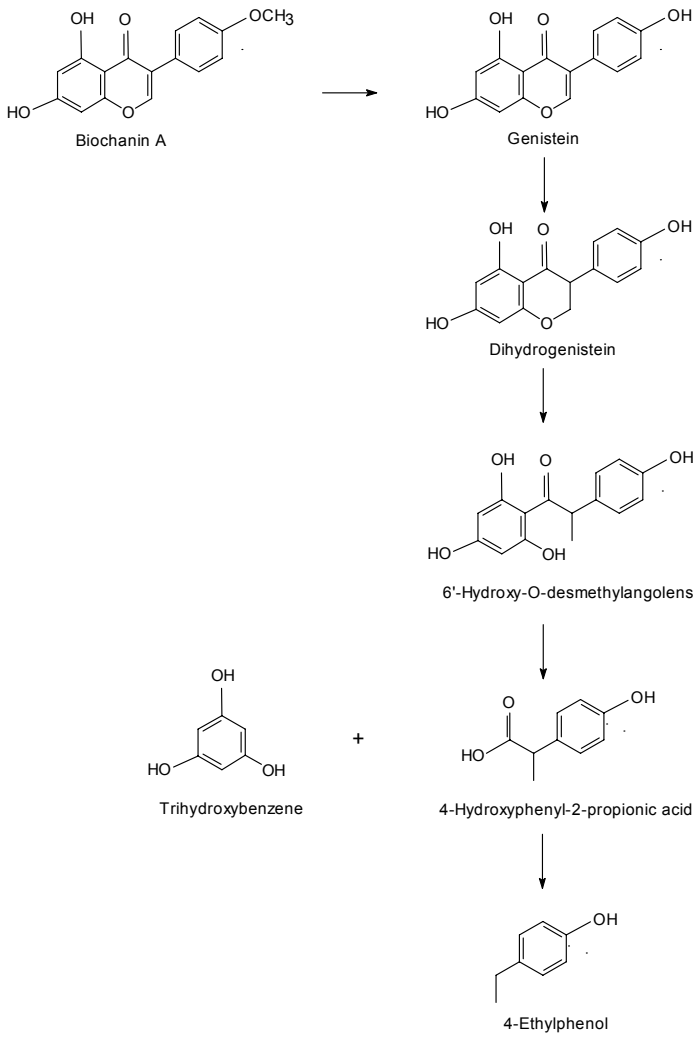


***Figure 1.3**: Potential metabolic pathways of biochanin A or genistein to 4-ethylphenol.*

suspected carcinogens potentially involved in the aetiology of breast cancer (Bolton et al., 2004). The hydroxylation reaction seems to be more important than the reaction of COMT. Each of the methylated catechols was found under *in vivo* conditions in only very small amounts, which suggests that the methylation by COMT does not play a major role in the biotransformation of isoflavones *in vivo* (Scalbert and Williamson, 2000; Kulling et al., 2001).

Phase II enzymes, such as uridine-5'-diphosphate-glucuronosyl transferase (UDPGT) and sulfotransferase catalyze the daidzein and genistein conjugation to glucuronide

and sulfate conjugates. In urine, genistein is mainly excreted, to a level of approximately 53-76%, as a monoglucuronide and to a much lesser extent as a diglucuronide (12-16%) and as a sulfoglucuronide (2-15%) (Adlercreutz et al., 1995). Daidzein occurred in the urine for about 80% as monoglucuronide and for 6-7% as sulfoglucuronide (Adlercreutz et al., 1995).

It is still uncertain what the role of all these metabolites of genistein and daidzein is in the disease aetiology. It can be assumed that metabolites of daidzein and genistein also have biological activities, which may, however, differ from those of the parent compounds. Better knowledge of the consumption and bioavailability of isoflavones and of the biological activity of the major metabolites formed will be essential to properly evaluate the role of isoflavones in the prevention of diseases and in other proposed health claims.

Isoflavone content in food and dietary intake

The main sources of isoflavones in food are soybeans and soy-based products (for instance tofu, tempeh, miso, natto, soy flour, soy milk, isolated soy protein, soy hot-dog and soy supplements) (Reinli and Block, 1996; Wakai et al., 1999; USDA-Iowa State University Database on the Isoflavone Content of Food, 2002). The predominant forms of isoflavones in soybeans and soy-based products are glycosides (Song et al., 1998). The content of isoflavones varies with the crop variety, geographic location, soil type, crop year and environmental factors (Wang and Murphy; 1994a; Hoeck et al., 2000). Processing can also affect the content and type of isoflavones in soy products (Wang and Murphy; 1996). Fermentation of soy into products such as tofu, miso and bean paste reduces the isoflavone content with a factor of 2-3 and cooking has also been shown to reduce the isoflavone content (Coward et al., 1993a). However, baking or frying does not seem to alter the isoflavone content of foodstuffs (Coward et al., 1998). The fermentation process increases the aglycone forms in soy products (Wang and Murphy, 1996; Coward et al., 1998). On the average cooked soybeans, and soy milk powder contain >95% of the total isoflavones as glycosidic forms, whereas soybean in fermented products such as tofu and tempeh contain approximately 20% and 40%, respectively of their isoflavones as aglycone (Wang and Murphy, 1994b). Dietary intakes of about 39 and 47 mg isoflavones/day in the Chinese and Japanese adult population, respectively, have been reported (Chen et al., 1999; Wakai et al., 1999; Arai et al., 2000). However, in the United States, the daily dietary consumption of soy isoflavones in the

general population is below 1 mg/day (De Kleijn et al., 2001; Setchell et al., 2003a). In Finland the mean daily isoflavone intake was estimated to be 0.9 mg/day for men and 0.7 mg/day for women (Valsta et al., 2003). The main sources in Finland appear to be legumes and processed meat products containing soy as an ingredient. The highest human intakes of isoflavones are reported for groups who consume a diet containing isoflavones as supplements (±40-100 mg/day), vegetarians (±75 mg/day), infants consuming soy-based formula (±40 mg/day) and consumers of a traditional South East Asian diet (±100 mg/day). The dietary isoflavone intake of the average Western (including Dutch) population and vegetarians is much lower, namely less than 1-2 mg/day and 3-12 mg/day, respectively (Van-Erp-Baart et al., 2003; Bakker, 2004).

Bioavailability of isoflavones

Food components must be bioavailable in some form to exert their biological effects. Therefore, bioavailability has to be studied in order to establish a relationship between isoflavone intake and its proposed biological activity. Bioavailability is defined as the actual amount available to the human body after administration of an oral dose. Bioavailability is the result of absorption and presystemic clearance, which are influenced by both genetic and life style factors, including intake of food. Information concerning the bioavailability of isoflavones could give insight into the effective dose needed to exert a claimed beneficial health effect such as the prevention of cancer or cardiovascular diseases. Upon soy consumption isoflavones reach micromolar concentrations in the blood (Setchell et al., 2003a). Which means that the bioavailability of isoflavones is low, since a single dose of 50 mg of isoflavone aglycones, leads to a maximum concentration of approximately 2 μM of total aglycones in plasma (reviewed by Manach et al., 2005).

In general, most of the studies report the total daidzein and/or genistein concentration, due to the analytical difficulties of measuring the low concentration of the various isoflavone aglycones and their different metabolites in plasma separately. In humans the peak plasma concentration of daidzein and genistein was reported to occur between 6-10 hours after consumption of soy based food (Setchell et al., 2000 and 2003a). One human study shows that two plasma peaks seem to appear at about 2 and 9 hours after oral dosing (Setchell et al., 2001). Studies with rats also demonstrated that two plasma peaks appeared, the first one observed between 0.5 and 2.5 hours and the second peak between 6-8 hours after oral administration (Coldham et al., 2000

***Table 1.2 A**: Bioavailability studies of isoflavones or isoflavone containing foods studied in humans and rat.*

Source	Species	Dose	Tmax (hour)	Maximum concentration in plasma (µM)	AUC (µmol h/L)	References
Soymilk powder (44% genistein and 56% daidzein)	Female humans (n=12)	0.7 mg soy /kg bw 1.3 mg soy /kg bw 2.0 mg soy /kg bw soy	6.5	0.74 ±0.44 genistein and 0.79 ±0.04 daidzein 1.07 ±0.63 genistein and 1.22 ±0.67 daidzein 2.15 ±1.33 genistein and 2.24 ±1.18 daidzein	n.d.	Xu et al., 1994
Soy protein	Male humans (n=20)	80.3 mg genistein 35.6 mg daidzein		0.90 ±0.25 genistein 0.50 ±0.10 daidzein	n.d.	Gooderham et al., 1996
Genistein and isoflavone-rich soy extract	Male Wistar rats (n-27)	20 mg genistein /kg bw 20 mg equivalent glycosides in soy /kg bw	2	11.0 ±2.3 genistein 4.93 ±0.22 glycosides	n.d.	King et al., 1996
Soy beverages	Male and female humans (n=4)	21.02 mg genistein 13.55 mg daidzein	6.5	0.50 ± 0.09 genistein 0.32 ± 0.09 daidzein	n.d.	Coward et al., 1996
Tofu, Texture vegetable protein (TVP)	Female humans (n=7)	Tofu: 34 mg genistein and 21 mg daidzein TVP: 30 mg genistein and 26 mg daidzein	6.5	1.36 ±0.49 genistein and 1.43 ±0.64 daidzein 1.29 ±0.37 genistein and 1.46 ±0.45 daidzein	n.d.	Tew et al., 1996
Soy flour in milk, single meal	Male humans (n=6)	0.97 mg genistein /kg bw 0.69 mg daidzein /kg bw	8.0 7.4	4.09 ±0.94 genisetin 3.14 ±0.36 daidzein	n.d.	King and Bursill, 1998
Baked soybean powder	Male humans (n=7)	27.8 mg genistein 28.5 mg daidzein	6	2.44 ±0.65 genistein 1.56 ±0.34 daidzein	n.d.	Watanabe et al., 1998
Diets with various concentration genistein	Male and female Sprague-Dawley rats (n=6)	5 µg genistein /g feed (=18.5 µmol /kg feed) 100 µg genistein /g feed (=380 µmol /kg feed) 500 µg genistein /g feed (=1852 µmol /kg feed)		male 0.06 ±0.01, female 0.10 ±0.008 genistein male 0.59 ±0.03, female 0.94 ±0.21 genistein male 6.00 ±0.65, female 7.94 ±2.47 genistein	n.d. n.d. male 22.3 ±1.2 and female 45.6 ±3.1	Chang et al., 2000
Soy diet (190 days)	Male Sprague-Dawley rats Female Sprague-Dawley rats (n=22-25)	30 µg genistein /g feed 30 µg daidzein /g feed 30 µg genistein /g feed 30 µg daidzein /g feed		0.35 ±0.03 genistein 0.20 ±0.02 daidzein 0.62 ±0.05 genistein 0.25 ±0.02 daidzein	n.d.	Doerge et al., 2000

***Table 1.2 B**: Bioavailability studies of isoflavones or isoflavone containing foods studied in humans and rat.*

Source	Species	Dose	Tmax (hour)	Maximum concentration in plasma (µM)	AUC (µmol h/L)	References
^{14}C genistein	Male (n=5)	4mg ^{14}C genistein (1.833 MBq) /kg bw	2 and 7	8.33 genistein	52.4 genistein	Coldham et al., 2000
	female Wistar rats (n=5)	4mg ^{14}C genistein (1.833 MBq) /kg bw	2 and 7	2.22 genistein	30.9 genistein	
Tablets aglycones (fermented, glycosides soybean extract)	Male (n=4) and Female humans (n=4)	0.78 mmol genistein 0.92 mmol daidzein 0.90 mmol genistin 0.80 mmol daidzin	4 4 4 4	21 genistein 16 daidzein 3 genistin 2.5 daidzin	n.d.	Izumi et al., 2000
Genistein, daidzein, genistin and daidzin	Premonopausal female humans (n=19)	50 mg genistein, 50 mg daidzein 50 mg genistin 50 mg daidzin	2 and 9.3 2 and 6.6 2 and 9.0 2 and 9.3	1.26 ±0.27 genistein 0.76 ±0.12 daidzein 1.22 ±0.47 genistin 1.55 ±0.24 daidzin	16.7±.5.2 genistein 11.6 ±0.9 daidzein 11.4 ±2.4 genistin 10.9 ±1.2 daidzin	Setchell et al., 2001
Formulated purified isoflavones	Postmenopausal female humans (n=24)	formulation A 100% unconjugated: 16 mg/kg bw formulation B 70% unconjugated: 16 mg/kg bw	3.1 free aglycones 5.6 total isoflavones	28.3 genistein and 3.1 daidzein 25.4 genistein and 8.9 daidzein	1.3 genistein and 48 daidzein 2.3 genistein and 135 daidzein	Bloedon et al., 2002
Purified soy	Male humans (n=30)	Formulation A(90% genistein) 16 mg kg bw Formulation B (43%genistein) 16 mg/kg bw	3.5 free 5.7 total	Formula A: 7.6 genistein and 1.3 daidzein Formula B: 27.5 genistein and 16.9 daidzein	0.180 genistein	Busby et al., 2002
Aglycone tablets and glucoside tablets	Female humans (n=15)	Aglycone tablets: 10.72 mg isoflavones (43% as genistein) Glucoside tablets: 17.24 mg isoflavones (53% as genistein derivate)	4 genistein and 5 daidzein 5 genistein and 4 daidzein	0.534±0.333 genistein and 0.530±0.205 daidzein 0.596±0.294 genistein and 0.396±0.104 daidzein	8.3±4.2 genistein and 8.3 ±2.6 daidzein 8.9±4.7 genistein and 6.2±1.7 daidzein	Zubik and Meydni, 2003
^{13}C genistein ^{13}C daidzein	Premenopausal female humans (n=16)	0.8 mg ^{13}C genistein /kg bw 0.8 mg ^{13}C daidzein /kg bw	5.5 ^{13}C genistein 7.4 ^{13} C daidzein	0.55 ±0.09 genistein 0.31±0.07 daidzein	6.75 ±1.29genistein 3.96 ±0.71daidzein	Setchell et al., 2003
Soy-derived phytoestrogens	Male Sprague-Dawley rats (n=3)	3 mg genistein /kg bw and 3 mg daidzein /kg bw	2 genistein and 2 daidzein	0.13 genistein and 0.20 daidzein	0.84 genistein and 1.08 daidzein	Mallis et al., 2003

AUC = area under the curve, Tmax= time to reach maximum plasma concentration, bw = body weight, n.d.= not detected

and 2002). The appearance of two plasma peaks may indicate that there is an enterohepatic circulation and that genistein or daidzein is absorbed for a second time. This enterohepatic circulation may have important implications, because it delays the excretion and increases the duration of exposure at the target tissues. Table 1.2 summarizes the bioavailability studies of isoflavones performed in humans and rats.

The table reveals differences in kinetics between humans and rats. Contradictory results have been obtained by studying the differences between the bioavailability of aglycones and glycosides. Some authors found a greater bioavailability of glycosides (Setchell et al, 2001), whereas others found a greater bioavailability of aglycones (Izumi,et al., 2000). Finally, no significant differences have been found by Richelle et al. (2002) and Zubik and Meydani (2003). Above all, the chemical structure of polyphenols determines their rate and extent of intestinal absorption and the nature of the metabolites circulating in the plasma. Understanding the various factors that influence absorption and metabolism of isoflavones and especially of their naturally occurring glycosides is essential to fully elucidate and understand the biological activity of these compounds.

Mechanism of absorption of isoflavones

In general, flavonoids are attached to sugar residues which affect the mechanism of absorption. For instance, flavonoid glycosides show a greater hydrophilicity than their respective aglycones (Brown et al., 1998). Aglycones are lipophilic molecules with an octanol/water partition coefficient between 0 and 4 (Rothwell et al., 2005). Lipohilic molecules can interact with the (lipo)proteins in the membrane of the intestinal cell and can pass by an energy-independent passive diffusion through the lipid bilayers. The flavonoid glycosides are hydrophilic and, therefore, can not readily pass the lipid bilayers. This implies that removal of the hydrophilic moiety is necessary before the passive diffusion across the small intestinal brush border membrane can occur. Therefore, it is postulated that a first step of metabolism should be a removal of the sugar by enzymes (glycosidases). Glycosidase activities can occur in the food itself (endogenous or added during processing) or can originate from the cells of the gastrointestinal mucosa or from the colonic microflora.

The originally proposed model for absorption of glycosides assumes that the glycosides were too polar to be absorbed by cells of the small intestine, but that the absorption has to occur in the large intestine after bacterial deconjugation (Griffiths

and Barrow, 1993). However, the rapid absorption of dietary flavonoid glycosides suggested a small intestinal absorption (Hollman et al., 1995; Walle et al., 2000; Sesink et al., 2001). Currently, it is postulated that a potential mechanism of glycoside absorption in the small intestines may proceed by the extracellular hydrolysis of the glycosides by a ß-glycosidase such as lactase phloridzin hydrolase (LPH) located on the brush border membrane of the intestinal cells. This ß-glycosidase may cleave off

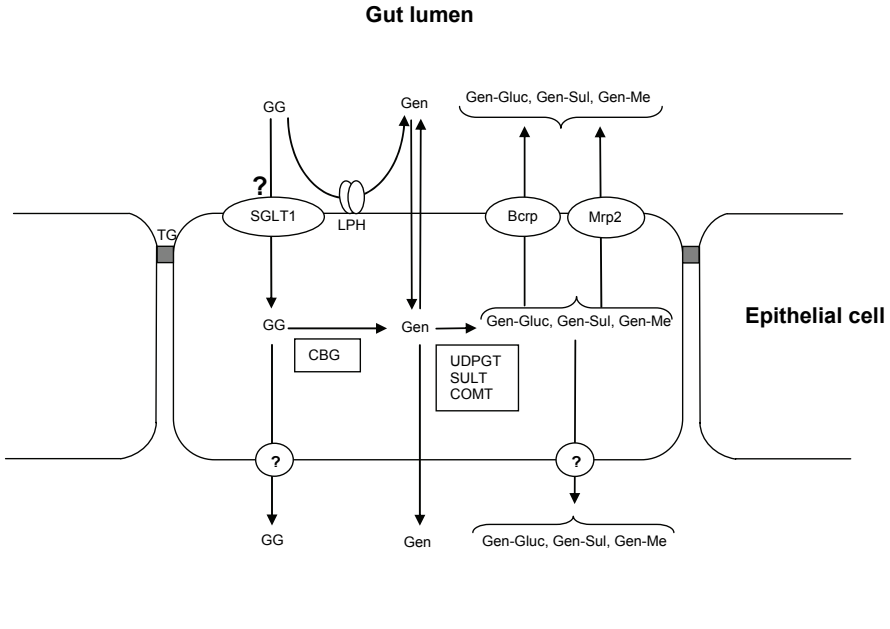


Figure 1.4*: Potential scheme for absorption of genistin by small intestinal cells without already taking the results of the present thesis into account. Genistin (GG) is hydrolysed by lactase phloridzin hydrolase (LPH) and free genistein (Gen) enters the cell by passive diffusion. On the other hand, it has been suggested that genistin is transported by the sodium dependent glucose transporter (SGLT1), and after entering the cell, genistin is converted by intracellular ß-glucosidases (CBG) to genistein. Once in the cell genistein is transported to the blood or conjugated, for example, with glucuronic acid (gen-Gluc) by uridine-5'-diphosphate-glucuronosyltransferases (UDPGT), sulfate (Gen-Sul) by sulfotransferases (SULT) and/or methyl groups (Gen-Me) by catechol-O-methyltransferases (COMT) and the conjugates are transported either to the blood or back to the intestinal lumen by various not yet fully identified transporters at the apical or basolateral membranes, such as breast cancer resistance protein (Bcrp) or multidrug resistance-associated protein 2 (Mrp2) which are located at the apical membrane at respectively the basolateral and apical membranes.*

the sugar moiety, after which the aglycone diffuses across the membrane into the entrocyte. On the other hand, glycosides may enter the intestinal cell as intact glycosides via the sodium dependent glucose transporters (SGLT1) (Gee et al., 1998; Walgren et al., 1998). When taken up in the intact form, the intracellular cytosolic ß-glycosidases (CBG) may convert the glycoside to generate its aglycon (Lambert et al, 1999; Ioku et al., 1998 and Day et al., 1998). In the enterocyte, the aglycon can be conjugated with glucuronic acid by uridine-5'-diphosphate-glucuronosyltransferases or with sulfate by sulfotransferases present in the enterocyte (Day et al., 1998). Subsequently, the aglycon and/or its metabolites enter the blood stream, or can be transported by apical intestinal membrane transporters like breast cancer resistance protein (BCRP1/ABCG2) or multidrug resistance-associated protein 2 (MRP2) back to the intestinal lumen (Walle et al., 1999; Imai et al., 2004; Sesink et al., 2005). Figure 1.4 presents a schematic overview of the mechanisms suggested for isoflavone glycoside absorption in the intestinal tract without taking the obtained results of the present thesis into account.

Research goals

Most isoflavones in plants are bound to sugars such as the glycosides genistin and daidzin. Generally, these glycosides are found to be less biological active than their corresponding aglycones (Setchell, 1998). Therefore, understanding the various factors that influence absorption and metabolism of isoflavones and especially of their naturally occurring glycosides is essential to fully elucidate and understand the activity of these dietary ingredients. This is also essential to fully explore the possible beneficial health effects of the isoflavones, so-called non-nutrients, which are considered to be promising as healthy constituents in enriched (novel) foods or supplements.

The aim of this thesis is to obtain data on the bioavailability and metabolism of the isoflavones genistein and daidzein and their glycosides genistin and diadzin. Special focus is directed to the possible role of SGLT1 in the mechanism involved in the transport and absorption of the isoflavonic glycoside genistin. Another objective is to explore, develop, validate and/or optimise *in vitro*, *in situ* and *in vivo* gastrointestinal model systems for studying the bioavailability and metabolism of genistein, daidzein and their glycosides. Validation of these models is based on a comparison with data from existing human and rat *in vivo* studies.

Upon oral intake, the gastrointestinal tract is the first organ system to interact with isoflavones. The epithelium structure of the intestinal wall is very complex because of its multifunctional characteristics (Ilett et al., 1990; Hillgren et al., 1995). It is very difficult to reproduce *in vitro* all of the characteristics of the intestinal mucosa (Kararli, 1995). Nevertheless, *in vitro* models permit a relatively rapid and mechanistic evaluation of the intestinal absorption and metabolism of compounds under well controlled circumstances. *In vivo* methods are ideally required to examine the bioavailability of a food compound, but there are also ethical aspects arguing in favour of reducing, replacing and refining the use of laboratory animals (Barr and Riegelman, 1970). In order to improve the effectiveness of food research, a balance between the *in vitro* and *in vivo* methods seems to be the most optimal and ethical model system. Therefore, testing in great detail the absorption characteristics of isoflavones across the intestine requires a combination of *in vitro* and *in vivo* techniques. Figure 1.5 shows the generally applied scheme for combining *in vitro* to *in vivo* studies and allowing an animal to human extrapolation ultimately aiming at predicting the human situation. The figure also indicates how these approaches link to the studies of the present thesis.

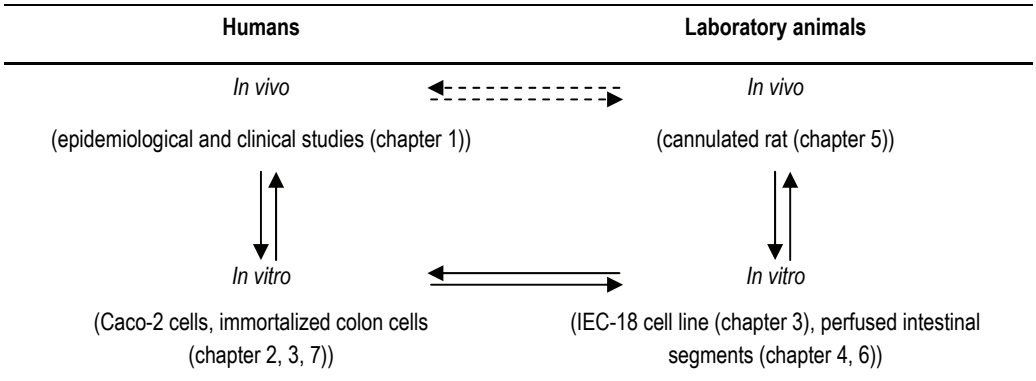


***Figure 1.5**: Comparative analysis of the toxicity and bioavailability by using in vivo and in vitro animal or human data. Solid lines represent data comparisons and dotted lines represent extrapolations (Huggett et al., 1996).*

Outline of the thesis

In chapter 1 an overview of the relevant literature is given and the objective of the thesis is described. First, the aim is focused on the bioavailability and intestinal metabolism of genistein and daidzein and their glycosides and the second objective is to explore, develop and/or optimise *in vitro, in situ* and *in vivo* gastrointestinal models for our bioavailability studies. To select the most optimal and reliable cellular model for these bioavailability studies in chapter 2 and 3 several *in vitro* models for human intestinal absorption and metabolism are compared. Human colonic Caco-2 cells, rat small intestinal IEC-18 cells and human immortalised colonic HCEC cells are chosen as well-organised monolayers grown on semi-permeable filters. In these three different cellular systems the transport and metabolism of genistein, daidzein and their glycosides are analysed and compared to each other. In the course of the further development and optimisation of intestinal transport models, various rat models are used in additional studies of the present thesis. In chapter 4 the fate and location of the intestinal absorption of genistein and its glycoside is studied in various isolated segments of the intestinal tract of the rat. In chapter 5 an *in vivo* rat study is conducted, describing an *in vivo* bioavailability experiment for genistein and its glycoside genistin. The rats are cannulated in the portal vein and can freely move. Genistein and genistin are dosed either as pure compounds or extracted from soy enriched protein isolate.

To study the mechanism of absorption of genistin and the role of SGLT1 in this process, *in situ* perfusion of intestines of rats is performed in chapter 6 with genistein and genistin in the absence or presence of phloridzin. Phloridzin is been used as a competitive inhibitor of SGLT1. Rat intestines with an intact vascular circulation are perfused and at the same time, a cannulation of the portal vein is carried out. In this model the intestinal transport of genistein and genistin in the absence or presence of phloridzin was characterized. In addition, the mechanism of absorption of genistin by SGLT1 in a selected *in vitro* model system is investigated in chapter 7 in order to assess if results from the *in vitro* model can match those obtained *in vivo* (chapter 6). To this end the Caco-2 cell line is used to confirm that the Caco-2 cells are a good model for mechanistic studies, and the transport of genistin or genistein through Caco-2 monolayers was studied in the presence or absence of phloridzin. Finally, the major results and conclusions achieved in this thesis are summarised in chapter 8.

Chapter 2

Bioavailability of genistein, daidzein and their glycosides in intestinal epithelial Caco-2 cells

Abstract

In this study information was obtained on bioavailability of genistein, daidzein and their glycosides in human intestinal epithelial Caco-2 cells grown on semi-permeable filters. The integrity of Caco-2 monolayers was confirmed by transepithelial electrical resistance measurements and by determination of the permeability of the radioactive marker polyethylene glycol (PEG4000). After 6 hours approximately 30 to 40% of genistein and daidzein added at the apical side was transported to basolateral side and this level was maintained for 24 hours. The glycosides were hardly transported through the Caco-2 cells. No significant metabolism of genistein and daidzein in the Caco-2 cells occurred, whereas the glycosides were mainly metabolised to their respective aglycones. Obviously, our data indicate that Caco-2 cells contain an endogenous glycosidase activity.

Introduction

Genistein and daidzein may help to prevent hormone-related cancer and cardiovascular diseases (Barnes et al., 1996; Kardinaal et al., 1997; Kurzer and Xu, 1997). Genistein and daidzein are naturally occurred estrogens in soy bean and soy foods and belong to the class of isoflavones (Barnes, 1997; Kurzer and Xu, 1997). Most isoflavones in plants are bound to sugar for instance the glycosides genistin and daidzin, respectively (Figure 2.1) (Adlercreutz et al., 1994; Messina et al., 1994). Data on the bioavailability of isoflavones and their glycosides are scarce (Messina et al., 1994). Caco-2 cells derived from human colon adenocarcinoma are being

Based on: Steensma, A., Noteborn, H.P.J.M., Van der Jagt, R.C.M., Polman, T.H.G., Mengelers, M.J.B. and Kuiper, H.A. (1999) Bioavailability of genistein, daidzein and their glycosides in intestinal epithelial Caco-2 cells. *Environmental Toxicology and Pharmacology* **7**, 209-211.

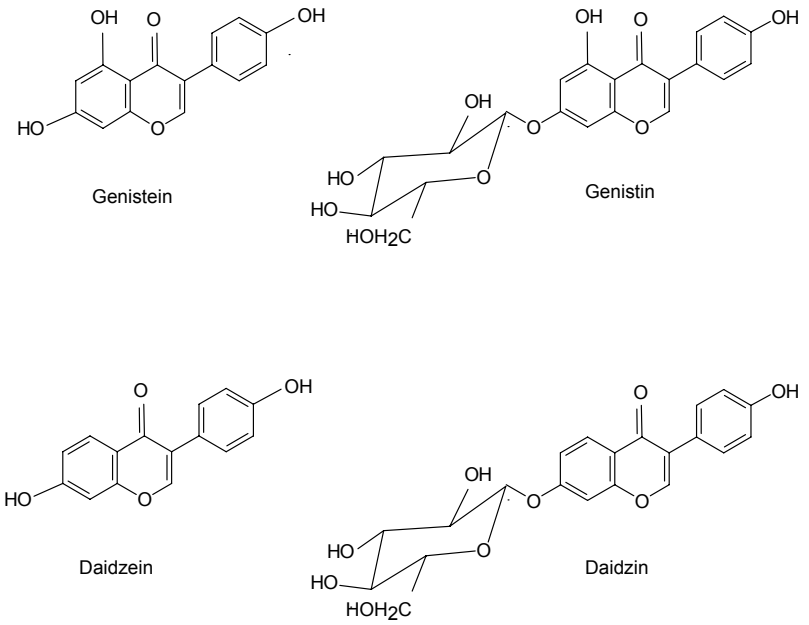


Figure 2.1. *Structure of genistein (4',5,7-trihydroxyisoflavone), daidzein (4',7-dihydroxyisoflavone) and their glycosides genistin (4',5,7-trihydroxyisoflavone 7-glucoside and daidzin (4',7-dihydroxyisoflavone 7-glucoside).*

frequently used for intestinal absorption studies (Hidalgo et al., 1989; Hilgers et al., 1990; Artursson and Karlsson, 1991b; Zucco et al., 1994).

The aim of the present study was to obtain data on transport and its mechanism of genistein, daidzein and their glycosides with Caco-2 cells grown on semi-permeable filters.

Materials and methods

Materials

Caco-2 cells originating from human colorectal carcinoma were obtained from the American Type Culture Collection (ATCC 37-HTB, Rockville, MD). Genistein, daidzein, genistin and tissue culture media were obtained from Sigma Chemical Co. (St. Louis, MO). Daidzin was purchased from Plantech (Reading, GB).

Cell culture

Caco-2 cells were grown at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity and in Dulbecco's modified Eagle's medium (DMEM) with high glucose (4.5 g/L) supplemented with 10% heat inactivated fetal calf serum (FCS), 2% penicillin /streptomycin solution and 1% non-essential amino acid. The cells were expanded in tissue culture flasks (75-cm^2) and when the cells were 90% confluent, the cells were detached from the flasks by treatment with trypsin (0.25% in Hank's balanced salt solution without calcium, magnesium and phenol red) and 0.05% w/v EDTA.

For transport experiments Caco-2 cells were seeded at a density of 2.10^6 on Transwell-clear, tissue culture treated polyester membrane filter inserts (pore size 0.4 mm, diameter 24 mm, apical volume 2 ml, basolateral volume 3 ml, Costar, Badhoevedorp, The Netherlands). Between passage number 35-40 cells were allowed to grow and differentiate to confluent monolayers for 20-22 days. The medium was changed twice a week.

Integrity of the monolayers

The integrity of the monolayers was checked from transepithelial electrical resistance across the layer (TEER) measurements and from permeability to the radioactive marker, polyethylene glycol (MW 4000) (^{14}C PEG_{4000}). The TEER values were calculated by subtraction of the intrinsic resistance of the filters without cells (Ohm cm^2).

^{14}C PEG_{4000} was added to the apical side of the monolayers and the transport of the labelled marker was followed for up to 24 hours at 37°C. Samples of 50 ml were measured in a liquid scintillation counter.

Transport experiments

All transport experiments were performed at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity and in Hank's balanced salt solution. The Hank's balanced salt solution contained calcium chloride.H_2O 0.185 g/L, magnesium sulfate (anhydrous) 0.09767 g/L, potassium chloride 0.4 g/L, potassium phosphate monobasic (anhydrous) 0.06 g/L, sodium chloride 8.0 g/L, sodium phosphate dibasic (anhydrous) 0.04788 g/L and D-glucose 1.0 g/L. The transport experiments were initiated by washing monolayers with buffer before the stock solutions were added to the apical side of the cells. Stock solutions of genistein, daidzein and their glycosides genistin

and daidzin were prepared in dimethylsulfoxide (DMSO) and were diluted in medium at a maximum of 0.2% (v/v) DMSO. Test solutions of genistein, daidzein and their glycosides were used separately at a substance concentration of 50 mM in 3 experiments. Samples of 100 ml were taken from the basolateral side for up to 24 hours. Transport of the test compounds across the cells was expressed as a fraction of initial amount applied to the apical side.

HPLC

Samples were analysed by reversed-phase HPLC equipped and an UV photodiode array detector with a Supelcosil LC-ABZ (250*4.6 mm, 5 mm) column (Supelco, Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). The column was eluted at a flow rate of 1 ml/min employing a concave gradient at 30°C for 60 minutes. The eluent was monitored at 260 nm.

Results and discussion

Integrity of the monolayer

The transmembrane resistance of Caco-2 monolayers was measured on Transwell tissue culture polyester filters during transport of genistein, daidzein and their glycosides. The transepithelial electrical resistance decreased in the first 3 hours, but reached a constant level at about 400 Ohm.cm^2 (Figure 2.2).

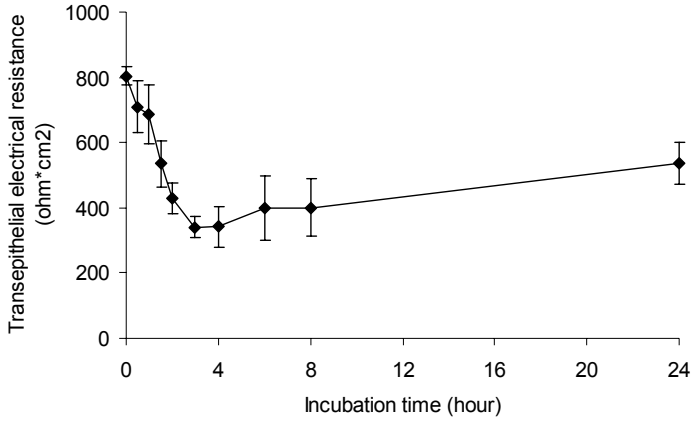


Figure 2.2: *Transepithelial electrical resistance over Caco-2 monolayers as a function of incubation time. Data are expressed as mean ± SD of threefold measurements.*

This initial decrease was subsequently observed throughout all time points during the 3 monolayers studies and have been reported also by others (Hilgers et al., 1990; Zucco et al., 1994; Pasternak and Miller, 1996).

Transport of ^{14}C PEG $_{4000}$ across the Transwell tissue culture polyester filters was studied in apical to basolateral direction (Figure 2.3).

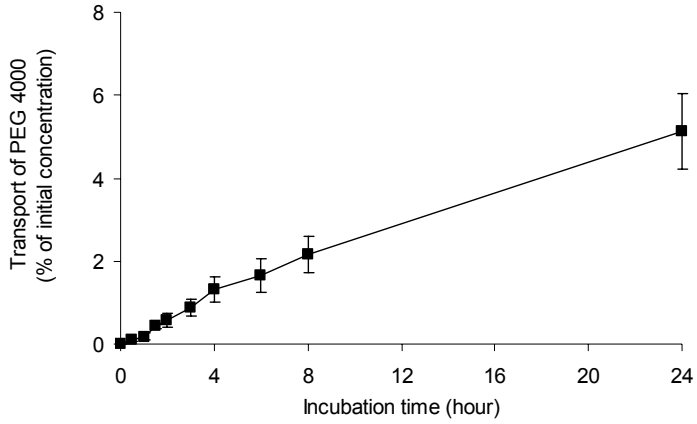


***Figure 2.3**: Transport of ^{14}C PEG $_{4000}$ (initial concentration of 5 mM) across Caco-2 monolayers. Data are expressed as mean ± SD of three fold measurements.*

The Caco-2 cells were almost impermeable to the radioactive marker as only small amounts of ^{14}C PEG_{4000} were transported across the Caco-2 monolayer. A result illustrative for an intact cell monolayer as the marker should not be transported across the enterocytes as has been reported before (Meunier et al., 1995; Pasternak and Miller, 1996; Murkies et al., 1998).

Transport experiments

Transport of genistein, daidzein and their glycosides in the apical to basolateral direction across the Caco-2 monolayers is shown in figure 2.4.

There was a significant difference in transport and metabolism of genistein, daidzein and their glycosides in Caco-2 cells (Figure 2.4). After about 6 hours 30 to 40% of genistein and daidzein added at the apical side was transported to the basolateral side and they remain constant for 24 hours. However, the glycosides were hardly transported across the Caco-2 monolayer. Genistein and daidzein was not significantly metabolised in the Caco-2 cells, whereas genistin and daidzin were mainly metabolised to genistein and daidzein a fraction of 20% ±2% was converted to

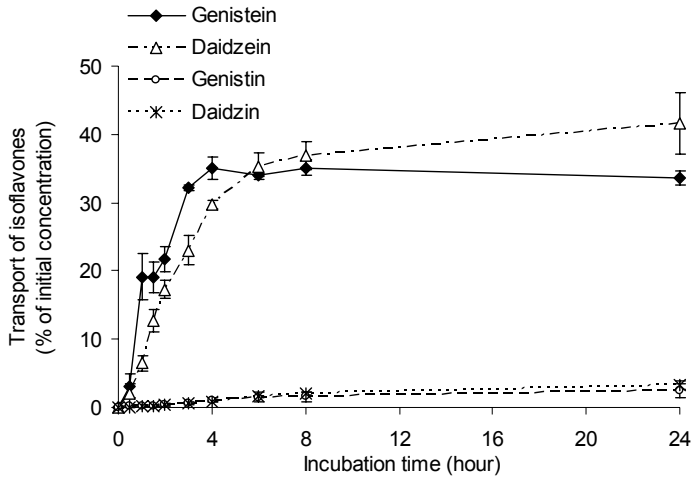


***Figure 2.4**: Transport of genistein, daidzein and their glycosides genistin and daidzin across Caco-2 cells. The initial substance concentrations were 50 μM for genistein,daidzein, genistin and daidzin. Data are expressed as mean ± SD of triplicate measurements.*

aglycones on the basolateral side. Polar metabolites of genistin, daidzin were also formed to a minor extent and preliminary results indicated the presence of phase II conjugates e.g. glucuronides and sulfates.

In summary, Caco-2 cells grown on semi-permeable filters represent a useful system for study of the transport and its mechanism of isoflavones *in vitro*. The integrity of the Caco-2 monolayer was satisfactorily as indicated by the transepithelial electrical resistance and ^{14}C PEG $_{4000}$ probing. Genistein and daidzein were transported through the Caco-2 cells, whereas their glycosides were hardly transported. Moreover, genistin and daidzin were metabolised in Caco-2 cells to genistein and daidzein, respectively. Obviously, our data indicating that Caco-2 cells contain an endogenous glycosidase activity. The relative contribution of the microflora to the deglycosylation process remains to be studied in more detail. The main site of absorption of the isoflavones in the gut will be studied with *in vivo* models.

Chapter 3

Comparison of Caco-2, IEC-18 and HCEC cell lines as a model for intestinal absorption of genistein, daidzein and their glycosides

Abstract

Genistein and daidzein receive much attention because of their potential to prevent hormone-related cancer and cardiovascular diseases. Limited information is available on the pharmacokinetics of these compounds like, for instance, intestinal uptake by humans and systematic bioavailability. In this study the transport and metabolism of genistein, daidzein and their glycosides has been compared in various cellular models for intestinal absorption such as human colonic Caco-2, rat small intestinal IEC-18 and human immortalised colon HCEC cell lines.

Genistein and daidzein were taken up by Caco-2, IEC-18 and HCEC cells and transported to almost same rate and extents. Glycosides were transported across IEC-18 and HCEC monolayers, but not across Caco-2 cells.

In Caco-2 and IEC-18 cells the glycosides were metabolised to their respective aglycones. Furthermore, it was shown that genistein and daidzein were glucuronidated and sulfated in Caco-2 cells, to glucuronidated forms in IEC-18 cells and to sulfated conjugates in HCEC cells.

The results of this study compared with reported *in vivo* data indicate that Caco-2 cells are a valuable model for studying intestinal transport and metabolism of isoflavones

Based on: Steensma, A., Noteborn, H.P.J.M. and Kuiper, H.A. (2004) Comparison of Caco-2, IEC-18 and HCEC cell lines as a model for intestinal absorption of genistein, daidzein and their glycosides. *Environmental Toxicology and Pharmacology* **16**, 131-139.

Introduction

Genistein and daidzein belong to the class of isoflavones and have been reported to exhibit a variety of biological effects on human health (reviewed by Barnes et al., 1996; Adlercreutz and Mazur, 1997; Setchell and Cassidy, 1999; Clarkson, 2000). *In vitro* experiments showed that these compounds decrease the risk of chronic (cardiovascular and cancer) diseases (Fotsis et al., 1993; Lethonen et al., 1996; Hodgson et al., 1996;) and inhibited enzymes like tyrosine kinase (Akiyama et al., 1987), topoisomerase II (Kaufmann et al., 1998) and steroid-metabolizing enzymes (Wong et al., 1997). On the other hand, results of *in vivo* studies of these compounds are controversial and it has not yet been able to confirm conclusively the positive health effects (McIntosh et al., 1995; Record et al., 1997; Rao et al., 1997; Davies et al., 1999; Gee et al., 2000b).

Soybeans and soy-derived foods contain a relatively high concentration of isoflavones (1-3 mg/g) (Coward et al., 1993b; reviewed by Reinli and Block, 1996). In crops and derived food products these compounds are bound to a sugar group primarily in the form of ß-glycoside conjugates and, therefore, called genistin and daidzin.

There is a need to study the pathways through which the compounds are absorbed in order to assess the systemic bioavailability and, thus, the efficacy of these compounds. The Caco-2 cell line is frequently used in drug absorption studies for intestinal absorption models (Artursson, 1991a). This cell line is derived from a human colon adenocarcinoma and differentiates spontaneously into morphological and functional characteristics of the human small intestinal cell. These cells form tight junctions and develop microvilli. They also express brush border enzymes and growth factor receptors (reviewed by Bailey et al., 1996; Delie and Rubas, 1997). Previous studies with Caco-2 cells showed that the isoflavones without a sugar group (i.e. aglycones) were readily transported, whereas the glycosides passed hardly through the monolayers of cells (Steensma et al., 1999; Walle et al., 1999).

On the other hand, the Caco-2 cell line in absorption model studies may have certain disadvantages as the tight junctions display a tightness commonly found in the colonic epithelium. Moreover, the cell shows a different cytochrome P450s pattern compared to human intestinal tissue (Delie and Rubas, 1997).

This could mean that the use of the Caco-2 cell line as a model in predicting the uptake of poorly absorbable compounds might have limitations. Certainly, natural compounds that are transported paracellularly would probably be inaccessible in this model. In contrast, IEC-18 cells seem to be more useful in an absorption model to obtain information on the uptake rates of paracellularly transported compounds (Ma et al., 1992; Quaroni and Hochman., 1996; Duizer et al., 1997). IEC-18 cells are a small intestinal crypt cell line derived from the rat ileac epithelium and are also used as a model to study small intestinal permeability. In addition, the IEC-18 cells closely resemble those of the small intestine, which could indicate a comparable leakiness/ tightness of the paracellular pathway as observed in human intestinal tissue.

A second deficiency is that intestinal cells in culture, like Caco-2cells, show a different cytochrome P450 pattern compared to human intestinal tissue. However, it was recognised that the immortalised human colonic epithelial cell line HCEC expressed cytochrome P450s (i.e. CYP1A1, 2C, 2D6, 2E1, 3A4/5 with CYP3A) at a similar level as observed in human colonic tissue (Macé et al., 1998). Another feature appears to be that this transformed cell line possesses a gut epithelial character by forming tight junctions and desmosomes. Moreover, the differentiation and intestinal properties of HCEC cells were confirmed by the expression of alkaline phosphatase and sucrose isomaltase after induction with TGF-b (Macé et al., 1998).

In the course of the development of valid *in vitro* models for human intestinal absorption and metabolism, these three cell lines (i.e. human colonic Caco-2, rat small intestinal IEC-18 and human immortalised colonic HCEC) were chosen as well-organised monolayers grown on semi-permeable filters (Steensma et al., 1999). Cell systems intended to be a simulation of the epithelial cell lining of the human gut. The aim of the present investigation was to select the most optimal and reliable cellular model. To achieve this goal, the transport and metabolism of genistein, daidzein and their glycosides was analysed in the three different cellular systems and compared to each other.

Materials and Methods

Materials

Caco-2 cells, originating from a human colorectal carcinoma and IEC-18 cells, originating from the rat ileac epithelium, were obtained from the American Type Culture Collection (Rockville, MD). HCEC, primary human adult colonic cells were

derived from scratch biopsy of a healthy donor and immortalised by infection with SV40 large T antigen. The HCEC cells were a generous gift from the Nestlé Research Centre (Lausanne, Switzerland). Genistein, daidzein, genistin, ß-glucuronidase, sulfatase and tissue culture media were obtained from Sigma Chemical Co. (St. Louis, MO). Daidzin was purchased from Plantech (Reading, UK). The radioactive labelled markers [^{14}C] polyethyleneglycol (PEG4000) (MW 4000, specific radioactivity 60 μCi/mmol) and D-[1-^{14}C] mannitol (MW 182, specific radioactivity 56 mCi/mmol) were obtained from Amersham (Buckinghamshire, UK).

Cell cultures

Caco-2 and IEC 18 cells were grown as described by the supplier in Dulbecco's modified Eagle's medium (DMEM) in tissue culture flasks (75 cm^2) at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity. DMEM contained glucose (4.5 g/l) supplemented with 10% (v/v) heat inactivated foetal calf serum (FCS), 100 units/ml penicillin, 0.1 mg/ml streptomycin and 1% (v/v) non-essential amino acids for Caco-2 cells. IEC 18 cells contained additionally 0.1 units/ml of insulin in DMEM and less (5% (v/v) FCS. When the cells were 90% confluent, they were detached from the flasks by treatment with trypsin (2.5 g/l) in Hank's balanced salt solution without calcium, magnesium and phenol red but supplemented with 0.5 g/L EDTA.

HCEC cells were a gift of E. Offord, K. Macé and A.M.A. Pfeifer from Nestlé Research Centre, Lausanne, Switzerland. HCEC cells were grown as described by the supplier in A52 medium of Biofluids (Rockville, MD) in tissue culture flasks (75 cm^2) coated with 0.17 mg matrigel basement membrane matrix (Becton Dickinson, Bedford, NJ) at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity. A52 medium was supplemented with 2 mM glutamine, 38 μg/ml vitamin C, 100 nM retinoic acid, 1 nM dexamethasone and 30 μg/ml of bovine pituitary extract. When the cells were 90% confluent, the cells were detached from the flasks by treatment with trypsin (0.25 g/L) in Hank's balanced salt solution without calcium, magnesium and phenol red but supplemented with 0.5 g/l EDTA. Trypsin activity was neutralised with soybean trypsin inhibitor of Biofluids (Rockville, MD).

Transport experiments

Transport experiments were carried out by a modification of the method described by Duizer et al. (1997). For transport experiments Caco-2 cells (passage 35-60), HCEC cells (passage 63-80) and IEC-18 (passage 20-30) were cultivated on Transwell-clear tissue culture treated polyester membrane filter inserts (pore size 0.4 mm, diameter 24

mm) of Costar (Badhoevedorp, The Netherlands). The apical volume was 2.0 ml and basolateral volume was 3.0 ml, and cells were seeded at a density of 2.10^5 cells/ml. Cells were allowed to grow and differentiate to confluent monolayers for 20-22 days by changing the medium twice a week. Transport studies with Caco-2 and IEC-18 cells were performed in Eagle's Minimum Essential Medium (EMEM) at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity. Transport studies with HCEC cells were performed in A52 medium under similar atmospheric and temperature conditions as with Caco-2 and IEC 18 cells. The transport experiments were initiated by washing monolayers with fresh medium before test solutions were added to the apical side of the cells. Stock solutions of genistein, daidzein, their glycosides genistin and daidzin, radioactive labelled markers [^{14}C] polyethyleneglycol (PEG4000) and D-[1-^{14}C] mannitol were prepared in dimethylsulfoxide (DMSO) and were diluted in medium at a maximum of 0.2% (v/v) DMSO. The solutions of genistein, daidzein, genistin, daidzin and the radioactive labelled markers were used separately at a concentration of 50 µM. Two experiments were carried out in triplo. Aliquot samples of 75 µL were taken from the basolateral side for up to 24 hours. The radioactive labelled markers were quantified on a DSA-based liquid scintillation counter of Wallac, EG&G Instruments (Nieuwegein, The Netherlands) after addition of counting scintillation fluid of Quickzint 130, Zinsser analytic (Berkshire, UK). For inhibition experiments, a combination of genistein, daidzein, genistin and daidzin with a concentration of 5 µM for the aglycones and 50 µM for the glycosides was added to confluent Caco-2 cells.

Isoflavone detection

A modification of the method described by Coward et al. (1996) was used. The isoflavones present in the aliquots of cell culture were analysed by reversed-phase HPLC of Waters (Etten-Leur, The Netherlands) equipped with a Supelcosil LC-ABZ column (250*4.6 mm, 5 mm, Supelco, Sigma-Aldrich Chemie BV (Zwijndrecht, The Netherlands) and an UV photodiode array detector of Waters (Etten-Leur, The Netherlands). The column was eluted at a flow rate of 1.0 ml/min employing a gradient of two eluens (eluent A: 10 mM ammoniumacetate pH 6.5 with 2.5% (v/v) acetonitril; eluent B: acetonitril) at 30°C for 60 minutes. The elution conditions were as follows: 0-5 minutes 10% eluent B, 5-10 minutes 10-32% eluent B, 10-25 minutes 32% eluent B, 25-35 minutes 32-40% eluent B, 35-40 minutes 40-50% eluent B, 40-45 minutes 50% eluent B, 45-50 minutes 50-10% eluent B and 50-60 minutes 10% eluent B. The eluent was monitored at 260 nm. Routinely 50 µL of media was injected onto the column support without further pre-treatment and metabolites were

identified by comparison with retention times of known standards. Glucuronides and sulfates were detected by treating the apical or basolateral media with ß-glucuronidase from Escherichia coli (500 units/ml) respectively sulfatase from abalone entrails (100 units/ml). These treated media were subsequently compared to media treated without enzymes.

Transepithelial electrical resistance (TEER)

Transepithelial electrical resistance across the cell monolayers was measured using the Millicell-ERS epithelial voltmeter (Millipore Co., Bedford, NJ) at each time the basolateral samples were taken. The TEER of the cell monolayers was calculated using the following formula: TEER = (R_{total} - R_{blanc}) * A (Ω cm^2).Where R_{total}= resistance measured, R_{blank}= resistance of control filters without cells (approximately 100 Ω) and A=surface of insert of the transwell (4.7 cm^2).

Enzyme activity

LDH leakage was determined using the Abott Vision lactate dehydrogenase (LDH) assay. This assay is based on a method described by Wacker et al. (1956) in which the enzyme catalyses the oxidation of L-lactate to pyruvate with NAD^+. The rate of the formation of NADH was determined spectrophotometrically at 340 nm and is proportional to the LDH activity. Aliqouts of 50 µL apical and basolateral media were taken at 24 hours and without further pre-treatment tested in the Abott Vision LDH test package.

Alkaline phosphatase (ALP) activity was determined according to Allain and modified using the method of Bessey et al. (1946) on the Abott Vision. *p*-nitrophenylphosphate was used as a substrate in the test, where alkaline phosphatase catalyses the magnesium-activated hydrolysis of this substrate. The product was measured at 418 nm, and the rate of hydrolyse is related to the activity of alkaline phosphatase. Aliqouts of 50 µL apical media were taken at 24 hours and without further pre-treatment tested in the Abott Vision ALP test package.

Transmission electron microscopy (TEM)

Caco-2 epithelial cells grown on semi-permeable inserts were washed with phosphate buffer solution at 4 hours after initiating a transport experiment and used for examination by transmission electron microscopy (TEM). The monolayers were fixed for 2 hours in 1% aqueous osmium tetroxide, rinsed with MQ-water and dehydrated

through a graded series of ethanol. Subsequently, the cell layers were embedded in LR white and examined using a Philips electron microscope operated at 80 kV.

Kinetic analysis

Data from the transport experiment were fitted according to an equation derived from a 2-compartment model described by Jacques (1985). The 2-compartment model for the transport experiment is previously described by Steensma et al. (2000). The kinetic values of the transport experiments of the different cells were subsequently compared to a transport experiment without cells.

Statistical analysis

Statistical evaluation of data was performed by one-way ANOVA ($p<0.05$) and comparison between groups were made using Tukey's Honestly Significant Difference test ($p<0.05$).

Results

Integrity of the monolayers

Upon the incubation of genistein, daidzein, their glycosides and the labelled markers the TEER across the cell monolayer was measured at each of the time points where samples were taken. Since fluctuations in TEER values between the various compounds were not significantly different, only the mean values have been presented in figure 3.1. It was observed that TEER values of the Caco-2 cell monolayer decreased within the first 2 hours for all compounds applied, where after the TEER value slowly increased within the next 2 hours towards its initial level. In contrast, the TEER values across HCEC and IEC-18 cell monolayers were constant over the whole range of sampling time points. TEER values of the IEC-18 cell monolayer were lower than those of the Caco-2 and HCEC cell monolayers. Examination of Caco-2 monolayers under the transmission electron microscope showed the presence of a proper morphogenesis and functional tight junctions and desmosomes (Figure 3.2).

Enzyme activity of the monolayers

LDH and ALP enzyme activities in media sampled at the apical side of the monolayers are given in Table 3.1. LDH release was highest in Caco-2 cells (140 ±52 IU/L) as compared to IEC-18 cells and HCEC cells. Whereas the release of alkaline

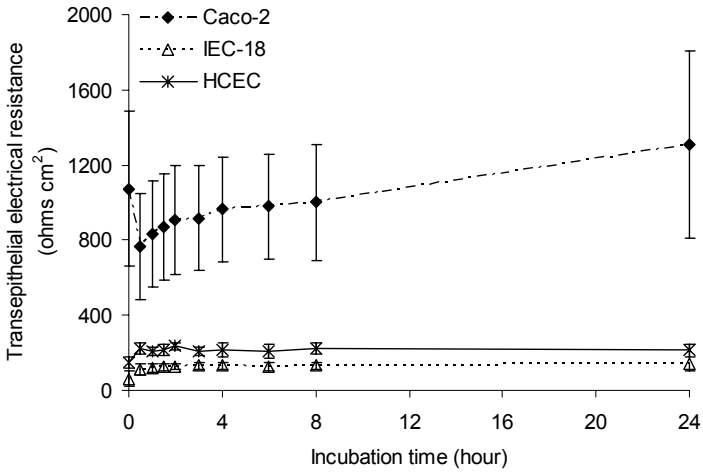


***Figure 3.1**: Transepithelial electrical resistance (TEER) across Caco-2 cells, IEC-18 or HCEC cells. TEER values are expressed as the mean ± sd of genistein, daidzein, genstin and daidzin incubations, initial concentration at apical side was 50 µM for two triplicate measurements.*

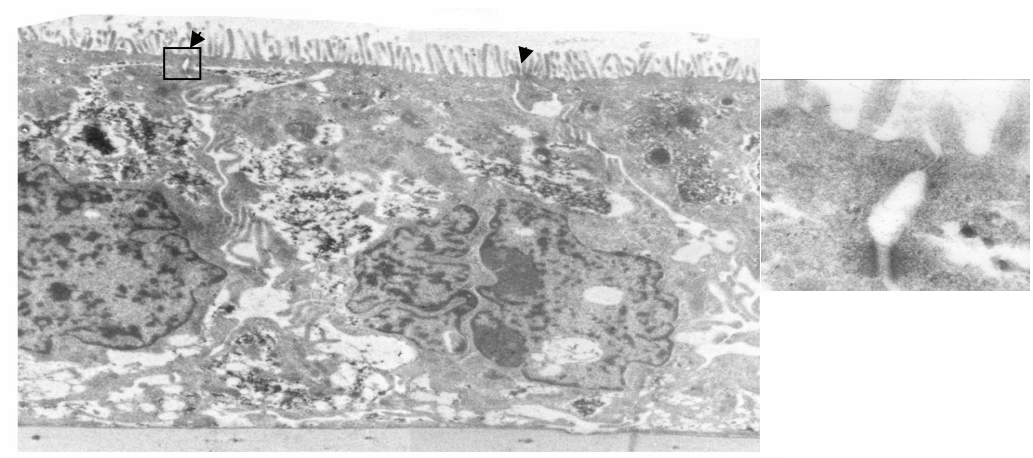


***Figure 3.2:** Transmission electron micrograph of Caco-2 epithelial cells. Caco-2 epithelial cells grown on semi-permeable inserts were examined at 4 hours after initiating a transport experiment. The original magnification of the cells was * 1850. The arrows are showing the tight junctions between two cells. On the right side the tight junction is tightly sealed, showed with magnification of *29500.*

***Table 3.1**: LDH and ALP enzyme activity at the apical side of the monolayer after 24 hours of incubation in the presence of genistein, daidzein or their glycosides.*

	LDH (IU/L)			ALP (IU/L)		
	Caco-2	IEC-18	HCEC	Caco-2	IEC-18	HCEC
Non-treated	139 ±29.2	48.4 ±7.7	34.0 ±20.0	209 ±13.5	n.d.	n.d.
Genistein	127 ±27.2	46.8 ±7.0	44.1 ±29.0	254 ±95.2	n.d.	n.d.
Daidzein	139 ±37.8	45.0 ±8.2	37.5 ±23.0	166 ±77.4	n.d.	n.d.
Genistin	130 ±24.7	43.3 ±0.8	20.1	227 ±24.6	n.d.	n.d.
Daidzin	140 ±51.9	43.8 ±0.1	20.5	194 ±28.3	n.d.	n.d.

The activities expressed are the mean ±SD of two triplicate experiments, n.d. means not detectable (i.e. detection limit of LDH: 15 IU/L and ALP: 10 IU/L).

phosphatase in the medium was low in both HCEC and IEC-18 cell monolayers. There was no significant difference for LDH and ALP release in all cell types between the various compounds compared with values of non-treated cells.

Transport of marker compounds

Figure 3.3A and B show the transport of the labelled markers PEG4000 and mannitol across the different cell monolayers. The transport of PEG4000 across Caco-2 the monolayer increases in time until approximately 2% (±1.8) of the initial concentration applied at the apical side. IEC 18 and HCEC cells transported PEG4000 much faster and to a greater extent reaching after 24 hours a level of up to 27% (±10) respectively 36% (±0.3) of the initial concentration at apical side.

Transport of mannitol across the monolayers of the investigated cell types is much higher compared to the transport of PEG4000. The transport of mannitol was relatively fast across HCEC cells and reached a constant level after 4 hours at 37% (±12) of the initial concentration, and up to 24 hours at 41% (±6.6). Across IEC-18 cells the transport of mannitol increased up to 43% (±6.6) of the initial concentration at 24 hours of incubation. However, mannitol was hardly transported across Caco-2 cells i.e. 8.5% (±1.9) of the initially applied concentration.

Transport of genistein, daidzein and their glycosides

Transport of genistein, daidzein and their glycosides across the Caco-2, IEC-18 and HCEC monolayers was studied in apical to basolateral direction. This is illustrated in

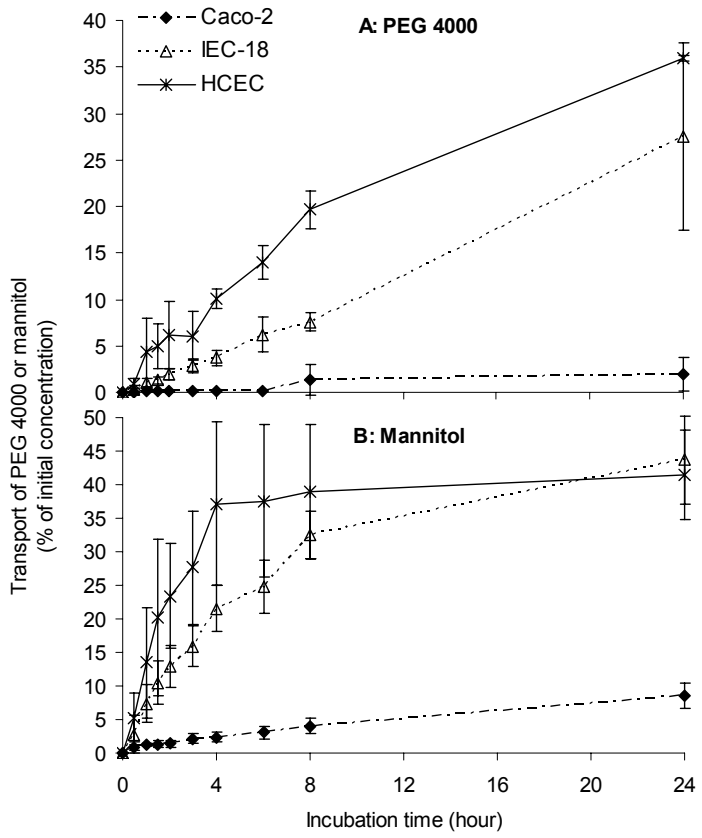


***Figure 3.3**: Transport of labelled PEG 4000 (figure A) or mannitol (figure B) across Caco-2 cells, IEC-18 or HCEC cells. Initial concentration at apical side was 5 µM. Data are expressed as mean ± sd of triplicate measurements.*

figure 3.4A, B and C. There was only a slightly difference in transport of genistein, daidzein and their glycosides in IEC-18 and HCEC cells. However, in Caco-2 cells a significant difference in the transport rate and metabolism of genistein and daidzein was observed as compared to their glycosides. After 6 hours 30-40% of genistein and daidzein added at the apical side was transported to the basolateral side of the model system. On the other hand, the glycosides were hardly transported through the Caco-2 cell monolayer, whereas the HCEC and IEC-18 transported the glycosides as well as their respective aglycones. Table 3.2 shows the kinetic values of the transport experiment.

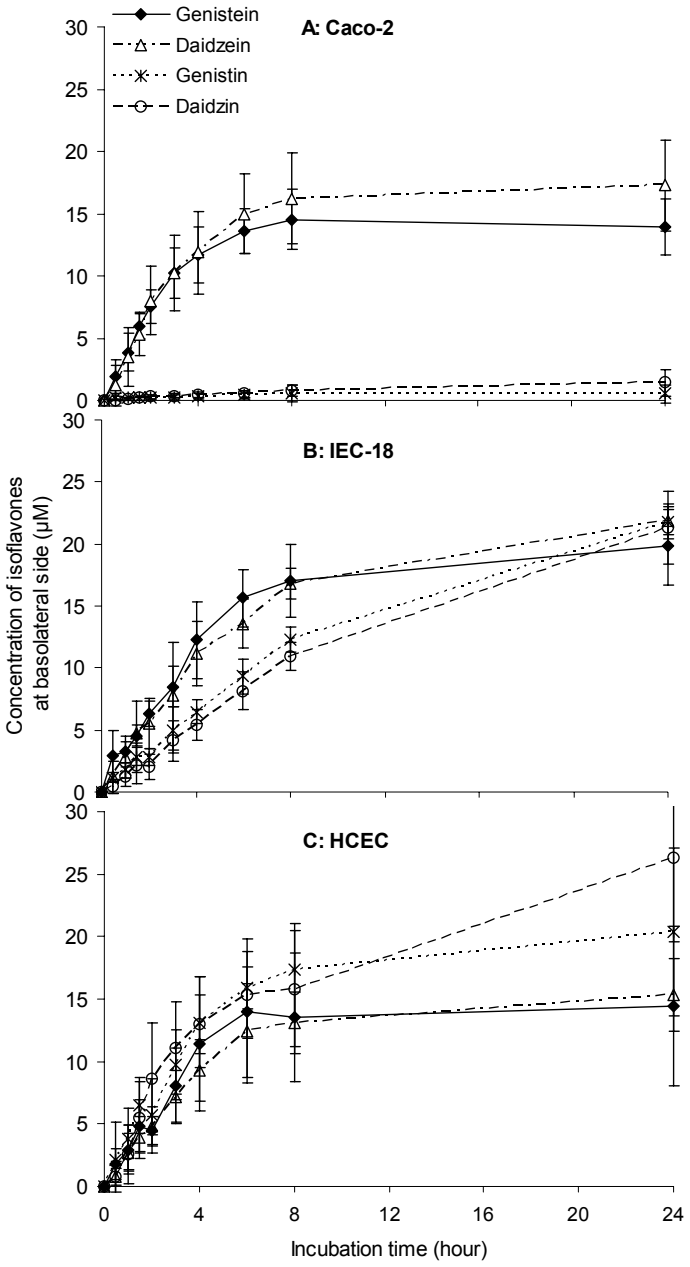


Figure 3.4*: Concentration of genistein, daidzein or their glycosides in basolateral medium (EMEM) across Caco-2 (figure A), IEC-18 (figure B) and HCEC(figure C) cells. Initial concentrations at apical side were 50 μM. The concentrations obtained at basolateral side are shown for genistein, daidzein, genistin or daidzin. Data are expressed as mean ± sd of two triplicate measurements.*

***Table 3.2**: Values of the kinetic constants (Kac in hour^{-1}) obtained from transport studies of genistein, daidzein, genistin and daidzin across Caco-2, HCEC or IEC cell monolayers. Initial concentrations of the compounds were 50 µM, except in the combination experiment. In the combination experiment the concentration of genistein and daidzein were 5 µM and for genistin and daidzin 50 µM.*

	Maximum concentration (µM)			Kac (hour^{-1})				
	Caco-2	IEC-18	HCEC	Caco-2	IEC-18	HCEC	Without cells	Caco-2 Combination Isoflavones
Genistein	17.9	30.1	25.3	5.02	4.02	3.79	10.0	0.40
Daidzein	22.8	34.5	26.0	4.56	3.43	3.17	8.73	0.46
Genistin	0.49	33.1	25.4	0.15	1.96	4.41	11.9	0.27
Daidzin	0.33	43.7	15.2	0.28	1.67	4.93	7.74	0.33

The maximum concentration of genistein and daidzein was shown in IEC-18 cells. A significant difference of the maximum concentration was seen for the glycosides transported across Caco-2 cells. The uptake rate of genistein and daidzein in the various cells was almost similar, whereas for the glycosides this was quite different. The uptake rate for the glycosides was comparable with that for the aglycones in HCEC cells. The combination of genistein, daidzein, genistin and daidzin added to confluent Caco-2 cells showed a factor of 10 lower uptake rate for aglycones. However, the concentration of the aglycones applied was a factor of ten lower compared to the concentration of aglycones separately used in the transport experiments (50 µM). The kinetic values for genistein and daidzein separately added to Caco-2 cells at a concentration of 5 µM was 0.7 and 0.4 hour^{-1}, respectively.

Metabolism of genistein, daidzein and their glycosides

Results have been summarised in table 3.3. The metabolites formed at the apical and basolateral side, and released into the media of the different cells, have been presented as a percentage of the concentration of test compound initially applied at the apical or basolateral side. The metabolites obtained have been depicted as aglycons (genistein or daidzein), glucuronides, sulfates and total. The total amount of metabolites is the sum of aglycones, glucuronide, sulfates and included also unknown metabolites (i.e. unknown metabolites have been estimated using labelled genistein, Steensma et al. (submitted), chapter 5). The application of ß-glucuronidase or sulfatase allowed the measurement of glucuronides and sulfates in apical or basolateral media by comparing HPLC-chromatographs with and without treatment.

***Table 3.3**: Metabolites detected at the basolateral and apical side of the cell monolayer after incubation with genistein, daidzein, genistin and daidzin in Caco-2, IEC-18 and HCEC cells for 24 hours.*

	Metabolites Basolateral Compartment (% of initial concentration)				Metabolites Apical Compartment (% of initial concentration)				Recovery (%)
	Aglycones	Glucuronides	Sulfates	Total	Aglycones	Glucuronides	Sulfates	Total	
Genistein									
Caco-2		2.23 ±0.24	1.57 ±1.04	**3.80** ±1.09		8.91 ±1.07	8.64 ±1.89	**17.9** ±2.41	81.0 ±12.0
IEC-18		0.86 ±0.44	n.d.	**0.86** ±0.44		1.48 ±0.64	n.d.	**1.49**±0.64	59.9 ±8.10
HCEC		0.07 ±0.07	2.16 ±1.68	**3.02** ±2.33		0.17 ±0.06	5.84 ±1.38	**7.00**±1.53	70.5 ±26.3
Daidzein									
Caco-2		0.41 ±0.41	3.33 ±1.98	**4.34** ±1.82		2.09 ±0.89	13 1 ±2.25	**16.2** ±1.14	92.4 ±15.8
IEC-18		0.40 ±0.12	n.d.	**0.40** ±0.12		0.78 ±0.10	n.d.	**0.78**±0.10	66.3 ±3.98
HCEC		0.19 ±0.13	1.92 ±0.86	**3.70** ±0.71		0.16 ±0.11	3.07 ±1.51	**4.46**±1.52	71.7 ±12.1
Genistin									
Caco-2	0.29 ±0.29	0.23 ±0.23	0.58 ±0.55	**1.11** ±1.11	1.60 ±1.60	2.27 ±2.27	7.71 ±5.88	**11.8** ±9.79	99.2 ±25.9
IEC-18	1.41 ±0.59	0.23 ±0.05	n.d.	**1.65** ±0.55	1.78 ±0.50	0.54 ±0.17	n.d.	**2.33**±0.34	69.7 ±3.95
HCEC	n.d.	0.10 ±0.10	0.19 ±0.16	**1.02** ±0.84	n.d.	0.10 ±0.07	0.54 ±0.30	**1.47**±2.53	98.0 ±18.2
Daidzin									
Caco-2	0.37 ±0.33	0.21 ±0.21	0.44 ±0.13	**1.08** ±0.79	1.54 ±0.80	0.66 ±0.33	4.95 ±2.36	**7.16**±3.01	90.7 ±15.2
IEC-18	0.79 ±0.27	1.67 ±0.16	n.d.	**2.46** ±0.43	1.13 ±0.20	1.58 ±0.36	n.d.	**2.71**±0.46	71.8 ±7.42
HCEC	n.d.	n.d.	2.11 ±0.80	**4.31** ±1.35	n.d.	n.d.	1.43 ±0.80	**3.23**±0.28	99.5 ±23.1

Initial concentrations at apical side were 50 µM, n.d. = not detectable (detection limit of genistein: 128 nM, daidzein: 111 nM, genistin: 64 nM and daidzin: 71 nM). The metabolites obtained are shown for aglycons (genistein or daidzein), glucuronides, sulfates and total. The total concentration of metabolites consists of the sum of aglycones (genistein or daidzien), glucuronides, sulfates and unknown metabolites (data not shown). Data are expressed as mean ± sd of two triplicate measurements.

In Caco-2 cells genistein and daidzein were only slightly metabolised into their respective sulfates and glucuronides. HPLC analysis revealed, however, that genistin and daidzin were also metabolised to genistein and daidzein. In Caco-2 cells this accounted for approximately 2% of the added parental glycoside compound. In the case of IEC-18 cells genistein and daidzein were primarily metabolised to their respective glucuronides. It was shown that the glycosides were also metabolised to their aglycones in this cell type (i.e. 1.9-3.2% of the initial concentration). In contrast to the Caco-2 and IEC-18 cell line, HCEC cells didn't metabolise the glycosides to their respective aglycones. It was also recognised that genistein, daidzein and their glycosides were hardly metabolised to sulfates in IEC-18 cells and hardly metabolised to glucuronides in HCEC cells.

Discussion

This study with Caco-2 cells clearly demonstrated that the monolayer was almost impermeable to the markers applied, indicating an intact cell monolayer. These findings confirm earlier observations by Artursson et al. (1996), Delie and Rubas. (1997) and Duizer et al. (1997), who reported that the marker PEG4000 or mannitol should not be transported across enterocytes (i.e. Caco-2).

However, this phenomenon was not observed with monolayers constituted of IEC-18 and HCEC cells. These cell types transported at a high rate the aforementioned markers, indicating that in these cell lines the paracellular route is permeable for the compounds under consideration. Generally, the increased transport of labelled markers and a decrease in TEER are regarded as a result of affected membrane integrity, rather than a specific effect on paracellular permeability (Fagerholm et al., 1996). Our experiment showed different results, because the TEER across the IEC-18 was lower than the TEER across HCEC, but kept constant in both model systems upon the addition of the test compounds. Duizer et al. (1997) reported similarly results showing that the TEER across IEC-18 cell monolayers appears to be lower compared to that of the Caco-2 cell monolayers. Based on our current observations, together with those of Ma et al. (1992) and Duizer et al. (1999), we concluded that the Caco-2, IEC-18 and HCEC possessed an intact membrane integrity. This conclusion has been confirmed applying transmission electron microscopy for Caco-2 cells. Ma et al. (1992) reported identical features in case of the IEC-18 cell monolayers. Addition of compounds in the apical compartment of the model system markedly impaired the TEER value across Caco-2 cell monolayers only. The results also

suggested that the TEER across Caco-2 slightly increased, reaching a level that was even higher than the level at the start of the experiment. These observations were also reported by a few other studies (i.e. Hashimoto et al., 1993; Pasternak et al., 1996; Madara, 1998). It is concluded that the initial levels of FCS in the culture media are seemingly responsible for the stabilisation of the polarisation (i.e. TEER) of the cell monolayer, because Caco-2 cells are normally cultured in DMEM containing 10% FCS. Thus, the change and addition of medium without FCS at the start of a transport experiment, therefore, disturbed the resistance of the membrane. Whereas, this effect did not occur in IEC-8 and HCEC cells as these cell lines are commonly cultivated in media that are already low in concentrations of FCS, namely 5% FCS for IEC-18 cells and no FCS for HCEC cells. On the other hand, other authors considered a lowered TEER value as an indicator of an increased permeability in transport experiments (Delie and Rubas, 1997), which is considered highly questionable regarding our current results.

In order to be sure that transport features were obtained from studies performed at only physiologic relevant dose ranges, the extent of cytotoxicity of genistein, daidzein and their glycosides was explored by using the LDH assay. In all the transport experiments that were carried out, there was no significant difference in the LDH levels of treated intestinal cell types compared to non-treated cell monolayers, indicating that no cytotoxicity had occurred.

To asses the stage of differentiation of the different cell types used ALP levels were measured as an indicator for a correct assembly of the brush-border morphogenesis (Delie and Rubas, 1997). It was demonstrated that the ALP levels were low in the IEC 18 and HCEC model systems. Therefore, it is suggested that these cells in monolayer cultures were not or did not fully differentiate, as they did not show a steady state level of the ALP enzyme expression.

The transport of genistein and daidzein were studied from apical to basolateral side in the Caco-2, IEC-18 and HCEC cells. There were almost similar rates and extents of transport observed, only the rate for IEC-18 cells was higher. This can be explained by the low metabolic activity found in these latter cells. The rates indicates that the uptake of the compounds tested is independent as it does not follow the paracellular route. As suggested by Oitate et al. (2001) the transport of genistein across Caco-2 cell monolayers could be mediated by a carrier system. This is in accordance with our present inhibitory experiment, where the kinetic values of genistein were significantly decreased by a combination of aglycones and glycosides in the transport experiment

across Caco-2 cells. However, in case of daidzein an inhibitory effect was not clearly seen. But, this effect has also been observed by Oitate et al. (2001) and they suggested that the uptake of isoflavones may only occur via a common transport system.

Our experimental evidence suggests that the Caco-2 cells transported genistein and daidzein at a much higher rate than their related glycosides. In the *in vitro* model system, the glycosides did not pass through the Caco-2 monolayer. Walgren et al. (1998, 2000a and b) and Walle et al. (1999) described similar findings in a Caco-2 system using the flavonoid glycosides such as quercetin-4-glycoside and genistin. In contrast, it appeared that in our hands the glycosides in IEC-18 and HCEC cells were readily transported. This observation can be explained by a paracellular transport of the glycosides in these cell lines. However, the tight junctions in these two cell lines appeared to be less tight than in case of Caco-2 cells. Moreover, the paracellular markers were also transported.

Our findings that glycosides are hardly transported across the Caco-2 cells are consistent with *in vivo* studies (Piskula et al., 1999; Setchell et al., 2001) and studies with perfusions of isolated rat intestinal segments (Spencer et al., 1999; Andlauer et al., 2000a; Sesink et al., 2001). These studies confirmed that no or only small amounts of flavonoid glycosides appeared in the plasma compartment. Furthermore, in these studies the plasma profiles of the glycosides showed similarities with their corresponding aglycones. Comparing these *in vivo* results to the results of our *in vitro* model systems; the Caco-2 cell line appears to be a better cellular model line for transport and metabolism studies than those systems using the IEC-18 or HCEC cell line.

The metabolic activities in the various cell lines studied appeared also to be significantly different. As expected, our data indicated that Caco-2 and IEC-18 cells show a glucosidase activity as aglycones (e.g. genistein or daidzein) could be detected in both the donor and acceptor compartment of the cellular model system. The appearance of genistein or daidzein in both compartments suggests strongly that genistin might be hydrolysed at or in the cellular monolayer. Because deglycosylation of genistin by cytosolic broad specific ß-glucosidases in cell free extracts of the human intestine has been demonstrated by Day et al. (1998). In addition, Walle et al. (1999) suggested that genistin could be hydrolysed in the culture medium. These authors demonstrated that fetal bovine serum contained a high ß-glucosidase activity

showing a KM value of 17 μM that was very similar to the results of Day and coworkers.

In our study, the aglycones were formed after about 6 hours of incubation of the glycosides as there was no FCS in the media. It is therefore postulated that the isoflavone glycosides can only be transported after deglycosylation at the side of the cell membrane and/ or within the cytosolic compartment. However, the relative contribution of both these possible exo- and endogenous enzymatic activities as well as the influence of the microflora in the deglycosylation process remains a subject for further research. Also the main site of absorption of the isoflavones within the gastro-intestinal tract must be studied in greater detail using other systems including animal models.

In conclusion, the results of this study demonstrated that Caco-2 cells are a good cellular model for studying the intestinal transport and metabolism of the glycosides genistin and diadzin. Generally, the observed transport and metabolism of the glycosides in Caco-2 monolayers agreed very well with existing and published *in vivo* data. Although the IEC-18 and HCEC monolayers have proven to be a powerful tool in absorption studies concerning the paracellular transport processes, however, their use as a model in predicting the uptake of glycosides is probably limited and, therefore, not recommended for use in model systems.

Acknowledgements

We would like to thank E. Offord, K. Macé and A.M.A. Pfeifer from Nestlé Research Centre, Lausanne, Switzerland for their kind donation of the immortalised human colon cells (HCEC). We would also thank J. Donkers from Agrotechnological Research Institute ATO B.V, Wageningen, The Netherlands for assisting with the transmission electron microscopy experiments.

Chapter 4

Intestinal uptake of genistein and its glycoside in the rat using various isolated perfused gut segments

Abstract

Genistein receives much attention because of its potential to prevent hormone-related cancer and cardiovascular diseases. Limited information is available on the pharmacokinetics of this compound like, for instance, the intestinal uptake by humans and systematic bioavailability. In this study the fate of the absorption of genistein and its glycoside has been analysed in various isolated perfused gut segments of the rat.

In all perfused gut segments the transport of genistein was higher compared to its glycoside. Furthermore, it appeared that the resorbate (i.e. serosal side) amount of genistein was the highest in ileac segments, whereas the transport of genistein in the various other segments tested showed no difference between intestinal compartments. Less than 0.2% of genistin appeared in the resorbate fluid of all isolated gut segments.

The main site of metabolism of genistein and its glycoside appears to be located in the jejunal compartment of the rat gut. About 38% of genistein and about 29% of genistin metabolised within 2 hours of perfusion. In the ileac and colonic intestinal segments, genistein metabolised for only 10%. For the first time, this study demonstrated that genistin could be metabolised by epithelial cells present in isolated colonic segments. However, the metabolites of genistin did not occur at the serosal side (the resorbate) of isolated colonic segments. We assume that there is no absorption of genistin and/or its metabolites in or through colonic tissue of the rat.

Based on: Steensma, A., Bienenmann-Ploum, M.E. and Noteborn, H.P.J.M. (2004) Intestinal uptake of genistein and its glycoside in the rat using various isolated perfused gut segments. *Environmental Toxicology and Pharmacology* **17**, 103-110.

Introduction

Soybeans and soy-derived foods contain isoflavones, which contains predominantly large amounts of ß-glycosides (1-3 mg/g product) and, in much smaller concentration, aglycones such as genistein (Coward et al., 1993a; Reinli and Block, 1996). In general, little is known about the biological activity of these glycosides. On the contrary, the aglycones have been studied extensively over the past decade. The aglycones have received this attention because pure compounds are readily available and have been reported to prevent hormone-related cancers and cardiovascular diseases (Barnes et al., 1996; Adlercreutz and Mazur, 1997; Setchell and Cassidy, 1999; Clarkson, 2000).

Although soy flavonoids consist mainly of ß-glycosides, it is still unknown whether the glycosides are absorbed in an intact form or are hydrolysed to their respective aglycones. The rapid absorption of dietary flavonoid glycosides, as observed in several human and animal studies, point at a possible role of a ß-hydrolytic activity of the small intestine (Hollman et al., 1995; Piskula, et al., 1999; Walle et al., 2000; Sesink et al., 2001). Experimental models of transport studies using Caco-2 cells demonstrated that the glycosides were hardly transported, but metabolised into their respective aglycones (Walgren et al., 1998; Steensma et al., 1999; Walle et al., 1999).

Until recently, the intake of dietary flavonoid glycosides was thought to result in a high flux towards the colon, where gut bacteria convert these glycosides into aglycones. However, the exact mechanism of action has not been reported so far, and is still unknown.

There are a few studies that have compared the absorption between glycosidic compounds and the aglycones. In these mainly *in vivo* studies, the plasma profiles of the administered glycosides showed similarities with their corresponding aglycones, but no glycosides could be detected in the systematic circulation (Piskula et al., 1999; Sesink et al., 2001; Setchell et al., 2001). However, the relative contribution of the glycosidase activities in the small intestine, as well as that of the microflora in the deglycosylation process remains to be studied in much greater detail.

The aim of the present study is to obtain information on the fate and location of the intestinal absorption of genistein and its glycoside. Therefore, various segments of the

intestinal tract of the rat were perfused with genistein and genistin to study the intestinal absorption and transport mechanism of these isoflavonic compounds

Materials and Methods

Materials

Genistein, genistin, ß-glucosidase (from Escherichia coli, 500 units/ml) and sulfatase (from abalone entrails, 100 units/ml) were obtained from Sigma Chemical Co. (St. Louis, MO). [4-^{14}C] Genistein (spec.act 0.2 mCi/ml) was obtained from Moravek Biochemical (Brea, CA) and [^{14}C] polyethyleneglycol (PEG4000) (MW 4000, radioactive concentration 50 µCi/ml, spec. act. 68 µCi/µmol) was obtained from Amersham, Buckinghamshire, UK. The Tyrode buffer contained 136.9 mM NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$, 4.2 mM NaH_2PO_4, 1.2mM $CaCl_2$*2 H_2O, 0.5 mM $MgCl_2$ *6 H_2O and 15 mM glucose. The Supelcosil LC-ABZ column was purchased from Supelco (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands).

Transport in isolated gut segments

The preparation of intestinal segments was carried out according to the method described by Richter and Strugala (1985). Briefly, male Wistar rats (200-250 gram) were anaesthetised by inhalation of ether and the rats remained unconscious throughout the operation. The abdominal cavity was opened by a midline incision and the flexura duodeno-jejunalis was identified. The intestine was divided at this point and the different types of segments were prepared as follows: 5 cm from the flexura duodeno-jejunalis for jejunum A (proximal) segments, 20 cm from flexura duodeno-jejunalis for jejunum B (distal) segments, 10 cm from valvula ileocoecalis for ileal segments and 10 cm from ceacum to rectum for colon segments. Two segments from each animal were randomly used and the length of each intestinal segment was approximately 10 cm. At each site of the 10 cm intestinal segment, two glass cannulas were inserted into the intestines and the intestinal segment was fixed on these cannulas. The endogenous contents of the cannulated segments were removed by intense flushing with 20 ml Tyrode buffer (37°C). Subsequently, the blood supply was interrupted by carefully dissecting the intestinal segment. Thereafter, segments were immediately mounted in the lower chamber of the perfusator. The perfusator was kept at a constant temperature of 37°C and the isolated intestinal segments were perfused with a constant flow of carbogen gas (i.e. bubbles). The perfusate contained either the marker [^{14}C] polyethyleneglycol or 50 µM [4-^{14}C] genistein or 50 µM

genistin dissolved in Tyrode buffer. During perfusion, absorbed fluid dropped through the intestinal wall of segments in the direction of the base of the perfusion chamber and called resorbate. Aliquots of resorbate and perfusate were taken at various time points during a total perfusion period of 2 hours and analysed by HPLC. After 2 hours perfusion was terminated and intestines were weighted, their length measured and aliquots of final perfusate and resorbate were taken and stored at –30°C until analysis.

Analysis of isoflavones and metabolites

The isoflavones and their metabolites were analysed by reversed-phase HPLC equipped with an UV photodiode array detector (Waters, Etten-Leur, The Netherlands) set at 260 nm. The solvents for the gradient elution were 10 mM ammonium acetate pH 6.5 with 2.5% (v/v) acetonitril (solvent A) and acetonitril (solvent B). Separation was performed on a Supelcosil LC-ABZ column (250 x 4.6 mm, 5 mm), maintained on 30°C using a Merck Hitachi column heater. The following gradient was used at a flow rate of 1 ml/min: 0-5 minutes 10% solvent B, 5-10 minutes 10-32% solvent B, 10-25 minutes 32% solvent B, 25-35 minutes 32-40% solvent B, 35-40 minutes 40-50% solvent B, 40-45 minutes 50% solvent B, 45-50 minutes 50-10% solvent B and 50-60 minutes 10% solvent B.

Routinely 50 µl of aliquots of perfusate and resorbate without further pre-treatment were injected onto the column support and metabolites were identified and qualified by comparison with the retention times of known reference compounds. To determine glucuronide and sulfate conjugates, aliquots of the perfusate or resorbate were pre-incubated with either ß-glucuronidase or sulfatase. After 1 hour of incubation the chromatogram of isoflavones were subsequently compared to resorbate aliquots treated without enzymes.

The metabolites of [4-^{14}C]-Genistein were analysed on a Waters HPLC system connected to both a photodiode array detector (Waters, Etten-Leur, The Netherlands) and an on-line radioactivity detector (LB506C, Berthold, Wildbad, Germany). Routinely 50 µl of medium was analysed as described above. Following the passage through the diode array detector the eluent was mixed with Maxifluor scintillation fluid (Baker, The Netherlands) at a flow rate of 1 ml/min and radioactivity detected.

Analysis of gut segment viability

Glucose levels were determined photometrically according to Klee (1992) in both perfusate and resorbate fluids after 2 hours perfusion using the Glucose/GOD-period test kit (Boehringen Mannheim GmbH, Germany). Standard viability criteria of the jenunal segments used in the experiments were 0.5 ml/cm resorbate, 12.15 µmol/cm glucose and a glucose ratio of resorbate to perfusate of 1.6. The standard viability criteria of respectively the ileac and colonic segments were 0.5 ml/cm resorbate, 4 µmol/cm glucose and a glucose ratio of resorbate to perfusate of 1.0 (Klee, 1992). If the intestinal segments did not reach these standard values, they were excluded from the experiment (i.e. of non-predictive value) and the samples were not considered further in the HPLC analysis.

Statistics

Results are presented as means ±SEM. Statistical evaluation of data was performed by one-way ANOVA ($p<0.05$) and the comparison of values between the treated groups and the control group was based upon the Tukey's Honestly Significant Difference test ($p<0.05$).

Results

Mucosal effects of genistein and genistin

Various intestinal segments were perfused with 50 µM of genistein or genistin. Whereas, the control intestinal segments were perfused with labelled PEG 4000 only. The total perfusion time was 2 hours and resorbate samples were taken between 0 and 2 hours. Control perfusions with PEG 4000 showed no isoflavones and metabolites in the HPLC diagram of the small intestinal fluids.

Figure 4.1 illustrates the volume of the resorbate samples measured at each sampling time points. The water transport was highest in proximal jejunal segment (A), after that in distal jejunal part (B), followed by the ileac and colonic segments, respectively. There was a significant difference of water transport in proximal jejunal segments if perfused with genistein and compared to the control segments.

The active transport of glucose through the wall of the gut segments i.e. from perfusate to resorbate is shown in figure 4.2. The active transport of glucose was

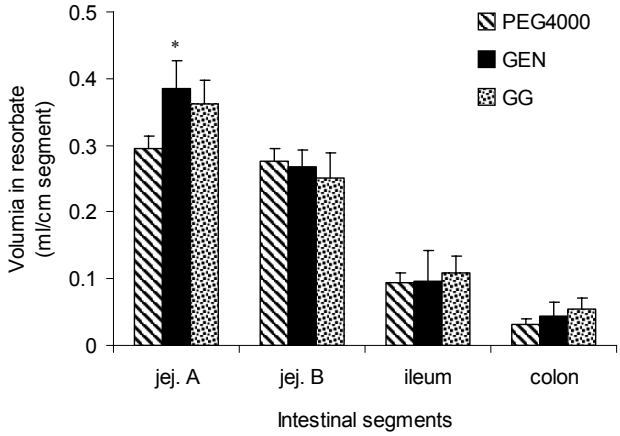


***Figure 4.1**: Waterflux across the different intestinal segments as described in the material and methods section. Intestinal segments were perfused with PEG4000, genistein or genistin. The initial concentration of PEG4000 was 2.5 µM and the initial amount of genistein and genistin was 50 µM (i.e. perfusate compartment). Data are expressed as mean ±SEM for jej. A (=jejunum A, proximal jejunum) n=5, jej. B (=jejunum B, distal jejunum) n=5, ileum n=4 and colon n=4 measurements. *Statistically significant different when compared with the PEG4000 perfusion p<0.05.*

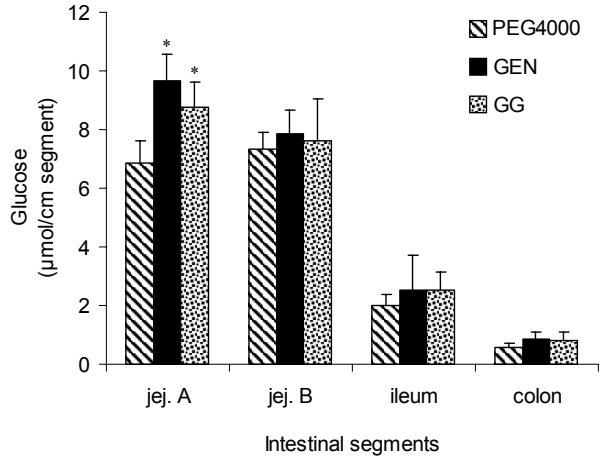


***Figure 4.2:** Glucose amount in resorbate of the perfused intestinal segments of rats (see materials and method section). The intestines were perfused with PEG4000, genistein) or genistin. The initial concentration of PEG4000 was 2.5 µM and the initial amount of genistein and genistin was 50 µM (i.e. perfusate compartment). Data are expressed as mean ±SEM for jej. A (=jejunum A, proximal part) n=5, jej. B (=jejunum B, distal part) n=5, ileum n=4 and colon n=4 measurements. *Statistically significant different when compared with the PEG4000 perfusion p<0.05.*

systematically increased in the proximal jejunal segments perfused with the isoflavones.

Transport of genistein and genistin

In all perfused gut segments the transport of genistein was higher compared to the transport of its glycoside. Figure 4.3 illustrates the cumulative amount of genistein, genistin and PEG4000 (marker) in the resorbate of the various perfused segments. Furthermore, it appeared that the resorbate amount of genistein was the highest when using ileac segments, but there was no difference in transport of genistein in the various other compartments tested. The transport of genistin across the various intestinal segments seems to be constant and almost comparable to the transport of PEG4000, which was used as a marker.

Metabolism of genistein and genistin

The segments of the various intestinal compartments showed a time dependent metabolism of genistein (Figure 4.4 and 4.5). Metabolism was most rapid in both the proximal and distal jejunal segments with about 38% of genistein being metabolised within 2 hours of perfusion (Figure 4.4). During this period, ileac and colonic segments metabolised only 10 and 11% of the genistein substrate, respectively (Figure 4.5).

The HPLC chromatograms of samples from the jejunum segments that were perfused with genistein showed three additional peaks of radioactivity of which one detectable just above the detection limit of the method (Figure 4.6). To further identify these peaks, treatment of the samples with ß-glucuronidase resulted in subsequent formation of genistein only. The HPLC chromatograms of ileac and colonic segments perfused with genistein showed only one glucuronide peak and, as expected the genistein peak. The HPLC chromatogram of a proximal jejunal segment A perfused with genistin is shown in figure 4.6. The same trend of formation of glucuronide metabolites in the various with genistin perfused segments was observed. However, an unknown component was detected in jejunal perfusate samples and two unknown components were analysed in the perfusate samples of the colonic segment.

Table 4.1 summarises the metabolites that appeared in the samples of perfusate and resorbate of the different intestinal segments perfused with genistein or genistin. Jejunum A segments showed the highest glucuronidation activity, whereas almost 24% of genistein appeared as a glucuronide in the resorbate fluid.

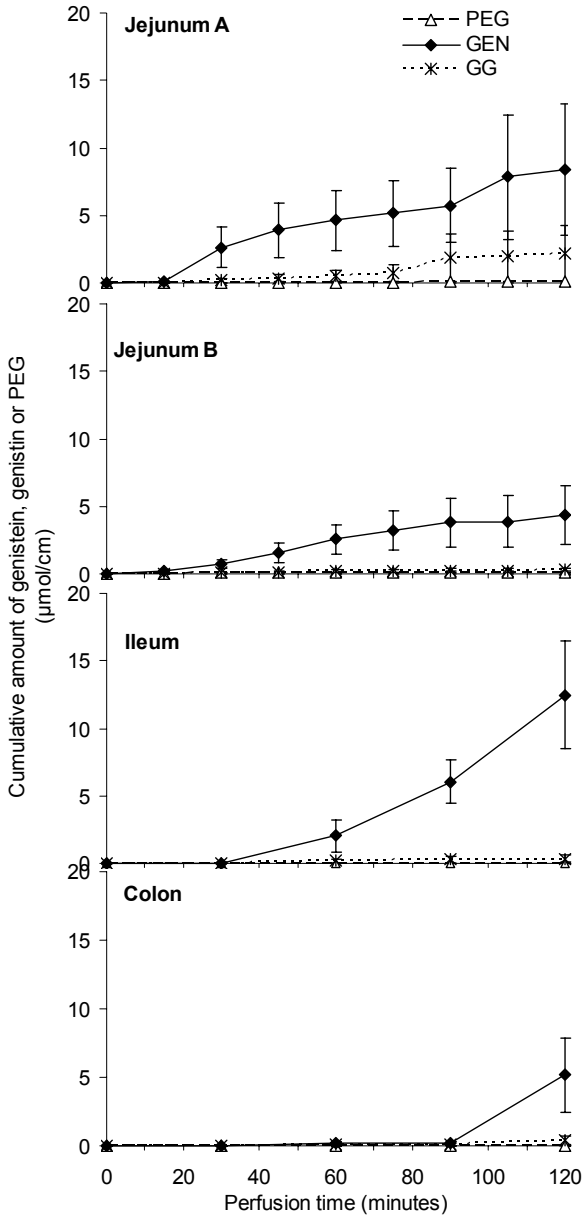


***Figure 4.3**: Cumulative amount of genistein, genistin and PEG4000 in resorbate of intestinal segments (jejunum A is proximal part, jejunum B is distal part). The intestinal segments were perfused with PEG4000, genistein (GEN) or genistin (GG). The initial concentration of PEG4000 was 2.5 µM of genistein and genistin was 50 µM (i.e. perfusate compartment). Data are expressed as mean ±SEM for jejunum A (proximal part) n=5, jejunum B (distal part) n=5, ileum n=4 and colon n=4 measurements.*

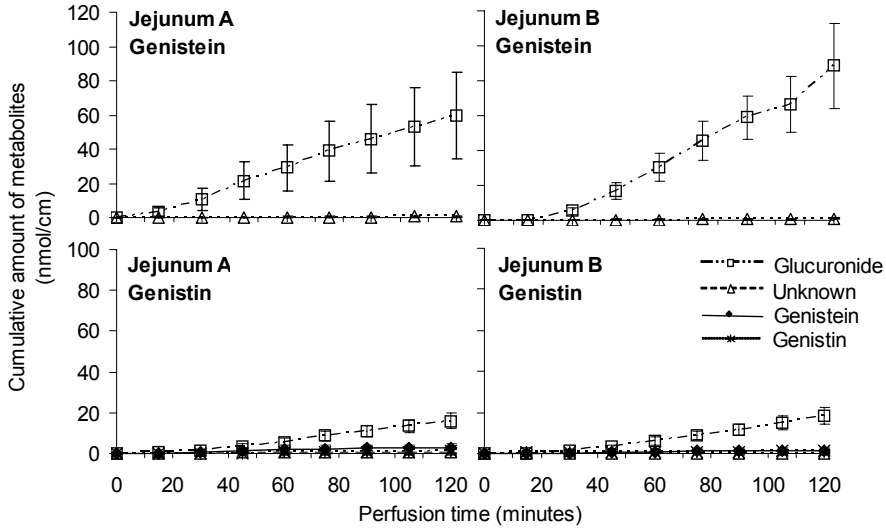


Figure 4.4: *Metabolism of isoflavone compounds in jejunum A (proximal part) and B (distal part) segments measured in resorbate fluid. The jejunal A segments were perfused with genistein (above figures) or genistin (down figures). The initial concentration of genistein and genistin was 50 µM (i.e. perfusate compartment). Data are expressed as mean ±SEM for n=5 measurements.*

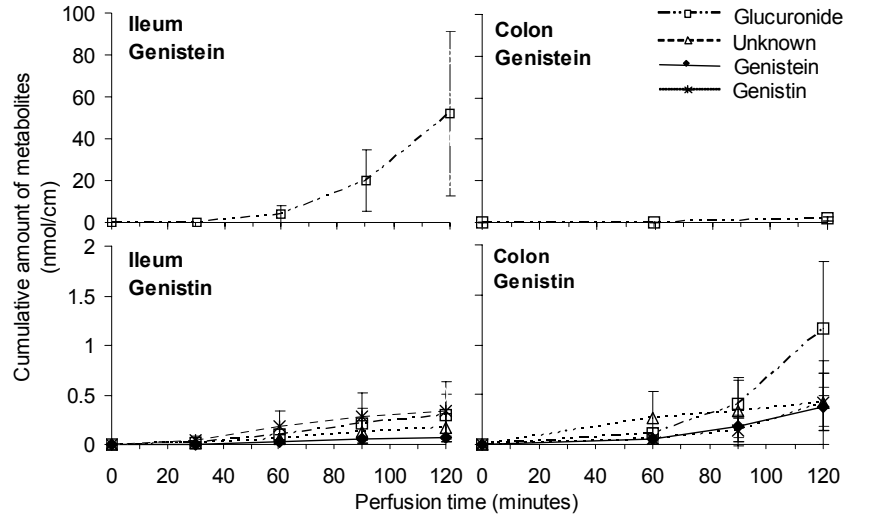


Figure 4.5*: Metabolism of isoflavones compounds in ileac and colonic segments measured in resorbate fluid. The ileac and colonic segments were perfused with genistein (above figures) or genistin (down figures). The initial concentration of genistein and genistin was 50 µM (i.e. perfusate compartment). Data are expressed as mean ±SEM for n=4 measurements*

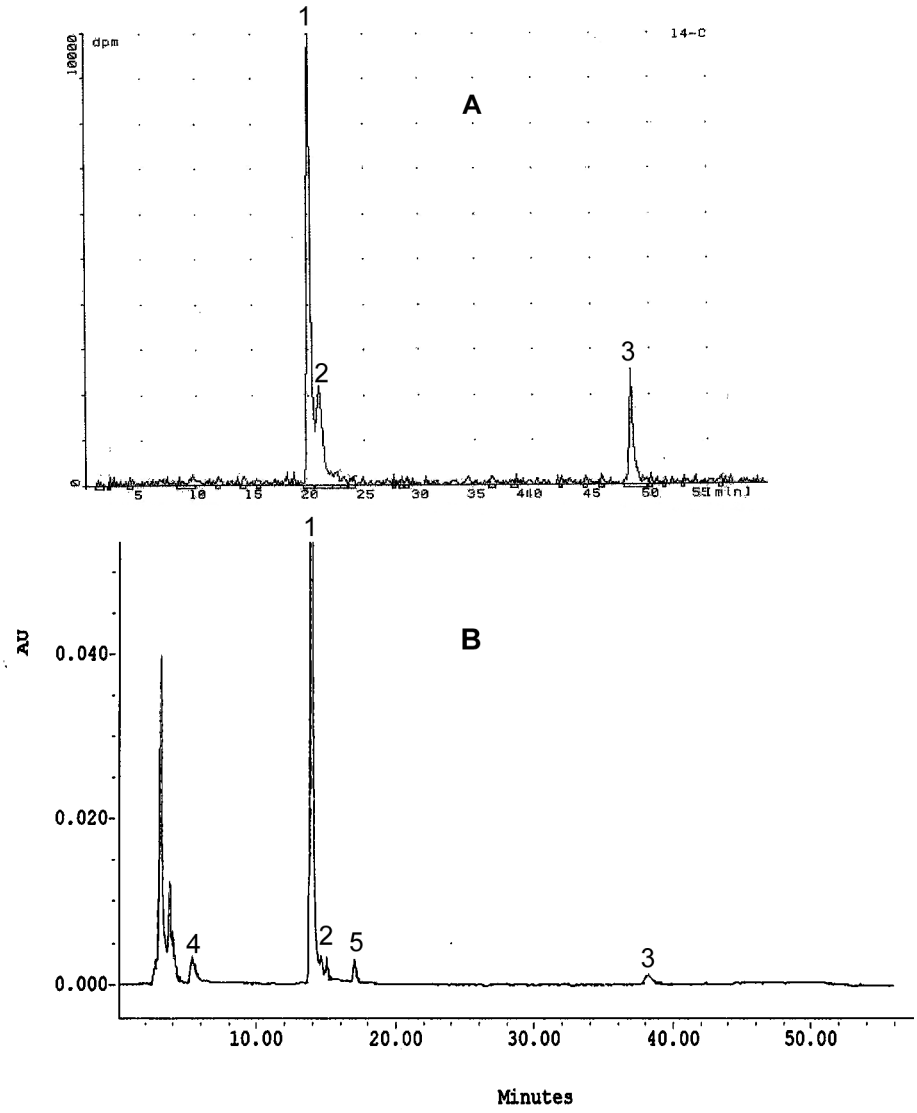


***Figure 4.6**: Representative HPLC chromatograms: A) radioactivity detection of [4-14C]-Genistein and B) photodiode array detection of genistin resorbate of jejunal A segments perfused with genistein or genistin. The peak 1 and 2 represent glucuronides, peak 3 is genistein, peak 4 is an unknown metabolite and peak 5 represents genistin.*

***Table 4.1**: Distribution of genistein and genistin metabolites in perfusate and resorbate after 2 hours of perfusion. Data are expressed as mean ±SEM.*

Applied compound / Segments	Metabolites in perfusate (%)				Metabolites in resorbate (%)				Recovery (%)
	Genistein	Genistin	Glucuronides	Unknown	Genistein	Genistin	Glucuronides	Unknown	
Genistein									
Jejunum A	58 ±22	n.p.	14.6 ±9.00	0.00 ±0.01	1.64 ±1.71	n.p.	24.1 ±17	0.19 ±0.20	98
Jejunum B	65 ±17	n.p.	9.34 ±9.75	0.03 ±0.05	0.94 ±1.25	n.p.	15.8 ±9.8	0.22 ±0.22	91
Ileum	62 ±14	n.p.	3.21 ±1.11	n.d.	2.17 ±0.55	n.p.	6.88 ±10.2	n.d.	74
Colon	77 ±34	n.p.	10.9 ±5.30	n.d.	1.05 ±0.85	n.p.	0.35 ±0.3	n.d.	89
Genistin									
Jejunum A	0.74 ±0.83	75 ±14	7.01 ±3.61	8.11 ±4.25	0.87 ±1.09	0.19 ±0.29	5.10 ±0.9	6.18 ±2.56	103
Jejunum B	0.46 ±0.31	65 ±17	7.22 ±3.78	8.02 ±3.90	0.55 ±0.65	0.18 ±0.29	6.04 ±4.1	6.63 ±4.52	87
Ileum	0.06 ±0.10	84 ± 6.9	0.05 ±0.04	0.30 ±0.22	0.02 ±0.01	0.07 ±0.07	0.01 ±0.0	0.06 ±0.05	85
Colon	14.6 ±18.6	56 ±23	0.09 ±0.05	36.4 ±41.9	0.12 ±0.12	0.12 ±0.12	0.39 ±0.5	0.64 ±0.74	108

n.p.=not pesent, n.d.=not detectable

Genistin was metabolised by all the various segments to its aglycone for only less than 1% of the initial amount applied in perfusate compartment. Glucuronides in the resorbate of jejunual segments accounted for 5.1% and 6.0% of the initial amount applied, respectively. The metabolism of genistin in the ileac segment was less than 0.1% of the initial amount applied in perfusate compartment. It was observed that two out of four segments of the colon possessed the metabolic activity that appeared to be very low. On the other hand, two other segments showed that almost 30% of genistein was metabolised and thereby two unknown compounds appeared in perfusate fluid (i.e. HPLC chromatogram). Although heavily flushed with Tyrode buffer, it could be possible that these latter segments still did contain a reasonable amount of microfloral contents (Figure 4.5). In the resorbate of these two colonic segments no extra metabolites occurred, however and, no significant differences were observed between all the colonic segments tested.

Discussion

This study clearly demonstrates that the main site of metabolic activity and absorption of genistein and its glycoside is located in the jejunal compartment of the rat gut. However, a significant difference was observed between the transport of genistein and its glycoside. The glycoside was hardly transported, which rate was in line with the limited rate of transport of PEG4000. PEG4000 was used both as a control compound for the viability of this gut system model, and as a marker of transport, indicating an intact and viable intestinal segment. Because an increased transport of labelled PEG 4000 would be a result of an affected membrane integrity (Fagerholm et al., 1996). In addition, control perfusions with PEG 4000 without isoflavones demonstrated that the small intestine of the experimental rat model did not contain any isoflavones (i.e. derived from dietary contamination), because no isoflavones and/ or metabolites were detected neither in the resorbate nor in the perfusate fluids of the control rats.

In order to be certain that at the functional and morphological integrity of the isolated gut segments were present upon perfusion, the extent of water and active glucose transport has been assessed. Our results of these parameters (i.e. water transfer and active transport of glucose) under control conditions were well within the range of physiological values reported in literature (Fisher and Parsons, 1949 and 1950; Richter and Strugula, 1985; Klee, 1992). As expected, the water transfer and glucose amount diminishes in descending order from the jejunal to the colonic compartment.

The water transfer continued at a constant flux rate for the 2 hours perfusion period in all intestinal segments of the rat. The glucose ratio of resorbate/perfusate was approximately 1.5 or higher, indicating the ability for active solute transport (Richter and Strugula, 1985; Klee, 1992; Spencer et al., 1999). Moreover, the advantage of this gut model is that it contains all the relevant metabolic enzymes in an environment that closely approximates that of the *in vivo* situation.

The amounts of genistein and genistin applied did affect the active transport of glucose in proximal jejunal segment, indicating that obviously the isoflavones enhanced the active transport of glucose. This effect seems to be in contrast to the studies of Walgren et al. (2000a) and Wolffram et al. (2002). Those authors showed that the presence of glucose inhibited the disappearance (metabolism) of quercetin-3-glucoside in perfusate. However, in these latter studies the amount of glucose in the resorbate was not measured. Hence, our results do not support their conclusion that the SGLT1 receptor is involved in the uptake of isoflavones glycosides.

The present study shows that genistein is mainly metabolised to two forms of glucuronides, which accounted for 24 and 16% of the applied amount present in the resorbate of jejunal proximal and distal segments, respectively. This observed rate is somewhat higher than reported results of experiments with genistein containing products in a vascular perfusion model and *in vivo* studies. More particularly, an absorption rate of 8% of genistein compounds from tofu (i.e. isolated small intestine) has been published by Andlauer et al. 2000b, which corresponded to rates of 14.6% (Lu et al, 1995), 9% (Xu et al., 1994), 10% (Xu et al., 1995) in human feeding volunteers studies with soy milk and of 16% with tofu (Xu, et al., 2000). Andlauer et al., (2000a) already showed that the absorption of genistein in the isolated small intestine was 41% and higher compared to genistein in soy products. However, in our study genistein was for only 10% metabolised in the ileac and colonic intestinal segments.

The present study clearly showed that less than 0.2% of genistin appeared in the resorbate fluid of all isolated gut segments. So far, only Sesink et al, (2001) reported a comparable result and demonstrated that there was no quercetin glycoside (i.e. parent compound) present in the circulation of humans. These latter results are in clear contrast to those of other studies, showing that phloretin and quercetin occur as glycosides in human plasma (Paganga and Rice-Evans, 1997). There are currently no reports of isoflavonic glycosides in human plasma besides those of studies using *in vitro* models. Piskula et al. (1999) compared the absorption of aglycones with that of

glycosides in rats. The glycosides were not absorbed, but their metabolites appeared in plasma with a few minutes delay as compared to an administration of aglycones only. Furthermore, Piskula showed a delay in absorption of metabolites of genistin when compared with the aglycones using a rat stomach model. This is in contrast to the study of Hollman et al. (1995) studying the uptake of quercetin glycosides in a model of ileostomy volunteers. They demonstrated that the glycosidic forms of quercetin were more efficiently absorbed than quercetin itself (Hollman et al., 1995; Olthof et al., 2000; Crespy et al., 2001)

Our previously reported *in vitro* studies using a Caco-2 cell lines based model also showed that the glycosides are hardly transported through an epithelial gut cell layer. These studies suggest that glycosides are not transported across the basolateral side of intestinal cells (Steensma et al, 1999; Walle et al, 1999). This is in agreement with our present results.

For the first time, we showed that genistin can be metabolised by microflora or epithelial cells present in the isolated colonic segments, but the metabolites of genistin did not occur in the resorbate of these isolated colonic segments. We believe that there is no transport of genistin and their metabolites in colonic gut wall tissue. This contradicts with the general assumption that the large intestines absorb the flavonoid glycoside after a bacterial deconjugation. Indeed, this study suggests that the use of various isolated perfused intestine are suitable tools for investigation of genistein and genistin transport, metabolism and mechanism.

Chapter 5

Bioavailability of genistein and its glycoside genistin as measured in the portal vein of freely moving unanaesthetized rats

Abstract

The present study describes an *in vivo* bioavailability experiment for genistein and its glycoside genistin using freely moving unanaesthetized rats with a cannulation in the portal vein. Genistein and genistin were dosed either as pure compounds or as extracted from a soy protein matrix.

The results show that genistein is readily bioavailable, already being observed in portal vein plasma at the first point of detection at 15 minutes after dosing, with a total maximum concentration of 16.5 μM. The results also reveal that genistin is partly absorbed in its glycosylated form. This result is different from that obtained in other studies. After analysing whole body plasma instead of portal vein plasma, it is concluded that deglycosylation of the absorbed genistin occurs upon first pass through the liver. Deglycosylation accompanying intestinal absorption also appears to be efficient because upon dosing the rats with genistin the portal vein plasma levels of genistin amount to an AUC that is generally 10-25 times lower than those of the deconjugated genistein and its metabolites.

Comparison of plasma metabolite levels, but also of metabolite patterns of faeces and intestinal contents of rats exposed to genistein, genistin or an extracted soy protein isolate showed that soy extract metabolite profiles resemble those of genistin more than those of genistein. This is in line with the natural distribution of isoflavones in the soy beans, where the glycosylated forms are dominant.

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Altogether the present study reveals that bioavailability studies based on portal vein plasma levels contribute to insight in the role of the intestine and liver in deglycosylation and uptake characteristics of glycosylated flavonoids. The glycosylated form in which these bioactive food ingredients generally occur in our diet can be absorbed to a limited extent.

Introduction

Several of the major diseases of Western populations are hormone dependent. Epidemiological data reveal that not only genetic factors but also life-style factors, such as diet, play an important role in disease aetiology. It has been estimated that about one third of all human cancers are related to the modern diet (Doll and Peto, 1981; Peto, 2001). Epidemiological and migration studies have shown that the low incidence of hormonally dependent cancers in Asian countries can be associated with a diet rich in soybean (Barnes et al., 1996; Adlercreutz and Mazur, 1997; Setchell and Cassidy 1999). Soybeans contain 1-3 mg/g of isoflavones. Asian people consume approximately 50 mg of isoflavones per day, whereas the average daily intake of isoflavones in Western countries has been estimated to be less than 1 mg per day (Kimira et al., 1998; Chen et al., 1999).

Isoflavones are structurally similar to estrogens and are able to bind to estrogen receptors either as agonist or antagonist (Mazur and Adlercreutz, 2000). Furthermore, isoflavones can inhibit enzymes involved in estrogen synthesis like 17 ß-steroid oxidoreductase and steroid sulfohydrolase (Mazur and Adlercreutz, 1998). In the last years, a protective effect of the isoflavone genistein against prostate tumours has been reported (Boersma et al., 2001).

In soybeans and soy-derived foods genistein is mainly present as a glycoside, genistin. In spite of this, most metabolism studies primarily focus on the metabolism of genistein in humans and animals, and there is limited information on the metabolism and bioavailability of the glycoside genistin (Setchell et al., 2001; Zubik et al., 2003; reviewed by Manach et al., 2005). One of the questions raised in flavonoid effect studies relates to the bioavailability of the glycoside forms and/or the need for deconjugation of the glycosides before absorption can actually occur. It is well known that colonic bacteria convert (iso)flavonoids glycosides into the corresponding aglycones. In addition, extra- and intracellular ß-glucosidase activity in the small intestine of human and animals provide an additional route for

deglycosylation and lead to the appearance of the aglycon and its metabolites in plasma with a few minutes delay compared to aglycon administered (Spencer et al., 1999; Setchell et al., 2001). Some studies have even reported that flavonoids may be absorbed in their glycosylated form (Hollman et al, 1995). Whereas in the case of genistin others have reported the lack of absorption of the isoflavonic glycosides in man (Piskula et al., 1999; Izumi et al., 2000; Setchell et al.,2001). On the other hand, it is well established that the aglycone genistein is absorbed from the intestine and conjugated with glucuronic acid during transport across the intestinal epithelial cells. After transport to the liver the glucuronide may be excreted in the bile, where after it could re-enter the small intestine allowing genistein to be deconjugated, absorbed and metabolised for the second time. This enterohepatic circulation may have important implications, because it delays the excretion and increases the duration of exposure.

In vivo, a primary location of glucuronidation appeared to be the intestinal wall, because an infusion of the rat duodenum with labelled ^{14}C genistein elucidated that the portal vein blood contains predominantly the 7-*O*-glucuronide of genistein (Sfakianos et al., 1997).

The aim of the present study was to obtain detailed information on the transport and metabolism of genistein and especially of its glycoside genistin, either dosed as pure compounds or extracted from a soy protein matrix, in an *in vivo* model, with special emphasis on the features of uptake of the glycosylated form. Our study also raises questions concerning the effects of anaesthetic on transport processes in the gut. For example, Uhing and Kimura (1995) investigating the transepithelial absorption of 3-*O*-methylglucose in the rat observed that acute effects of anaesthesia and surgery diminished carrier mediated (SGLT1 and GLUT2) but not paracellular or non-carrier mediated transport processes. The inference that carrier-mediated processes are diminished by anaesthesia and surgery in rat is also strongly supported by the experiments described by Ugolev et al. (1986).

To this end, rats with a permanent cannulation of the portal vein were therefore used as a model for studying the intestinal transport and metabolism. Permanent cannulation of the portal vein allowed for repeated blood sampling, while the rats were unanaesthetized and freely moving. Using this model, time dependent genistein and genistin derived portal vein plasma profiles could be obtained and compared to the metabolite pattern recovered in the small intestine, colon and faeces. This provided a detailed insight in the bioavailability of genistein and its glycoside genistin.

Materials and Methods

Materials

Genistein, genistin, polyethylene glycol, ß-glucuronidase (from *Escherichia coli*, 2000 units/ml) and sulfatase (from *Abalone entrails*, 250 units/ml) were obtained from Sigma Chemical Co. (St. Louis, MO). [4-^{14}C] Genistein (specific activity 0.2 mCi/ml) was purchased from Moravek Biochemical (Brea, CA). All other reagents used were of analytical grade and obtained from Merck (Darmstadt, Germany). An isoflavone-enriched soy protein isolate was derived from Protein Technology International (St. Louis, MO).

Animals

The experimental protocol was approved by the Animal Welfare Committee of Wageningen University (Wageningen, The Netherlands). Male rats (wistar, 200-250 gram) were obtained at 7 weeks of age from Charles Rivers and housed at the Laboratory Animals Centre (Wageningen University, The Netherlands). The rats were kept under standard conditions, temperature of 22 ±1°C, 12 hours light/dark cycle and humidity of 55% for 1 week before surgical operations and fed *ad libitum* with a standard diet (Hope Farms, Woerden, The Netherlands). After one week of acclimatisation, the surgical operation leading to the cannulation of the portal vein was carried out according to the method of Van Dongen et al. (1990). During the surgical operation, rats were anaesthetized by inhalation of isoflurane using oxygen as carrier. Then the abdominal cavity was opened and a cannula was inserted into the portal vein towards the liver. The cannula was fixed and placed in the abdominal cavity and was anchored to the internal abdominal muscle near the xiphoid cartilage. Subsequently, the abdominal wall was closed and the cannula was tunnelled subcutaneously and fixed together with an L-shaped adapter at the crown of the head. Twice a week the cannulas were checked if blood appears and closed with viscous PVP-solution until the experiment started. Starting one day after the operation rats were fed with a commercially available soy-free semi-purified diet (containing standard AIN-76 g/kg vitamin and mineral mixtures, 540 g/kg dextrose, 50 g/kg cellulose, 100 g/kg cornstarch, 200 g/kg casein and 50 g/kg corn oil (Hope Farms, Woerden, The Netherlands)). The experiment was started one week after the surgical operation.

Animal experiment

Rats had limited access to soy free food (10 g) 16 hours before the experiment started. Throughout the experiment rats could freely move and were given free access to tap water. The experiments were initiated by oral administration per gavage of the test solutions. Test solutions of genistein and genistin were prepared in ethanol and were diluted in polyethylene glycol at a maximum of 0.1% (v/v) ethanol. Soy protein isolate was concentrated by diluting in ethanol (1g/4 ml), shaken overnight and sonificated for 30 minutes. Subsequently the soy protein isolate was extracted three times by adding ethanol, vortexed and centrifugated at 2000*g for 10 minutes. The obtained supernatants were pooled and evaporated to dryness under a stream of nitrogen. The volume of the concentrate was adjusted to 1 ml with polyethylene glycol. At the start of the experiment, soy protein isolate was mixed with extracted soy protein isolate (1 g/4 ml), also called soy extract.

Table 5.1 gives an overview of the results of the analytical analysis of the isoflavone composition of the stock solutions, used to prepare the various test solutions.

***Table 5.1**: Isoflavone composition of the various stock solutions administered to the rats by oral gavage*

Compound analysis	Concentration in stock solution (mM)		
	Genistein	Genistin	Soy extract
Genistein	13.8 ±1.38		5.52 ±2.36
Genistin		9.36 ±1.61	12.1 ±1.62
Daidzein			6.01 ±2.48
Daidzin			6.53 ±2.15
Glycitein			7.87 ±1.07
Glycitin			3.53 ±1.19

The test solutions were administered by oral gavage at a dose level of 15 mg of genistein or genistin/kg body weight at a maximum volume per dosage of 1 ml/rat. The control rats received a solution of polyethylene glycol and 0.1% ethanol only. Before the experiment started, rats were weighted and divided by weight in four treatment groups: 1) a control group, 2) a genistein group, 3) a genistin group and 4) a soy protein isolate group. Ten rats were used per experimental group. Aliquot samples of 0.3 ml blood were taken from the portal vein via the cannula at 0, 0.25, 0.5, 1, 2.5, 4, 5, 6 and 7 hours after administered of the isoflavones. 24 Hours after administration of the various isoflavones the animals were sacrificed by total

(heparinised) blood collection via aortal puncture under isofluran anaesthetics. Faeces were collected during the whole duration of the experiment (24 hours). Luminal contents was collected from five 10 cm parts of the intestine (i.e. 5 cm from the pyloric sphincter for duodenum, 5 cm from the flexura duodeno-jejunalis in the jejunum proximal part, 20 cm from the flexura duodeno-jejunalis in the jejunum distal part, 10 cm from the valvula ileocoecalis in the ileum and 10 cm from ceacum to rectum in the colon). All samples were stored at –80°C until analysis of genistein, genistin and their respective metabolites.

Extraction of genistein, genistin and metabolites from plasma

Aliquots of 0.3 ml of portal venous blood samples were collected in tubes pre-treated with heparin 0, 0.25, 0.5, 1, 2.5, 4, 5, 6 and 7 hours after dosing. Plasma was prepared by centrifugation of the blood samples for 10 minutes at 12000*g and stored at –80°C until analysis.

The isoflavones were extracted by passage of the plasma over an activated Oasis HLB-30 cartridge (Waters, Etten-Leur, The Netherlands), pre-equilibrated with 3 ml methanol and 3 ml double distillated water. The cartridge was washed with 3* 3 ml of 10 mM ammonium acetate (pH 6.5), 3 ml 10% methanol in 10 mM ammonium acetate (pH 6.5) and isoflavones were eluted with 4 ml methanol. The methanol fraction was evaporated to dryness under a stream of nitrogen at 37°C. Residues were sonificated for 10 minutes in 0.1 ml acetonitril/10 mM ammonium actetate (pH 6.5) (1:1; v/v) and stored at –20°C until analysed by HPLC.

Extraction of genistein and genistin metabolites from faeces

The faeces were lyophilised, and powdered using a pestle and mortar. Samples of 0.05 g were taken in duplo and 1 ml ice-cold methanol was added. The samples were shaken for 1 hour and sonificated for 30 minutes. Subsequently samples were extracted three times by adding 1 ml of ice-cold methanol, vortexing and centrifugating at 2000 *g for 10 minutes. The three methanol supernatants thus obtained, were pooled and the volume adjusted to 10 ml with 10 mM ammonium acetate (pH 6.5). The faecal isoflavones were further extracted by passage over an activated Oasis HLB-30 cartridge (Waters, Etten-Leur, The Netherlands), as described above.

Extraction of genistein, genistin and metabolites from luminal contents

The various lyophilised intestinal contents were extracted three times by adding 1 ml of ice-cold methanol, vortexing and centrifugating at 2000 *g for 10 minutes. The three supernatants obtained, were pooled and the volume adjusted to 5 ml with 10 mM ammonium acetate (pH 6.5). The isoflavones in the contents of the intestinal were further extracted by passage over an activated Oasis HLB-30 cartridge (Waters, Etten-Leur, The Netherlands), as described above.

Detection of genistein, genistin and their metabolites

The extracted plasma, faecal or intestinal samples were analysed by HPLC equipped with an autosampler (Waters, Etten-Leur, The Netherlands), an UV photodiode array detector (Waters, Etten-Leur, The Netherlands) and an electrochemical detector (Decade, Antec Leyden BV, Leiden, The Netherlands). Separation was performed on a Supelcosil LC-ABZ column (25 cm x 4.6 mm ID, 5 µm particles) from Supelco (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands), maintained at 30°C (Merck Hitachi column heater). The column was eluted at a flow rate of 1.0 ml/min employing a gradient of two eluens. The solvents for the gradient elution were 10 mM ammonium acetate (pH 6.5) with 2.5% (v/v) acetonitrile (eluent A) and acetonitrile (eluent B). The following gradient was used: 0-5 minutes 10% eluent B, 5-10 minutes 10-32% eluent B, 10-25 minutes 32% eluent B, 25-35 minutes 32-40% eluent B, 35-40 minutes 40-50% eluent B, 40-45 minutes 50% eluent B, 45-50 minutes 50-10% eluent B and 50-60 minutes 10% eluent B. The eluent was monitored both at 260 nm and 76 mV.

Routinely 50 µl of extracted plasma, faecal or intestinal samples were injected onto the HPLC column and genistein, genistin and their metabolites were identified by comparison of retention times and UV spectra of the respective peaks to those of the known standards. The unknown metabolites were identified on the basis of [4-^{14}C] genistein used in a pilot experiment. In this pilot experiment two rats were orally administered 15 mg [4-^{14}C] genistein (0.375 kBq) / kg bw and they were sacrificed by total (heparinised) blood collection via aortal puncture under isofluran anaesthetics after 0.5 hours. Plasma, faecal and intestinal samples were collected and extracted by the method described above. The radioactive samples were analysed on a Waters HPLC system connected to both a photodiode array detector (Waters, Etten-Leur, The Netherlands) and an on-line radioactivity detector (LB506C, Berthold, Wildbad, Germany) by a method described elsewere (Steensma et al., 2004). The UV and electrochemically established chromatograms were compared to the radiochemical

chromatograms in order to verify and identify retention times of the (unknown) metabolites.

The amount of genistein glucuronides and sulfates was calculated from the increase in the amount of genistein upon treatment of the samples with respectively ß-glucuronidase and/or sulfatase. To this end, aliquots of extracted plasma were incubated with either ß-glucuronidase (2000 U/ml) in 100 mM potassium phosphate (pH 7.0) or sulfatase (250 U/ml) with 17 mM D-saccharic acid 1,4-lactone in 0.5 M sodium acetate (pH 5.0) in a dilution of 2:1 (plasma:enzyme buffer v/v). After overnight incubation at 37°C the isoflavones were measured as describe above.

Statistics

Results are presented as means ± SEM. Statistical evaluation of data was performed by one-way ANOVA ($p<0.05$) and the comparison of values between the treated groups and the control group was based upon the Tukey's Honestly Significant Difference test ($p<0.05$).

Results

Genistein, genistin and their metabolites in portal vein plasma

Figure 5.1 presents the time dependent concentration of genistein (free (Figure 5.1A) and conjugated genistein (Figure 5.1B)) in the portal vein plasma of the rats administered with either genistein, genistin or soy extract.

The pharmacokinetic data are summarised in table 5.2. For all the test solutions a significant peak in the portal vein plasma concentration of genistein and its conjugates was detected 15 minutes after administration. Portal vein plasma genistein levels were highest in rats receiving genistein, amounting to a peak level (Cmax) that was about 6 times higher than the peak level detected in portal vein plasma of rats exposed to similar amounts of genistin or the soy extract isoflavones (Figure 5.1A, Table 5.2). The maximum concentration (Cmax) for genistein observed 15 minutes after dosing was 5.49, 0.91 and 0.78 µM for genistein, genistin and soy extract exposed rats respectively (Figure 5.1A and Table 5.2). Whereas, the area under the curve ($AUC_{(0\text{-}24\ hr)}$) for genistein was 9.76, 2.65 and 1.14 µM h for genistein, genistin and soy extract administered, respectively. Portal vein plasma genistein patterns of genistin and soy extract exposed rats appeared to be similar. For rats exposed to genistin and soy extract a second genistein portal vein plasma peak was observed at

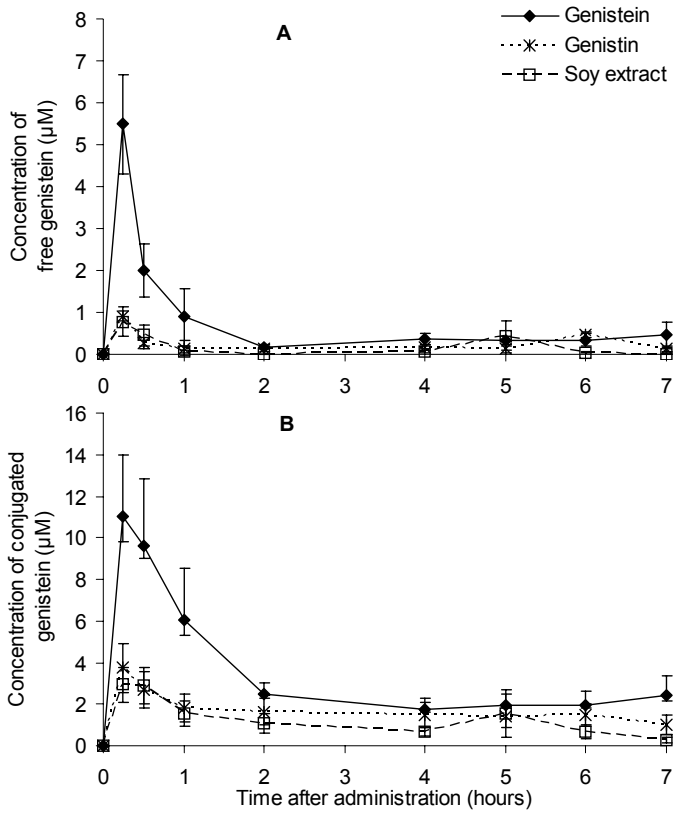

***Figure 5.1**: Concentration of A) unconjugated (free) genistein and B) conjugated genistein (i.e. glucuronides and sulfates) in portal vein plasma of cannulated rats. The rats were administered with genistein, genistin or the soy extract. The dose of genistein and/or genistin was 15 mg/kg BW. Data are expressed as mean ± SEM for n=10 rats for genistein and genistin and n=9 for soy extract.*

***Table 5.2**: Plasma pharmacokinetic variables of genistein, conjugated genistein and genistin of rats who received a single oral dose of genistein, genistin or soy extract.*

	Genistein			Conjugated genistein			Genistin		
Compound administered	AUC (µmol h/l)	Cmax (µM)	Tmax (h)	AUC (µmol h/l)	Cmax (µM)	Tmax (h)	AUC (µmol h/l)	Cmax (µM)	Tmax (h)
Genistein	9.76	5.49	0.25	44.6	11.0	0.25			
Genistin	2.65	0.91	0.25	20.9	3.79	0.25	0.85	0.15	2
Soy extract	1.14	0.78	0.25	10.5	2.93	0.25	1.42	0.52	5

Note: AUC was calculated over a time period of 0 to 24 hours after dosing.

respectively 6 and 5 hours amounting to about 50% and 55% of the plasma peak concentration at 15 minutes after dosing. In our hands the administration of genistein did not show a clear second peak at later time points.

The HPLC chromatograms of plasma samples from the rats that were administered with genistein showed several other peaks. Treatment of these samples with ß-glucuronidase and sulfatase resulted in the loss of five peaks, with a concomitant formation of genistein. Figure 5.1B shows the time dependent plasma concentration of conjugated genistein as measured, upon deconjugation with sulfatase and glucuronidase, after dosing with genistein, genistin or soy extract. This figure shows a similar trend as presented in figure 5.1A for unconjugated genistein, with the concentration of conjugated genistein being highest in plasma of rats administered genistein and amounting to a Cmax of approximately 11 μM at 15 minutes after administration. The Cmax of conjugated genistein in portal vein plasma of rats dosed with genistin and soy extract was 3.76 and 2.93 μM, respectively. As observed in the portal vein plasma for free genistein, the time-dependent portal vein plasma profile of conjugated genistein also revealed a second peak at time points similar to those observed in figure 5.1.A., that is at 5 hours for soy and 6 hours for a pure genistin administration.

Figure 5.2 shows the portal vein plasma concentration of genistin after administration of genistin or soy extract. The Cmax for genistin appeared at 2 and 5 hours having a level of 0.15 and 0.52 μM after genistin or soy extract dosing, respectively and the $AUC_{(0\text{-}24\ hr)}$ or genistin was 0.85 and 1.42 μmol h/l for genistin and soy extract administration, respectively (Table 5.2). From these data it follows that the portal vein plasma levels of genistin amounted to a level that is generally 10-25 times lower than those of deconjugated genistein including its metabolites. This points at an efficient deconjugation of the glycosylated form before absorption occurs but also indicates intestinal uptake of the glycoside genistin.

Figure 5.3 presents the time dependent portal vein plasma profile of different conjugated genistein metabolites (i.e. glucuronidated and sulfated forms of genistein and an unknown genistein-like metabolite). From these profiles it is concluded that independent of the form in which genistein is administered, more particularly in its unconjugated form, in its glycosylated form as genistin or as a soy extract in which genistein is glycosylated to a large extent (Table 5.1), genistein glucuronides appear to be the major metabolites. Although a Cmax for the glucuronidated metabolites occurred at the same time, i.e. 15 minutes for all administrations, the levels of Cmax

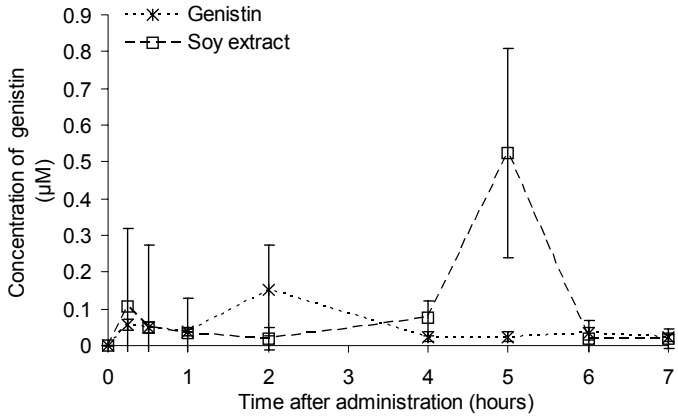

***Figure 5.2**: Concentration of genistin in portal vein plasma of cannulated rats. The rats were administered with genistin or soy extract. The dose of genistin was 15 mg/kg BW. Data are expressed as mean ± SEM for n=10 rats for genistin and n=9 for soy extract.*

were lower when the isoflavone was administered as genistin or soy extract, pointing at a less efficient formation of these metabolites.

Genistein, genistin and their metabolites in faeces

Figure 5.4 present the genistein metabolite patterns detected in 24 hours faeces of rats exposed to genistein, genistin or soy extract. In addition to the detection of genistein and genistin themselves, the faeces appeared to contain several additional genistein derived metabolites, including equol, O-desmethylangolensin (ODMA) as well as four unidentified metabolites. Several of these metabolites could be identified as genistein glucuronides and sulfates because following treatment with ß-glucuronidase or sulfatase some of the peaks disappeared, accompanied by an increase in the intensity of the peak at the position of genistein.

The amount of equol and ODMA detected in the 24 hours faeces of rats receiving soy extract were approximately 11 µmol of equol and 1 µmol of ODMA, respectively. This appeared to be significantly higher if compared to the amounts detected in 24 hours faeces of rats administered with genistein and genistin, where the amounts of equol and ODMA were approximately 1.0 µmol and 0.2 µmol for genistein administration and 1.2 µmol and 0.5 µmol for genistin administration, respectively. The faecal amount of genistein was the highest in rats administered with genistein, as approximately 1.8 µmol genistein was recovered in those faeces. An amount of

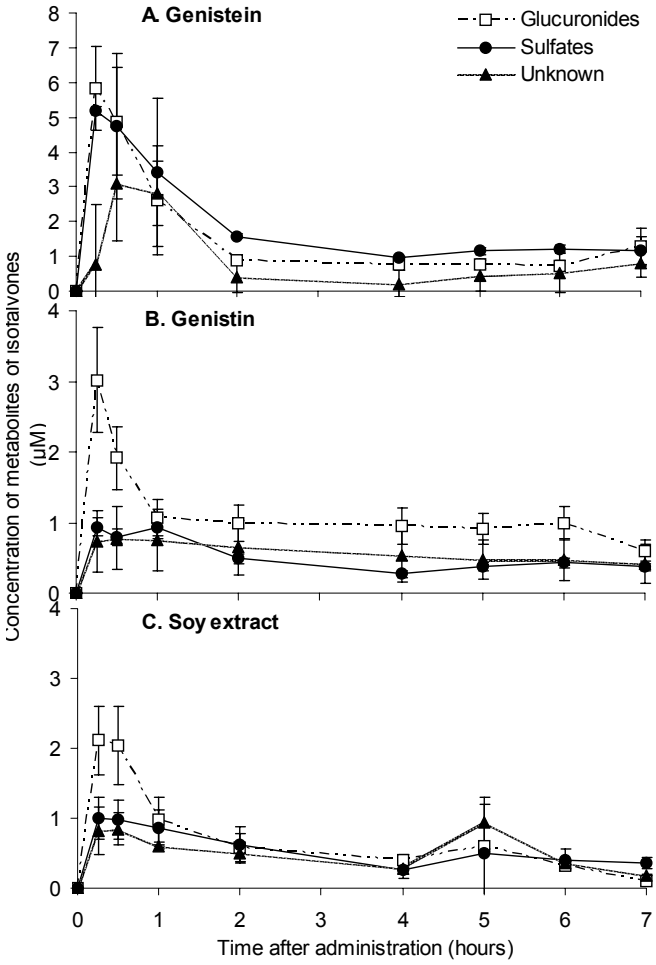

***Figure 5.3**: Metabolite profiles in portal vein plasma of cannulated rats. The rats were administered with A) genistein, B) genistin or C) soy extract. The dose of genistein and/ or genistin was 15 mg/kg BW. Data are expressed as mean ± SEM for n=10 rats for genistein and genistin and n=9 for soy extract.*

approximately 0.4 µmol and 0.2 µmol genistein was detected in faeces of rats administered with genistin or soy extract, respectively. The total recovery of genistein, genistin and their metabolites in the faeces was 29, 36 and 35% of the dose administered with genistein, genistin or soy extract, respectively.

Genistein and its metabolites in intestinal contents

Figure 5.5 shows the luminal amount of unconjugated genistein detected for rats of the three dose groups. For comparison amounts detected in the faeces are also

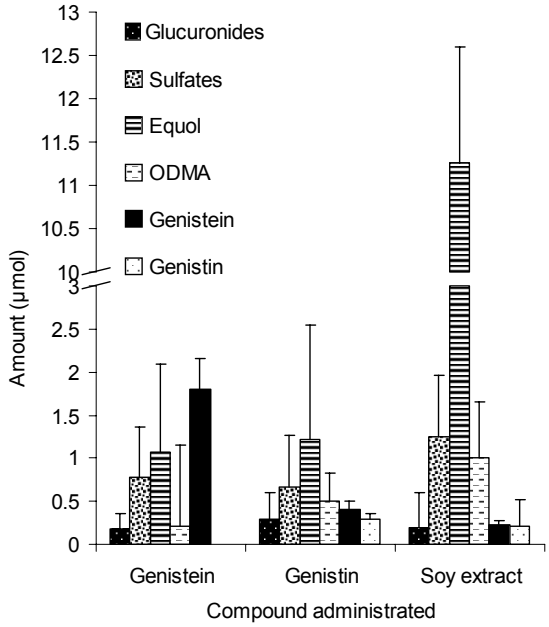

***Figure 5.4**: Metabolite amount in 24 hours faeces of cannulated rats. The rats were administered with genistein, genistin or soy extract and the dose of genistein and/or genistin was 15 mg/kg BW. Data are expressed as mean ± SEM for n=10 rats for genistein and genistin and n=9 for soy extract.*

included. As expected, the genistein amount was the highest in rats dosed with this compound. The intestinal contents of rats administered with genistein contained an almost factor 3 times higher amount of genistein than the intestinal contents of rats dosed with genistin or soy extract (Figure 5.5A). Furthermore, the amount of unconjugated genistein in all parts of the intestine was more or less identical in rats administered with genistin or the soy extract. The highest amount of conjugated genistein metabolites recovered in the luminal contents was in the ileum part of the intestine for all the compounds administered (Figure 5.5B).

Discussion

This study focuses on the *in vivo* intestinal transport and metabolism of the isoflavone genistein in both its aglyconic and glycosidic form, when dosed either as pure compounds or extracted from a soy matrix (i.e. enriched protein isolate). To this end, rats were used that were permanently cannulated in their portal vein, but could freely

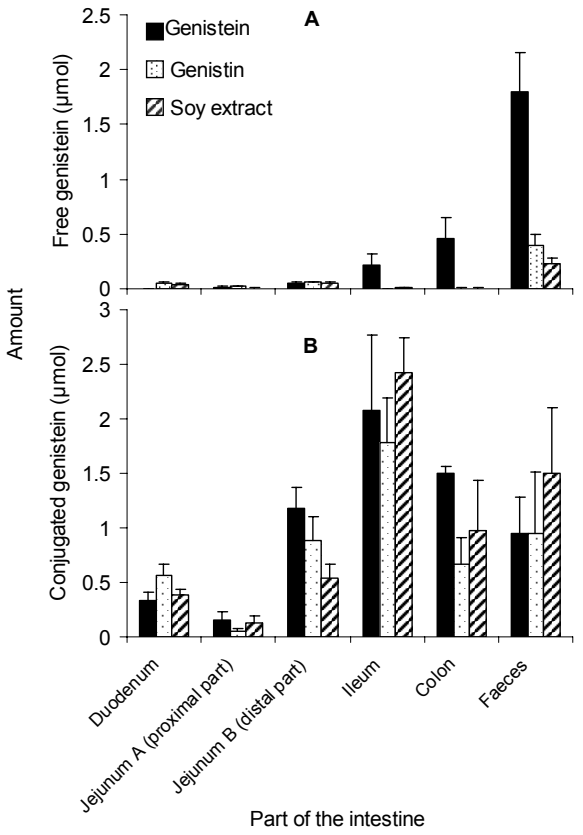

Figure 5.5*: Amount of free and conjugated genistein in contents of the intestines and 24 hours faeces of cannulated rats. The rats were administered with genistein, genistin or soy extract. The dose of genistein and/or genistin was 15 mg/kg BW. Data are expressed as mean ± SEM for n=10 rats for genistein and genistin and n=9 for soy extract.*

move. Our experiments show that the maximum plasma concentration (Cmax) and the time at which this peak level appeared (Tmax) was identical among all three test groups and observed at the first sampling point (i.e. 15 minutes). In contrast, most studies with human volunteers report a first sampling point after approximately 1 or 2 hours (Setchell et al. 2001,2002b,2003b; Bloedon et al., 2002; Busby et al., 2002; Zubik et al., 2003) or even later with a mean first sampling time of 4-6.5 hours after dosing (reviewed by Manach et al., 2005). Coldham et al., (2000 and 2002) reported an *in vivo* bioavailability study of genistein in rats with a first sample point and a Cmax at 30 minutes after an oral administration. The observations of the present study point to a swift absorption of free and conjugated genistein within already the

first 15 minutes after administration. However, the exact Cmax and Tmax values of genistein or genistin could even not be estimated due to experimental limitations, whereas this model did not allow an earlier first sampling point. Therefore, these parameters remain still to be studied in more detail. Nevertheless, the present study demonstrates in all three test groups a Cmax at 15 minutes upon dosing. It might therefore be concluded that the initial hydrolysis of genistin to its aglycon genistein is relatively fast and not a major rate-limiting step for the absorption of genistin.

It is postulated that the appearance of two subsequent plasma peaks might be in line with the general speculation that upon first passage through the intestinal wall, the isoflavones are readily conjugated with glucuronic acid. After transport to the liver the glucuronides are excreted in the bile, allowing this isoflavone after re-entering the small intestine to be absorbed and metabolised for the second time yielding the second peak observed in plasma (Coldham et al., 2000 and 2002).

For the first time, it is shown that shortly after oral intake of the glycoside genistin, the compound is also present as an intact molecule in plasma of the portal vein, albeit at concentrations that are approximately 10-25 times lower if compared to the levels of the deglycosylated form genistein or its metabolites. These observations are in contrast with those of Sesink et al. (2001) and Setchell et al. (2002b). Sesink et al. (2001) studying the intestinal metabolism of quercetin-3-glucoside, reported that no quercetin-3-glucosides was found in blood samples taken from the mesenteric vein and Setchell et al.(2002b) communicated that there is a lack of absorption of the isoflavone glycosides in humans. In this latter study adults ingested 50 mg of genistin and blood samples were obtained by a vein puncture. In both these studies, liver metabolism could significantly have contributed to the observed plasma metabolite patterns. In our study the blood samples were taken from the portal vein, eliminating as much as possible the contribution of liver metabolism. Therefore, our results suggest that limited amounts of the glycoside are able to enter the portal vein, and are subsequently fully deglycosylated and metabolised upon first pass through the liver. This conclusion is in line with results of *in vitro* studies using an isolated rat small intestinal perfusion model or Caco-2 cell models (Andlauer et al., 2000a; Andlauer et al., 2004; Steensma et al., 2004). These authors showed that a small amount of genistin indeed crosses the intestinal cellular monolayer.

In the present study the $AUC_{(0\text{-}24\ hr)}$ for total genistein was 54, 24 and 13 µmol h/l upon dosing of genistein, genistin and the enriched protein soy extract, respectively. These results indicate that the bioavailability of genistein is higher for the aglycon

compared to the glycosidic form. This is in line with the study of Piskula et al., (2000) and likely to represent the relative bioavailability to be expected in human (Setchell et al., 2003a). In addition, comparing our genistein bioavailability data for genistin and soy extract exposed rats reveals that the bioavailability is not significantly influenced by the presence of other compounds present in the soy enriched protein isolate such as for example other isoflavonic aglycones and/or glycosides like daidzein and glycitein. Andlauer et al. (2000b) reported a higher genistein absorption in rats after dosing with tofu products if compared to genistin applied as a pure compound. *In vitro* and *in situ* studies demonstrated a possible interaction of genistein and its conjugates with apical intestinal efflux-transporters, such as MRP2 (multidrug resistance protein 2) as shown by Walle et al. (1999) and BCRP1/ABCG2 (breast cancer resistance protein) as reported by Imai et al. (2004). These interactions may provide a possibility for higher genistein plasma concentrations upon inhibition of the MRP2 and BCRP mediated efflux from intestinal cells back into the intestinal lumen. One could therefore hypothesize that tofu contains inhibitors of these efflux-transporters. Obviously, in our extracted soy protein isolate these inhibitors may not be present. This statement is supported by the fact that daidzein, the major other isoflavone present in the soy extract, shows a low interaction with the BCRP1/ ABCG2 transporter (Imai et al., 2004).

In the present study the major amount of genistein and its metabolites are found in the luminal contents of the small intestine and faeces up to a total recovery of approximately 73 and 80% of the genistein and genistin administered, respectively. The recovery of genistein and its metabolites in faeces is in line with the recovery reported by Coldham et al. (2000). These authors used radioactive labelled genistein in rats.

In conclusion, the present study, using freely moving rats with a permanent cannulation in the portal vein, reveals that measurement of the isoflavone metabolite profiles in portal vein plasma contributes to an insight in the role of (de)glycosylation during the uptake. It is demonstrated that the glycosylated form in which these bioactive food ingredients generally occur in our diet can be absorbed to a limited extent, and also that intestinal deglycosylation is highly efficient. It appears likely that changes in perfusion techniques might have significant effects on the isoflavone profiles at the serosal side. Changing to a different intestinal absorption model, modifying the vascular circulation of the gut segment preparation or manipulations of isolated intestinal segments during surgery (Steensma et al., 2004b) all have the

potential to exert an effect, possibly a major one, on the absorption of the glycosides. With many potential variations in the formulations of dosed compounds and the perfusion designs used, it is likely that different authors have reported different mechanisms at work: in some instances regulation will occur only after hydrolytic activities, in others by changes in transporter levels, and in yet other cases by some combination thereof. It is therefore essential to bear such considerations in mind when studying the absorption of glycosidic isoflavones.

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Chapter 6

Bioavailability and mechanism of absorption of the glycoside genistin in the *in situ* perfused intestine of the rat

Abstract

Genistein is primarily present as its glycoside genistin in soybeans and soy derived food and the bioavailability and mechanism of absorption of isoflavonoid glycosides is largely unknown. The present study investigates whether the sodium dependent glucose transporter (SGLT1) plays a crucial role in the metabolism and absorption of genistein and genistin, the glycoside derivate of genistein. To study this mechanism, *in situ* single pass perfusion of small intestines of rats has been performed with genistin or genistein in the absence or presence of phloridzin, a competitive inhibitor of SGLT1. Thus, rat intestines with an intact vascular circulation were perfused and at the same time blood samples were taken from the cannulated portal vein.

Coperfusion with phloridzin increased both the concentration of genistein and of the glycoside genistin in the portal vein plasma. In addition, the glucuronidation activity is inhibited if phloridzin is present in the perfusion fluids. The results suggest that the SGLT1 transporter is not involved in the transport and absorption of genistin in the small intestinal tract of the rat. Instead, the large amount of deglycosylated genistin in the perfusion fluid reveals that genistin absorption is facilitated by a hydrolase in the brush border membrane of the intestine catalyzing conversion of genistin to genistein before actual absorption occurs.

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Introduction

The isoflavone genistein is primarily present as a glycoside derivate also called genistin in soybeans and soy-derived foods (Coward et al., 1993; Reinli and Block, 1996; Munro et al., 2003). In most of the studies reported over the past decade on the bioavailability of the isoflavone, the aglycone genistein has been studied (reviewed by Manach et al., 2005). Genistein intake is associated with a lower incidence of hormone-related cancers and cardiovascular diseases (Barnes et al., 1996; Adlercreutz and Mazur, 1997; Setchell and Cassidy, 1999; Clarkson, 2000). The reported biological activities of genistein are numerous and have been explained by different mechanisms of action (Record et al., 1997; Boersma et al., 2001). For example, genistein has been shown to inhibit different kind of enzymes, like tyrosine kinase (Akiyama et al., 1987), topoisomerase II (Kaufmann, 1998) and steroid-metabolising enzymes, such as 17 ß-hydroxysteroid oxidoreductase (Mäkelä et al., 1995) and aromatase (Wong et al., 1997; Kao et al., 1998). On the other hand, the cardiovascular effects of genistein are coupled to the inhibition of lipoprotein oxidation (antioxidant activity) and a lowering of serum cholesterol (Fotsis et al., 1993; Hodgson et al., 1996). However, harmful effects of genistein have also been reported (Rao et al., 1997; Record et al., 1997 and Gee et al., 2000b).

Most studies in humans and animals concentrate on the bioavailability of genistein, but there is very limited information on the fate of the respective glycoside. The originally proposed model of glycoside absorption assumes that the glycosides are too polar to be absorbed by the small intestine, and that therefore assumes that the absorption occurs in the large intestine after bacterial deconjugation (Griffiths and Barrow, 1972). Nowadays, several human and laboratory animal studies point at a possible role of ß-hydrolytic activity of the small intestine, because of, for instance, the rapid absorption of dietary flavonoid glycosides (Hollman et al., 1995; Spencer et al., 1999; Walle et al., 2000; Setchell et al., 2002b). Contradictory results have been obtained by studying the differences between the bioavailability of aglycones and glycosides. Some authors found a greater bioavailability of glycosides (Setchell et al, 2001), others found a greater bioavailability of aglycones (Izumi,et al., 2000) and finally, some did not found any significant differences (Richelle et al., 2002; Zubik and Meydani, 2003). It has also been concluded that no glycoside appeared in the systematic circulation (Piskula et al., 1999; Setchell et al, 2001, Sesink et al., 2001; Day et al., 2003). Furthermore, studies with isolated intestinal segments, which did not contain their usual bacterial contents, showed that genistin decomposes into

genistein before absorption, followed by conjugation of the aglycone, for instance, with glucuronic acid (Andlauer et al, 2000a and b). Sfakianos et al. (1997) reported a reappearance of the ß-glucuronide in the bile after rats were administered a ß-glucuronide conjugate of genistein. Thus, the relative contribution of glycosidase activities in the intestinal epithelium cell lining remains to be studied in more detail. It is postulated that a potential mechanism of absorption of genistin might proceed by a route identical to that of flavonoid glycosides including hydrolysis by a ß-glucosidase (i.e. lactase phloridzin hydrolase LPH). This ß-glucosidase activity is located at the brush border membrane of the small intestine (Scalbert and Williamson, 2000; Day and Williamson, 2001). LPH may cleave the glycoside, after which the aglycone diffuses across the membrane into the cytosolic compartment of the epithelial cell (Day et al., 2003; Sesink et al., 2003). On the other hand, glycosides might enter the intestinal cell as intact glycosides via the sodium dependent glucose transporter (SGLT1) localized in the small intestinal brush border membrane (Toggenburger et al., 1992; Gee et al., 1998). Inside the cellular compartment cytosolic ß-glycosidases may subsequently cleave the glycoside, after which the aglycones and/or their respective metabolites enter the blood stream (Day et al., 1998).

The aim of the present study is to investigate if SGLT1 plays a crucial role in the absorption and metabolism of genistin. Thereto, single-pass *in situ* perfusions of the small intestines of rats under conditions of low mechanical stress (i.e. no restricted blood supply and a low flow rate of the perfusate) were performed with the glycoside genistin or its aglycone luminally administered both in the absence and presence of 200 µM phloridzin. Phloridzin has been used as a competitive inhibitor of SGLT1 (K_m = 180 µM for phloridzin, Ramaswamy and Radhakrishnan (1975)). A repeated blood sampling via the portal vein was used in order to study the transport and metabolism of the isoflavone glycoside (i.e. genistin).

Materials and Methods

Materials

Genistin, genistein, phloridzin, ß-glucuronidase (from *Escherichia coli*, 2000 units/ml) and sulfatase (from *abalone entrails*, 250 units/ml) were obtained from Sigma Chemical Co. (St. Louis, MD). Tyrode buffer contained 136.9 mM NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$, 4.2 mM NaH_2PO_4, 1.2 mM $CaCl_2$*2 H_2O, 0.5 mM $MgCl_2$ *6 H_2O and 15 mM glucose. Supelcosil LC-ABZ column was purchased from

Supelco (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). Oasis HLB-30 cartridge was obtained from Waters (Etten-Leur, The Netherlands). All other reagents used were of analytical grade and obtained from Merck (Darmstadt, Germany).

In situ perfusion studies

The preparation of the perfused intestine was carried out according to the method as described by Sesink et al. (2003) and Van Dongen et al. (1990). Male rats (Wistar, 200-250 gram) were obtained at 7 weeks of age from Charles Rivers and housed at the Laboratory Animals Centre (Wageningen Agricultural University, The Netherlands). The rats were acclimated and kept under standard conditions, temperature 22 ±1°C and 12 hours light/dark cycle, humidity 55% for one week before start of operations and fed ad libitum on a soy-free based diet (Harlan, The Netherlands).

Rats had limited access to food (10 grams) 16 hours before surgical treatment and start of the experiment. Rats were anaesthetized by inhalation of isoflurane using oxygen and a mixture of nitrous oxide and oxygen (1:1) as carrier and animals remained unconscious throughout the whole experiment. First, the abdominal cavity was opened and a cannula was inserted into the portal vein towards the direction of the liver. Subsequently, two cannulas (internal diameter 1.52 mm) were inserted in the small intestine (i.e. jejunum and ileum) as previously described by Sesink et al. (2003). Finally, the luminal contents of the cannulated segment was withdrawn by flushing gently with Tyrode buffer (37°C) at adequate rinse volumes. Low mechanical stress perfusion was started by injecting 10 ml of test solution (37°C) in the cannulated segment, and connecting the intestine immediately to a single-pass perfusion system. Constant perfusion took place at a flow rate of 1 ml/min at 37°C, using Tyrode buffer. The test perfusates were: Tyrode buffer containing either 50 µM genistin, 50 µM genistein, 50 µM genistin plus 200 µM phloridzin or 50 µM genistein plus 200 µM phloridzin. Stock solutions of genistin, genistein and phloridzin were prepared in ethanol and diluted in Tyrode buffer at a maximal level of 0.1% (v/v) ethanol. Control solution contained Tyrode buffer with 0.1% (v/v) ethanol only. Control perfusions with Tyrode buffer without isoflavones confirmed that the small intestine of the experimental rat model did not contain any isoflavones sequestered from their food. The stability of the compounds was confirmed by measuring the concentration of the compounds in the perfusion fluid before passage through the intestine at the start and the end of the perfusions. Aliquots of 0.3 ml blood from the portal vein were collected in tubes pre-treated with heparin from the portal vein

through the cannule at respectively 0, 7.5, 15, 25, 40 and 60 minutes after initiating the perfusion. At the same time points aliquots of perfusate were taken after having been gently flushed through the small intestine (i.e. ileac side). After 1 hour of perfusion the animal was sacrificed and a final sample of heparinised blood was collected via aorta puncture.

Extraction of genistein and its metabolites from plasma

Plasma was prepared by centrifugation of blood samples for 10 minutes at 12000*g and the supernatants were stored at –80°C until analysis. The isoflavones from the plasma were extracted by passing them over an activated Oasis HLB-30 cartridge (Waters, Etten-Leur, The Netherlands). The cartridge was equilibrated with 3 ml of methanol and 3 ml of double distilled water. The cartridge was washed with 3* 3 ml of 10 mM ammonium acetate pH 6.5, 3 ml 10% methanol in 10 mM ammonium acetate pH 6.5 and eluted with 4 ml of methanol. Methanol was evaporated to dryness under a stream of nitrogen at 37°C. Residues were sonificated for 10 minutes in 100 µl acetonitril containing 10 mM ammonium actetate pH 6.5 (1:1 v/v) and stored at –20°C until HPLC analysis.

Detection of genistin, genistein and its metabolites

A Waters HPLC pump and autosampler (Waters, Etten-Leur, The Netherlands) was used, equipped with an UV photodiode array detector (Waters, Etten-Leur, The Netherlands) and with an electrochemical detector (Decade, Antec Leyden BV, Leiden, The Netherlands). Solvent A consisted of 10 mM ammonium acetate pH 6.5 with 2.5% (v/v) acetonitril and solvent B was acetonitril. Separation was performed on a Supelcosil LC-ABZ column (250 X 4.6 mm i.d., 5 µm particles), maintained at 30°C (Merck Hitachi column heater). The following gradient was used at a flow rate of 1 ml/min: 0-5 minutes 10% solvent B, 5-10 minutes 10-32% solvent B, 10-25 minutes 32% solvent B, 25-35 minutes 32-40% solvent B, 35-40 minutes 40-50% solvent B, 40-45 minutes 50% solvent B, 45-50 minutes 50-10% solvent B and 50-60 minutes 10% solvent B. The eluent was monitored at 260 nm using an UV photodiode array detector and electrochemically at 76 mV.

Routinely 50 µl of aliquots of perfusate or extracted plasma were injected onto the HPLC column and metabolites were identified by comparing the results with the retention times of known standards or with those of profile where rats were dosed with genistein spiked with [4-^{14}C] genistein (i.e. previous radioactive labelled experiments (Steensma et al., submitted, chapter 5)). To determine glucuronide and or

sulfate conjugates, aliquots of perfusate or extracted plasma were incubated with either ß-glucuronidase in 0.1 M potassium phosphate pH 7.0 or sulfatase with 17 mM D-saccharic acid 1,4-lactone in 0.5 M sodium actetate pH 5.0 at 37°C for 1 hour. After overnight incubation the deconjugated isoflavones were measured as described above.

Inhibition of SGLT1

After 60 minutes of perfusion glucose levels were determined photometrically in the portal venous plasma samples using the Glucose/GOD-perid test kit (glucose oxidase, peroxidase, ABTS Boehringen Mannheim GmbH, Germany).

Statistics

Results are presented as means ± SEM. Statistical evaluation of data was performed by one-way ANOVA ($p<0.05$) and the comparison of values between the treated groups and the control group was based upon the Tukey's Honestly Significant Difference test ($p<0.05$).

Results

Efficacy of inhibition of SGLT1

The glucose concentration in portal vein plasma is shown in figure 6.1, measured in the *in situ* intestinal rat model perfused with Tyrode buffer without or with the test compounds genistin or genistein in the presence or absence of phloridzin. As expected, a significant decrease ($p \leq 0.05$) of approximately 40% in the concentration of glucose in plasma was observed when phloridzin was added to the perfusion media and this indicates that intestinal SGLT1 present in the brush border membrane of the small intestine is effectively inhibited by the high affinity inhibitor phloridzin in our perfusion model.

Isoflavones levels in perfusion media

The initial concentration of genistin or genistein in the perfusion fluid of about 55 ±2 µM for both test compounds did not significantly differ from that of the groups perfused in the presence of phloridzin. After passage through the cannulated small intestinal segment the initially added concentration of genistin has dropped to approximately 26 and 25 µM for genistin in the absence or presence of phloridzin, respectively. Whereas, a level of approximately 39 and 42 µM for genistein was

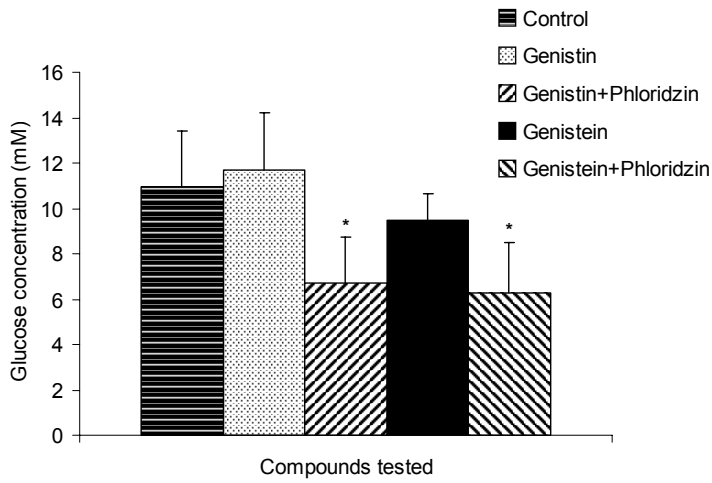

***Figure 6.1**: Glucose concentration in plasma of rats after one hour perfusion. The small intestine of rats was perfused with Tyrode buffer (=control), genistin, genistin with phloridzin, genistein or genistein with phloridzin. The initial isoflavone concentration of genistein and genistin amounted to 50 µM with or without a concentration of phloridzin of 200 µM. Data are expressed as mean ±SEM (n=6 perfusions per test solution). *Different from the control group ($p \leq 0.05$).*

recovered in the perfusion fluid with or without phloridzin, respectively. This indicated a significant hydrolysis or metabolism of these isoflavonic compounds. HPLC analyses of the intestinal perfusion samples demonstrated the presence of the aglycone genistein as well as the presence of genistein phase II metabolites like glucuronides and sulfates. Figure 6.2 presents the concentrations of free genistein and of conjugated forms (i.e. glucuronide and sulfate metabolites) recovered in the perfusion media after a single pass perfusion of the rat small intestines with genistin or genistein in the presence or absence of phloridzin. In case of a perfusion with only genistin the aglycone genistein appeared to be the major metabolite in the perfusion fluid. Moreover, the concentration of genistein detected in the perfusion media after intestinal passage (i.e. perfusate) of experiments with genistin was not affected by phloridzin (Figure 6.2). In the presence of phloridzin, however, the concentration of glucuronides in the perfusate appeared to be statistical lower ($p \leq 0.05$) both in case of genistin and of genistein. At the end of the perfusion time the concentration of sulfates recovered in the perfusion fluids was comparable among all compounds tested either in the presence or the absence of phloridzin.

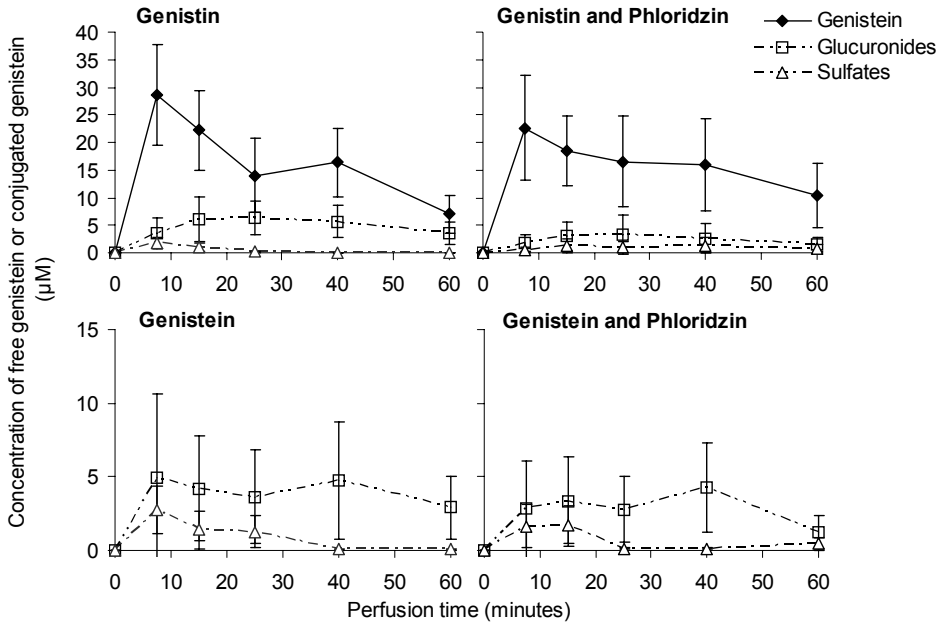

***Figure 6.2**: Relative distrubution of metabolites in perfusates containing genistin (above left figure) or genistin and phloridzin (above right figure), genistein (down left figure) or genistein and phloridzin (down right figure). The initial concentration of genistin or genistein was 50 µM with or without 200 µM phloridzin (i.e. perfusate). Data are expressed as mean ±SEM (n=6 perfusions per test solutions). In perfusates with genistein with and without phloridzin the residual level of genistein is not included in the figure and amounted to 39 and 42 µM respectively.*

Isoflavone levels in portal vein plasma

A representative HPLC analysis (i.e. UV and electrochemical detection) of extracted portal vein plasma of rats after a perfusion with genistin and phloridzin is presented in Figure 6.3. All electrochemical chromatograms of rats perfused with genistin or genistein showed a similar profile. In case of a perfusion with genistin, a genistin peak also appeared in the chromatograms of the extracted portal vein plasma. The genistin concentration in the portal vein plasma of rats perfused with genistin in the presence of phloridzin was almost 3-times higher ($p \leq 0.05$) in comparison with the perfusion of genistin without phloridzin. Following treatment with the enzymes ß-glucuronidase or sulfatase some of the peaks in the chromatogram disappeared (Figure 6.3A, peak no. 1, 2, 3, 6 and 7), accompanied by a two to five fold increase in the intensity of the peak at the position of genistein. Figure 6.4 reveals that the highest portal vein plasma concentrations and area under the curve ($AUC_{(0\text{-}1\ hr)}$) of genistein

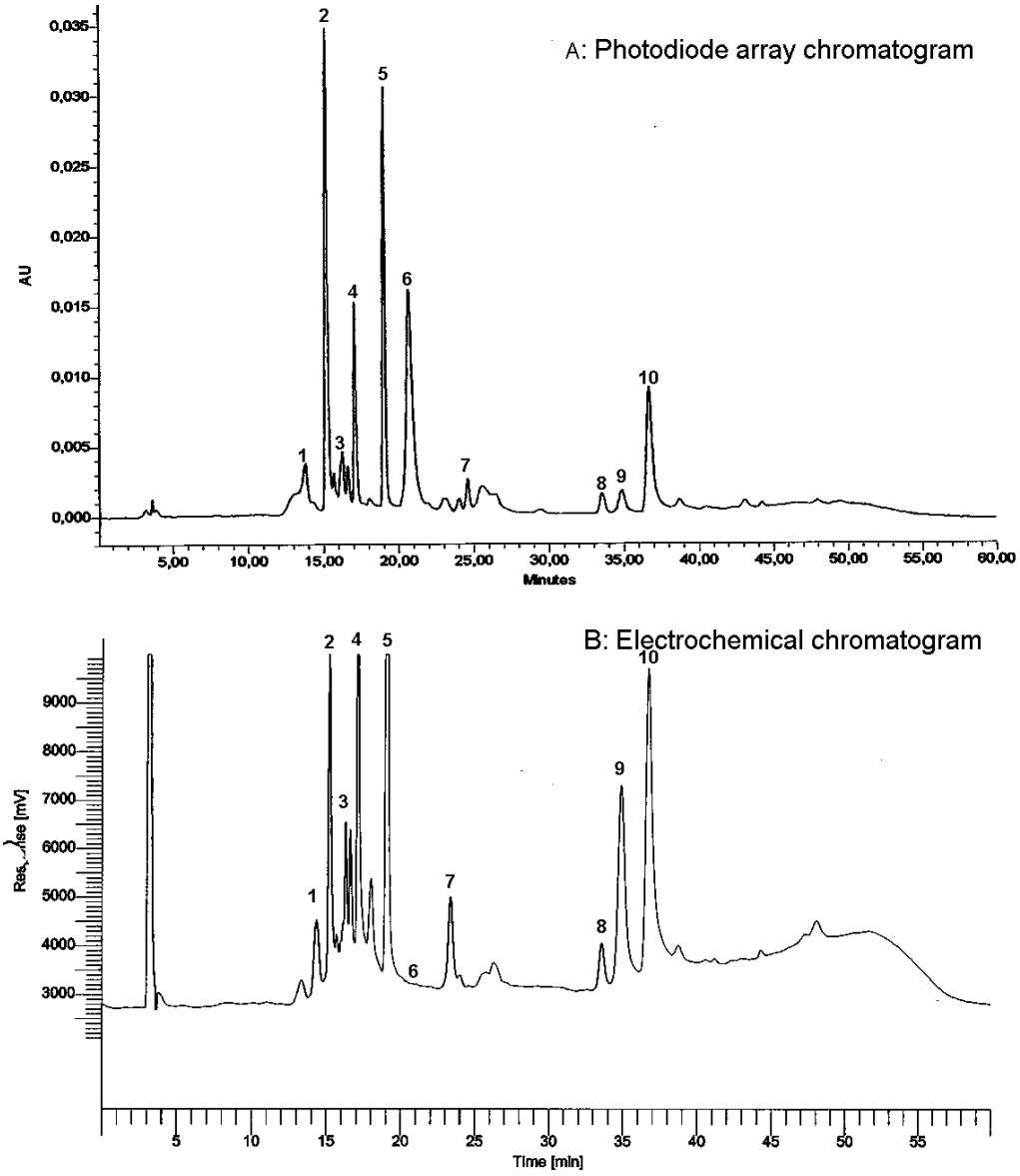

***Figure 6.3**: Representative photodiode array chromatogram (A) and electrochemical chromatogram (B) of plasma of rats perfused with genistin and phloridzin at 40 minutes. The peaks 1 and 2 represent glucoronides, peak 3 is phloridzin, 4 is the parent compound genistin, peak 5 is an unknown metabolite, peaks 6 an 7 represent sulfates and peak 8 is equol, peak 9 is a metabolite of phloridzin and peak 10 is genistein.*

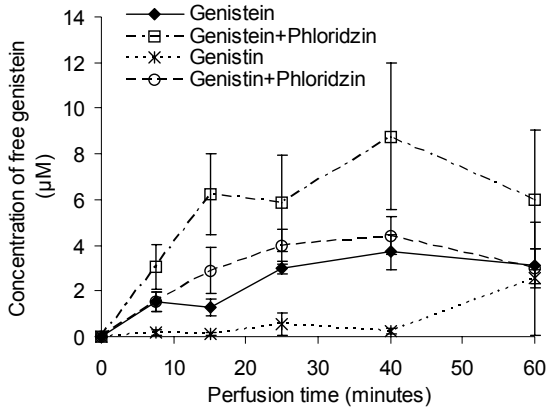

***Figure 6.4**: Levels of genistein in the plasma of in situ perfused rats. The small intestines were perfused respectively with 50 µM genistein, 50 µM genistein and/or 200 µM phloridzin. Data are expressed as mean ±SEM (n=6 perfusions per test solutions).*

appeared to be present after a coperfusion of genistein and phloridzin. The $AUC_{(0\text{-}1\ hr)}$ of the various compounds in the portal vein plasma are tabulated in table 6.1. A perfusion with only genistin yielded rather low levels of genistein in the portal vein plasma. However, phloridzin statistically increased ($p \leq 0.05$) the portal vein plasma genistein levels ($AUC_{(0\text{-}1\ hr)}$) when it was coperfused with genistin or genistein. Obviously, the presence of phloridzin in the perfusion fluids enhanced the transfer of free genistein into the portal vein plasma. The levels of conjugated genistein detected in portal vein plasma are presented in figure 6.5. The portal vein plasma concentration of conjugated genistein (i.e. glucuronides and sulfates) of rats upon a coperfusion of genistin and phloridzin appeared almost comparable to perfusions of genistin or

***Table 6.1**: Area under the curve ($AUC_{(0\text{-}1\ hr)}$) for portal vein plasma levels of genistein, conjugated genistein and genistin for rats who were perfused with genistin, genistein in the presence or absence of phloridzin.*

	AUC of the compound in plasma (µmol h/l)			
Compound administered	Genistein	Conjugated genistein	Genistin	Total
Genistin	0.65	3.20	0.37	4.22
Genistin + Phloridzin	3.22	3.69	1.02	7.93
Genistein	2.61	5.52		8.13
Genistein+ Phloridzin	6.06	4.48		10.5

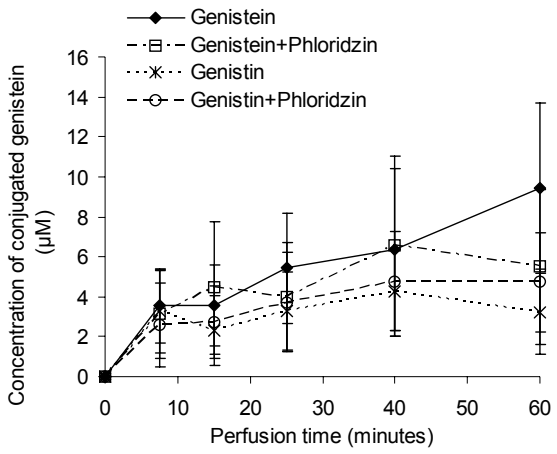

***Figure 6.5**: Levels of conjugated genistein (i.e glucuronides and sulfates) recovered from plasma of in situ perfused rats. The small intestines were perfused respectively with 50 µM genistein, 50 µM genistein in the presence or absence of 200 µM phloridzin. Data are expressed as mean ±SEM (n=6 perfusions per test solutions).*

genistein only. The total amount of isoflavonolic metabolites (aglycones and conjugates) transported through the brush border membrane of the epithelial cell lining of the small intestine is significantly lower for those perfusates containing genistin ($p \leq 0.05$) as compared to perfusates containing its aglycon genistein.

Discussion

This study focussed on the small intestinal transport and mechanism of absorption of the glycosidic isoflavone genistin with special emphasis on the role of the sodium dependent glucose transporter SGLT1 localized in the intestinal brush border membrane. Therefore, rat small intestines with an intact vascular circulation were perfused *in situ* under low stress conditions with genistin (or genistein for comparison) in the presence or absence of phloridzin. At the same time, a cannulation of the portal vein of the rat allowed to exclude the contribution of liver metabolism upon repeated blood sampling. Glucose was added in the perfusion fluid, because glucose is an essential nutrient for natural epithelia, rather than eliminate it from the perfusate entirely. This is also allowed glucose measurements in the plasma providing the possibility for a positive control for phloridzin mediated inhibition of the apical SGLT1 transporter. Phloridzin competes for the sugar binding site of SGLT1

(K_m = 180 µM for phloridzin) and thus serves as a specific inhibitor of SGLT1. As phloridzin does not inhibit basolateral facilitated glucose transport via the glucose transporter isoform GLUT2, the epithelial cells were not nutrient deprived. It is not likely that SGLT1 was competitively and preferentially occupied by the 15 mM glucose present in the Tyrode buffer. Since SGLT1 starts to saturate at concentrations around 30-50 mM glucose (Kellett and Helliwell, 2000). The presence of phloridzin in the perfusion fluid resulted in a significant decrease in glucose concentration in portal vein plasma, indicating that the SGLT1 transporter is inhibited. Phloridzin is a well known high-affinity inhibitor for SGLT1 and has been shown to prevent the interaction with SGLT1 of a 100-fold excess of quercetin glucoside (Gee et al., 2000a). Thus, the effects of phloridzin on the intestinal absorption of isoflavones as shown in this study are not affected by the presence of glucose in the perfusion media and this is in line with others (Wolffram et al., 2002; Cermak et al., 2004).

In the presence of phloridzin, there was a significant increase in the portal vein plasma concentrations of the aglycon (i.e. free genistein) after coperfusion with genistin or genistein. On the other hand, the concentration of genistin in the perfusion fluid was not significantly affected by phloridzin perfusions. Interestingly, the concentration of genistin in portal vein plasma was statistically raised after a coperfusion with genistin and phloridzin. Hence, it has demonstrated that inactivation of SGLT1 on the apical membrane of epithelial cells by addition of phloridzin leads to an increased flux of isoflavones i.e. genistin and genistein.

Oitate et al. (2001) suggested that the transport of isoflavones could be mediated by a carrier system. In addition, *in vitro* and/or *ex vivo* studies report that flavonolic glucosides could have an interaction with the SGLT1 transporter (Gee et al, 1998, Walgren et al, 2000b, Wolffram, 2002, Day et al, 2003). For instance, Gee et al. (1998) used rat jejunal everted sacs as a model, which were preloaded with labelled galactose and exposed to quercetin glucosides. The galactose efflux observed from the jejunal mucosa lead these authors to suggest that quercetin glucosides are capable of interacting with SGLT1. However, this observation is not a direct prove for a transportation by SGLT1. The measurements of Walgren et al. (1998) and Day et al. (2003) showed that quercetin 4'-ß-glucoside could be transported by SGLT1 across the apical membrane of the Caco-2 cell and in the rat everted-jejunal sac model, respectively. They also used the inhibitor phloridzin in these experiments. In addition, Wolffram's experiments with Ussing chambers also suggested a role of SGLT1 in the mucosal uptake of quercetin-3-glucoside. However, our current observations are in

contrast with the general expectation derived from the aforementioned studies that the small intestinal uptake of genistin should be lower and therefore, the concentration of genistein in the plasma should decrease when the transporter SGLT1 is inhibited by the use of phloridzin. On the other hand, our results confirm the results of the study of Andlauer et al. (2004). These authors reported that the amount of total genistein increased 17-fold in the presence of phloridzin compared to perfusions with genistin alone using an isolated preparation of the rat small intestine. However, our intestinal model possessed an intact vascular circulation. The present results may rather imply that phloridzin either increases the passive permeability of isoflavones including genistin or interferes with other enzymes in the brush border membrane of the small intestine. Whereas, Crespy et al. (2001) and Day et al. (2003) reported that SGLT1 is not involved in the intestinal transport of isoquercetrin (i.e. quercetin-3-*O*-glucoside). Crespy et al. (2001) based their statement on their findings after *in situ* perfusion of the jejunal and iliac compartment of rat guts. Because a coperfusion with phloridzin induced no decrease in the concentration of quercetin-3-glucoside in perfusion media. In our opinion, our results and those of, for instance Grespy et al. (2001) indicate that the SGLT1 transporter is not involved in the transport and absorption of genistin if performed *in situ*, although, this is possible not the case for other flavonoid glycosides.

Furthermore, our observation of a rather high concentration of free genistein in perfusion media containing genistin suggests that membrane-bound glycosidases are obviously active in the epithelial cell lining of the small intestines of the rat having no microfloral contents. Our findings are in line with the study of Day et al. (1998) showing that the enzyme LPH localized on the brush border of the small intestine can hydrolyse a broad range of flavonoid glycosides. This is also confirmed by other studies of Arts et al. (2002), Day et al. (2003) and by Sesink et al. (2003), who showed that LPH is involved in the uptake of the flavonoid glycoside quercetin-3-glucoside.

In this model statistical lower levels of glucuronides were observed in perfusion media containing phloridzin as compared to perfusion media without phloridzin for both genistin and genistein. This is in accordance with previous reports of Crespy et al. (2001), Mizuma et al. (1998) and Andlauer et al. (2004). Andlauer et al. (2004) stated that this could be due to an interaction of genistein and its conjugates with apical efflux-transporters, such as MRP2 (multidrug resistance protein transporter) and BCRP1/ABCG2 (breast resistance protein-mediated transporter). These

transporters could also be responsible for the transport of genistein and/or its metabolites back into the intestinal lumen as demonstrated by Walle et al. (1999) and Imai et al. (2004). Walle and Walle (2003) showed that phloridzin is transported by MRP2 and MRP1 and thus competitive inhibition is possible. It seems that phloridzin might be capable to inhibit these apical efflux-transporters. Therefore, the concentration of genistein glucuronides in the perfusion fluid is decreased and subsequently the concentration of genistein in the plasma is increased. However, in our studies the disappearance of genistein from the perfusates was not enhanced in the presence of phloridzin. This is in contrast with general understanding that, when an efflux transporter is inhibited, the absorption of its substrates could be enhanced. Therefore, the role of these apical efflux transporters in intestinal absorption of genistein remains to be studied in more detail. The present results may rather imply that phloridzin interferes with some secretory transporter located on the basal membrane of the epithelium. Notwithstanding these limitations, Sesink et al. (2005) showed that BCRP1/ABCG2 limits the net intestinal uptake of flavonoid quercetin in rats by mediating the apical efflux of its conjugates. In their experiments, genistein showed a stronger interaction with BCRP1/ABCG2 than quercetin (Imai et al., 2004).

The present study clearly showed that the glycoside genistin appears to be present as intact, native molecule in plasma of the portal vein if the small intestines were perfused with genistin in the presence or absence of phloridzin. These observations are in contrast with those of Sesink et al. (2001) and Setchell et al. (2002b). However, in both these latter studies liver metabolism could significantly have contributed to the plasma metabolite profiles observed. In our study blood samples were directly obtained from the portal vein, eliminating as much as possible the first-pass effects of liver metabolism. It is postulated that genistin transport to the plasma appears via the tight junctions of the epithelium cells. The transport of genistin via the tight junction is an efficient transport pathway, because the AUC of genistin in the portal vein plasma is a factor of 10 and 7.5 lower compared to the AUC of free genistein and its conjugated metabolites for perfusion with only genistin and coperfusion of genistin with phloridzin, respectively.

In our study, the bioavailability of genistin and genistein in portal vein plasma is increased in the presence of phloridzin. The AUC of total genistin, genistein and their metabolites were approximately 1.9 times higher for genistin with phloridzin compared to perfusions in its absence. These data observed are not as high as the 2.5 times increase of uptake rates reported by Andlauer et al. (2004). This could be due to

the fact that our concentration of phloridzin used in the perfusion experiments was lower. However, the uptake rates of approximately 9 and 16% of genistin and genistein, respectively observed in the present study are well in line with those of previously reported human and rat studies. In particular, an absorption rate of 8% of genistein-like compounds sequestered from tofu in the isolated small intestine model has been published by Andlauer et al. (2000a), which corresponded to rates published by others, for example 14.6% (Lu et al, 1995), 9% (Xu et al., 1994), 10% (Xu et al., 1995) in volunteers studies consuming soy milk and of 16% eating tofu (Xu, et al., 2000). However, in contrast to these *in vivo* studies, our results did not show a delay in the absorption and metabolism of genistin when compared to its aglycone genistein. Therefore, it is concluded that deconjugation of genistin is highly efficient.

To summarize, the present study using an *in situ* perfusion of the small intestines of the rat with an intact vascular circulation suggests that SGLT1 is not involved in absorption of genistin in the small intestinal tract of the rat. Instead, the large amount of deglycosylated genistein in the perfusion fluid, suggests that genistin absorption is facilitated by a hydrolase localized in the brush border membrane of the small intestine catalyzing conversion of genistin to genistein before the actual absorption occurs.

Acknowledgements

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Chapter 7

On the role of the sodium-dependent glucose transporter (SGLT1) in the absorption of the glycoside genistin in Caco-2 cells

Abstract

Genistein is predominantly found as the ß-glycosidic compound genistin in soybeans and soy derived food. The present study investigates the contribution of the sodium dependent glucose transporter (SGLT1) to the absorption of genistein and genistin, the glycoside derivate of genistein. To this end Caco-2 cells were grown on semi-permeable filters and incubated with genistin or genistein in the absence or presence of phloridzin, a competitive inhibitor of SGLT1.

Unexpectedly, the concentration of genistin recovered at the basolateral side of the Caco-2 monolayer with genistin added at the apical side was not significant different compared to that of transport experiments with genistin in the presence of phloridzin added at the apical side. Furthermore, phloridzin added at the apical side of a Caco-2 monolayer significantly increased instead of decreased the concentration of the aglycon genistein and its glucuronidic metabolites at the basolateral side.

The concentration of glucuronides and sulfates at the apical side appeared to be significant lower in media containing phloridzin as compared to media without phloridzin, both in case of incubation with genistin and genistein. It is suggested that phloridzin inhibits apical efflux-transporters at the brush border membrane of the enterocyte, which would normally facilitate transport of genistein and its metabolites back to the apical side of the Caco-2 cells. Upon inhibition of this process by phloridzin, the concentration of genistein at the basolateral side of the Caco-2 monolayer is increased.

Based on: Steensma, A., Noteborn, H.P.J.M. and Rietjens, I.M.C.M. On the role of the sodium-dependent glucose transporter (SGLT1) in the absorption of the glycoside genistin in Caco-2 cells. *To be Submitted.*

In conclusion, the results indicate that SGLT1 is not involved in genistin (or genistein) absorption. The current study confirms the existing and published *in vivo* and *in situ* data, which indicates that Caco-2 cells are a valuable *in vitro* model for studying the mechanism underlying the role of SGLT1 in genistin absorption.

Introduction

Genistein is an isoflavone that occurs especially in soybean and soybean derived food products and is widely available in herbal tablets. Many epidemiological studies have confirmed the health promoting action of genistein in preventing cardiovascular diseases, cancer and osteoporosis (Barnes et al., 1996; Adlercreutz and Mazur, 1997; Setchell and Cassidy, 1999; Clarkson, 2000; Munro et al., 2003; Setchell et al.,2003c). Genistein has an whole array of postulated biological mechanisms of action, including binding to estrogen receptors, inhibition of protein tyrosine kinase, lipoprotein oxidation, and of DNA topoisomerase I and II and ribosomal S6 kinase and affecting the metabolism of hormones (Akiyama et al., 1987; Fotsis et al., 1993; Mäkälä et al., 1995; Kao et al., 1998; Kaufmann, 1998). To properly evaluate the role of genistein in the prevention of diseases, knowledge of the consumption and bioavailability of genistein is needed.

In nature, genistein is predominantly found as a ß-glycoside, also called genistin. Genistin is less bioactive than its aglyconic form genistein. The extent and mechanism by which genistin reaches the systemic circulation from dietary sources are controversial. However, recent studies suggest that flavonoid glycosides are actively transported by a sodium dependent glucose transporter (SGLT1) in intact form (Hollman et al., 1995; Wolffram et al., 2002; Day et al., 2003). On the other hand, flavonoid glycosides may be hydrolysed by a ß-glucosidase (i.e. lactase phloridzin hydrolase LPH) bound to the brush border membrane of the intestinal cells (Scalbert and Williamson, 2000; Day and Williamson, 2001). This means that LPH removes the glycoside, after which the aglycone passively diffuses across the membrane into the enterocyte (Day et al., 2003; Sesink et al., 2003). When taken up in the intact form, the intracellular cytosolic ß-glycosidases may convert the glycoside to generate its aglycon, after which the aglycon can be conjugated with glucuronic acid by uridine-5'-diphosphate-glucuronosyltransferases or with sulfate by sulfotransferases present in the enterocyte (Day et al., 1998). Subsequently, the

aglycon and/or its metabolites enter the blood stream, or, alternatively, can be transported by apical intestinal membrane transporters like breast cancer resistance protein (BCRP1/ABCG2) or multidrug resistance-associated protein 2 (MRP2) back to the intestinal lumen (Walle et al., 1999; Imai et al., 2004; Sesink et al., 2005).

The objective of the present study is to obtain information on the transport and absorption mechanism of genistin (and genistein for comparison) with special emphasis on the role of SGLT1. Mechanistic studies on intestinal transport can be performed *in vitro*, using the Caco-2 cell line which is frequently used for intestinal absorption studies (Artursson and Karlsson., 1991b; Walgren et al., 1998; Walle et al., 1999).). The human colon adenocarcinoma cell line Caco-2 differentiates spontaneously into morphological and functional characteristics of the human small intestinal cell (Artursson and Karlsson, 1991b). These cells form tight junctions and develop microvilli when grown on filters. They also express brush border enzymes and transporters, including SGLT1, MRP2 and Brcp1/Abcg2 (Delie and Rubas., 1997; Hirohashi, et al., 2000; Walgren et al., 2002b).

To investigate the role of SGLT1 in genistin absorption and transport, Caco-2 cells were incubated with genistin in absence or presence of phloridzin, a competitive inhibitor of SGLT1. For comparison studies similar experiments were performed with the aglycon genistein.

Materials and Methods

Materials

Caco-2 cells, originating from a human colorectal carcinoma were obtained from the American Type Culture Collection (Rockville, MD). Genistein, genistin, phloridzin and tissue culture media were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell cultures

Caco-2 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) in tissue culture flasks (75 cm^2) at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity. DMEM contains glucose (25 mM) and was supplemented with 10% (v/v) heat inactivated foetal calf serum (FCS), 100 Units/ml penicillin, 0.1 mg/ml streptomycin and 1% (v/v) non-essential amino acids for Caco-2 cells. When the cells were 90% confluent, they were detached from the flasks by treatment with trypsin

(2.5 g/l in Hank's balanced salt solution without calcium, magnesium and phenol red supplemented with 0.5 g/l EDTA), and used for transport experiments.

Transport experiments

For transport experiments Caco-2 cells (passage number between 30-40) were cultivated on Transwell polyester membrane filter inserts (pore size 0.4 mm, diameter 24 mm) of Costar (Badhoevedorp, The Netherlands). The apical volume was 2 ml and the basolateral volume was 3 ml, and cells were seeded at a density of 2.10^5 cells/ml. Cells were allowed to attach and differentiate to confluent monolayers for 21 days while changing the medium three times a week. The integrity of the cell monolayers was checked before the transport experiment by measurement of the transepithelial electrical resistance (TEER) and after the transport experiment by measurement of the TEER, lactate dehydrogenase (LDH) leakage and alkaline phosphatase (ALP) activity, the method for these assays being described elsewhere (Steensma et al., 2004a). Transport studies with Caco-2 cells were performed in Eagle's Minimum Essential Medium (EMEM), with 1 g/l glucose and without foetal calf serum at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity. The transport experiments were initiated by washing the cell monolayers twice with fresh medium and leaving them for 30 minutes on fresh medium before test solutions were added to the apical side of the cells. Stock solutions of genistein, genistin and phloridzin were prepared in dimethylsulfoxide (DMSO) and were diluted in medium at a maximum of 0.2% (v/v) DMSO. The final concentration of genistein and genistin was 50 μM and phloridzin was used at a final concentration of 200 μM. Control solutions contained EMEM with 0.2% (v/v) DMSO only. Each experiment was carried out in six fold. Aliquot samples of 75 μL were taken from the basolateral side for up to 24 hours (at 0, 0.5, 1, 2.5, 4, 8 and 24 hours) and after 24 hours also two 75 μl samples from the apical side were taken.

Isoflavone detection

The isoflavones present in cell culture samples were analysed by reversed-phase HPLC (Waters, Etten-Leur, The Netherlands) equipped with a Supelcosil LC-ABZ (250*4.6 mm, 5 mm) column of Supelco (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) and an UV photodiode array detector (Waters, Etten-Leur, The Netherlands). The column was eluted at a flow rate of 1.0 ml/min employing a gradient of two eluens (eluent A: 10 mM ammonium acetate pH 6.5 with 2.5% (v/v) acetonitril; eluent B: acetonitril) at 30°C for 60 minutes. The elution conditions were as follows: 0-5 minutes 10% eluent B, 5-10 minutes 10-32% eluent B, 10-25 minutes

32% eluent B, 25-35 minutes 32-40% eluent B, 35-40 minutes 40-50% eluent B, 40-45 minutes 50% eluent B, 45-50 minutes 50-10% eluent B and 50-60 minutes 10% eluent B. The eluent was monitored at 260 nm. Routinely 50 µl of sample was injected without further pre-treatment and metabolites were identified by comparison with retention times and UV-VIS spectra of known standards. The amount of genistein glucuronides and sulfates was calculated from the increase in the amount of genistein upon treatment of the samples with respectively ß-glucuronidase or sulfatase. To this end, aliquots of media were incubated with either ß-glucuronidase (2000 U/ml) in 100 mM potassium phosphate (pH 7.0) or sulfatase (250 U/ml) with 17 mM D-saccharic acid 1,4-lactone in 0.5 M sodium acetate (pH 5.0) in a dilution of 2:1 (plasma:enzyme buffer v/v). After 1 hour incubation at 37°C the isoflavones were measured as describe above. HPLC chromatograms of treated media were compared to chromatograms of media incubated without enzymes.

Statistics

Results are presented as mean values ± SEM. Statistical evaluation of data was performed by one-way ANOVA ($p<0.05$) and the comparison of values between the treated groups and the control group was based upon the Tukey's Honestly Significant Difference test ($p<0.05$).

Results

There was no significant difference between the integrity of the Caco-2 cell monolayers incubated with the various compounds at the end of the transport experiment as measured by transepithelial electrical resistance (data not shown). There was also no significant difference in LDH and ALP activity detected in the basolateral media of Caco-2 cell monolayers treated with the various compounds and of non-treated cells, indicating that no cytotoxicity had occurred (Steensma et al., 2004a).

Figure 7.1 illustrates the time-dependent occurrence of genistin at the basolateral side of the Caco-2 monolayers after 0 to 24 hours incubation of the cells with genistin in the presence or absence of phloridzin at the apical side. The transport is calculated as a percentage of the amount of test compound initially added at the apical side. Genistin added at the apical side in the presence or absence of phloridzin was transported through the Caco-2 cell monolayer to a very limited extent, that is less than 3% of the amount added at the apical side. There were no significant differences

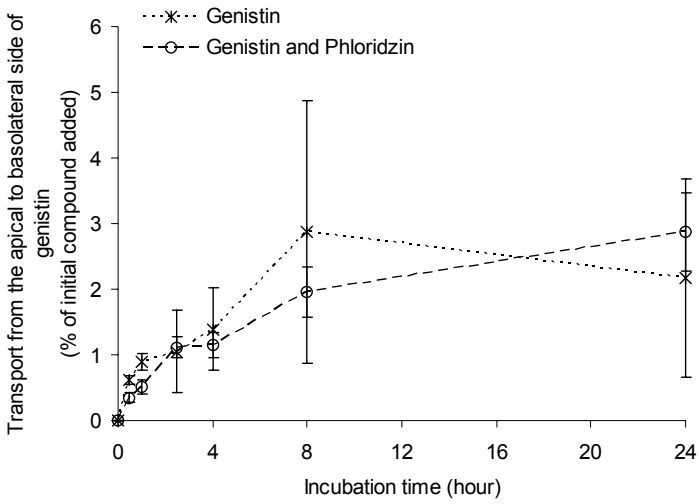

***Figure 7.1**: Transport of genistin in the absence or presence of phloridzin across Caco-2 cells from apical to the basolateral side. Initial concentrations at apical side were 50 µM of genistin and 200 µM of phloridzin. Data are expressed as mean ± S.E.M. of six fold measurements.*

in the concentration of genistin at the basolateral side, when the cells were incubated with either genistin or genistin with phloridzin.

The metabolites formed and released into the basolateral compartment after 24 hours of incubation of genistin with or without phloridzin, are presented in figure 7.2 as a percentage of the amount of test compound initially applied at the apical side. The metabolites obtained have been identified as genistein and genistein glucuronides and sulfates. The concentration of free and conjugated genistein in the basolateral medium of cells incubated with genistin with or without phloridzin was almost negligible, i.e. less than 0% of free genistein and 0.07% (±0.05) of conjugated genistein for cells incubated with genistin and less than 1.40% (±0.13) of free genistein and 2.29% (±0.36) of conjugated genistein for cells incubated with genistin in the presence of phloridzin, respectively.

At the apical side, the concentrations of genistein (i.e. 0.43% (±0.40)) and its glucuronides (i.e.1.30% (±0.24)) were significant lower in the incubations with genistin than in incubations of genistin in the presence of phloridzin (i.e. 1.86% (±0.12) and 3.41% (±0.77) of the initial added compound, respectively).

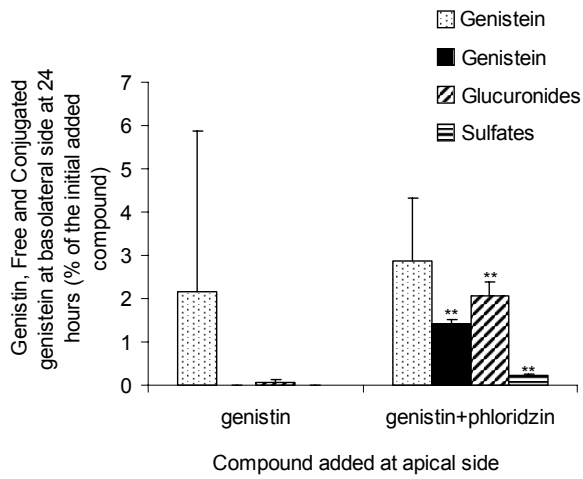

***Figure 7.2**: Genistin, free and conjugated genistein detected at the basolateral side of the Caco-2 cell monolayer incubated with genistin in the absence or presence of phloridzin after 24 hours. Initial concentrations at apical side were 50 µM of genistin and 200 µM of phloridzin. Data are expressed as mean ± S.E.M. of six fold measurements, values are statistical different with ** $p < 0.001$ from groups compared to groups incubated with phloridzin.*

Figure 7.3 shows genistein and its metabolites formed and released into the basolateral compartment after 24 hours of incubation of genistein with or without phloridzin. Genistein and its metabolites are presented as a percentage of the amount of test compound initially applied at the apical side. In the basolateral side after 24 hours, genistein was metabolised into approximately 2.55% (±0.78) and 6.98% (±1.34) genistein conjugates in the absence and presence of phloridzin, respectively. The concentration of genistein recovered at the basolateral side after 24 hours was significant higher in the incubations with genistein in the presence of phloridzin than in the absence of phloridzin.

Discussion

The objective of the present study was to obtain detailed information on the genistin (and genistein) transport mechanism and the role of SGLT1 in this transport and to what extent an *in vitro* Caco-2 cell monolayer could be used to study these objectives. It is of considerable value to have reliable and usefully predictive models to quantify transport across the intestinal epithelium. The Caco-2 monolayer model has achieved

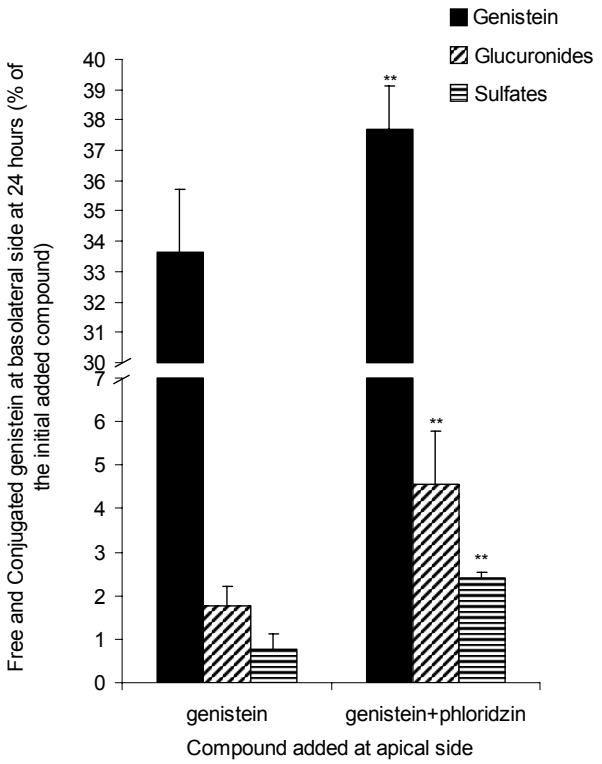

***Figure 7.3**: Free and conjugated genistein detected at the basolateral side of the Caco-2 cell monolayer incubated with genistein in the absence or presence of phloridzin after 24 hours. Initial concentrations at apical side were 50 µM of genistein and 200 µM of phloridzin. Data are expressed as mean ± S.E.M. of six fold measurements, values are statistical different with ** $p < 0.001$ from groups compared to groups incubated with phloridzin.*

pre-eminence as a transport system on studying the (paracellular) transport of hydrophilic (polar) molecules, and the effect of potential absorption modifiers on this route of absorption (Artursson and Karlsson, 1991).

In this study Caco-2 cells grown on semi-permeable filters were used as a model cell line for studying the role of SGLT1 in the mechanism underlying the transport of genistin. These cell monolayers were incubated with genistin or for comparison with genistein, in the presence or absence of phloridzin added in the apical compartment. Unexpectedly, the concentration of genistin recovered at the basolateral side of the Caco-2 monolayer when genistin was added at the apical side was not significantly

different if compared to the level of transport when genistin was incubated in the presence of phloridzin. This contradicts with the expectation that the inhibition of apical SGLT1 transporter by phloridzin causes generally a decrease in paracellular tight junction permeability and, thus, restricts the transport of hydrophilic molecules such as genistin. Our results are however in accordance with results of the study of Andlauer et al. (2004) and Steensma et al. (submitted, chapter 6) in rats and suggest that the SGLT1 transporter is not involved in the intestinal transport and absorption of genistin.

In addition, the presence of phloridzin increased the amount of genistein and its conjugated metabolites transported to the basolateral side in 24 hours incubations in Caco-2 cell monolayers for either genistin and genistein. This is in line with the results of the study of Andlauer et al. (2004) also showing that phloridzin considerably increased genistin in the form of genistein absorption based on a perfusion of these compounds in an isolated preparation of the rat small intestine. Moreover, results of the previous study of Steensma et al. (submitted, chapter 6) using an *in situ* single pass perfusion of small intestines of rats with an intact vascular circulation also pointed at an increased instead of decreased transport of genistin and its metabolites when coperfused in the presence of phloridzin. Significantly higher plasma concentrations of genistein were observed compared to those measured after perfusions in the absence of phloridzin. This increased transport of genistin in the form of genistein and its metabolites in the presence of phloridzin also indicate that the SGLT1 transporter is not involved in the intestinal transport and absorption of genistin. Thus, the results obtained in the Caco-2 *in vitro* model are in line with those obtained in animal based models. The lack of the role of SGLT1 in the absorption of genistin is in contrast with results on the role of SGLT1 in the mechanism of absorption of other flavonoid glycosides such as for example quercetin-4-glucoside which was demonstrated to involve both an interaction with SGLT1 and luminal hydrolysis by LPH (Walgren et al., 2002b; Day et al., 2003). Day et al. (2003) reported that in the presence of phloridzin, transfer of the aglycone and quercetin metabolites to the serosal side was significant reduced when quercetin-4-glycoside was added at the mucosal side in a rat everted-jejunal sac model. In addition, Wolffram et al. (2002) suggested a role of SGLT1 in the mucosal uptake of quercetin-3-glycoside. However, regarding this experiment it could also be postulated that quercetin-3-glucoside might be hydrolysed by LPH (Arts et al, 2002). Indeed, some studies suggested that quercetin-3-glucoside is hydrolysed by LPH (Crespy et al., 2001; Day et al., 2003; Sesink et al., 2003).

It is important to note that the Caco-2 cells have a low expression of LPH if compared to the intact, native intestinal cell lining (i.e. enterocytes) (Day et al., 2003). This is probably the reason why the formation of the aglycone genistein at the apical side of the Caco-2 cell monolayer is very low when these cells are incubated with genistin.

The effects of phloridzin within the intestine are complex (Day et al., 2003). Phloridzin is not only a competitive inhibitor of SGLT1, but it is also suggested that phloridzin inhibits efflux-transporters, such as MRP2 (multidrug resistance protein transporter). As stated by Walle and Walle (2003) MRP2 would normally facilitate transport of genistein and its metabolites back to the apical side of the Caco-2 cells. It is therefore postulated that phloridzin is capable of inhibiting apical efflux transporters such as MRP2 and BCRP1, which interfere with the absorption of genistein and its glucuronide, and, thus, concentrations of genistein and its glucuronide significantly increase when genistein and genistin are co-incubated with phloridzin. As shown in this study, the disappearance of genistein from the apical side of the Caco-2 monolayer was enhanced in the presence of phloridzin. Because it is a generally understanding that, when an efflux transporter is inhibited, the absorption of its substrate could be enhanced. Furthermore, it might be suggested that phloridzin competes with genistin and genistein for both UDP-glucuronosyltransferases in the enterocyte as well as with the phloridzin hydrolase domain of LPH on the brush border membrane (Day et al., 2003). According to our observations, the suggested effects of phloridzin explain the elevated genistein and its conjugated metabolites concentration found at the basolateral side of the Caco-2 monolayer.

In conclusion, the present study using Caco-2 cells as a model system reveals that SGLT1 is not involved in genistin (or genistein) absorption. Comparison of current observations with existing and published *in vivo* and *in situ* data, it can be concluded that Caco-2 cells are a valuable model for studying the role of SGLT1 in intestinal genistin transport.

Chapter 8

Summary and concluding remarks

This chapter summarises the major results and conclusions obtained in this thesis. Chapter 1 introduces the topic of the thesis and gives an overview of the literature supporting that genistein and daidzein may help to prevent hormone-related cancer, cardiovascular diseases and osteoporosis (Barnes et al., 1996; Kurzer and Xu, 1997; Setchell et al., 2003c; Williamson and Manach, 2005). Soybeans and soy derived foods contain relatively high concentrations of genistein and daidzein (1-3 mg/g), primarily in the form of their corresponding ß-glycoside conjugates, also called genistin and daidzin respectively (Coward et al., 1993; Reinli and Block, 1996). There is a need to study the pathways through which these compounds are absorbed and metabolised in the human gut in order to assess the systemic bioavailability and therefore, the efficacy of these compounds at the target tissues.

The aim of this thesis was twofold. A first objective was to define a suitable gastrointestinal model system for studies on the transport and bioavailability of the isoflavones genistein and daidzein and their glycosides genistin and daidzin as the model compounds. To this end *in vitro* (chapter 2, 3 and 7), *in situ* (chapter 4) and *in vivo* rat (chapter 5 and 6) intestinal models for metabolism and absorption were applied and compared. A second objective of the thesis was to study the mechanism of absorption of genistin, the glycoside of genistein with special emphasis on the deglycosylation and the role of the glucose transporter SGLT1 in absorption (chapter 6 and 7). To study the role of SGLT1 in genistin absorption, *in situ* perfusion of intestines of rats and *in vitro* transport studies across Caco-2 cells were performed with genistein and genistin in the absence or presence of the naturally compound phloridzin, a competitive inhibitor of SGLT1 present in apples (chapter 6 and 7).

In the course of the development of valid *in vitro* models for human intestinal absorption and metabolism, in chapter 2, Caco-2 cells grown on semi-permeable filters were used to study the transport of genistein, daidzein and their glycosides across these cells. The Caco-2 cell line is frequently used in drug absorption studies for intestinal absorption models (Artursson and Karlsson, 1991b). This cell line is derived from a human colon adenocarcinoma and differentiates spontaneously into morphological and functional characteristics of the human small intestinal cell (Bailey et al., 1996; Delie and Rubas, 1997). The results obtained in chapter 2 reveal that the integrity of Caco-2 monolayers was satisfactory as indicated by

transepithelial electrical resistance measurements and by determination of the permeability of the radioactive marker polyethylene glycol (PEG4000). The aglycones genistein and daidzein added at the apical side were readily transported to the basolateral side to a level of approximately 30 to 40% after 6 hours. The glycosides were hardly transported through the Caco-2 cells. No significant metabolism of genistein and daidzein was observed, whereas the glycosides were metabolised to their respective aglycones along with their transport to the basolateral side. Furthermore, because hardly deglycosylated isoflavones were observed at the apical side, it could be concluded that the deglycosylated metabolites observed at the basolateral side could be formed due to the fact that the Caco-2 cells contain an endogenous glycosidase activity.

In chapter 3, three cell lines (i.e. human colonic Caco-2, rat small intestinal IEC-18 and human immortalised colonic HCEC) were used for transport studies in order to compare various *in vitro* model cell lines. IEC-18 cells are a small intestinal crypt cell line derived from the rat ileac epithelium (Ma et al., 1992). IEC-18 cells in monolayer display tight junctions that closely resemble the tight junctions of the small intestine, which indicates a comparable leakiness/tightness of the paracellular pathway comparable to that observed in human intestinal tissue (Quaroni and Hochman, 1996; Duizer et al., 1997 and 1998). Furthermore, a deficiency of intestinal cells in culture, including Caco-2 cells, is that these intestinal cells show a different cytochrome P450 pattern compared to the normal human intestinal tissue. The immortalised human colonic epithelial cell line HCEC expresses cytochrome P450s (i.e. CYP1A1, 2C, 2D6, 2E1, 3A4/5) at a similar level as observed in human colonic tissue (Macé et al., 1998). In chapter 3, the transport and metabolism of genistein, daidzein and their glycosides was analysed in these three different cellular systems grown on semi-permeable filters and compared to each other. The membrane integrity, cytotoxic effect of the compounds and the stage of differentiation was checked with transepithelial electrical resistance, LDH leakage and alkaline phosphatase activity, respectively, for each cell line and compound. There was no unexpected effect seen, indicating no effect on the integrity of the cells and no cytotoxic effect of the compounds. The results obtained in this chapter show that Caco-2, IEC-18 and HCEC cells transport genistein and daidzein to almost the same rate and extent. The inhibition experiment showed that the kinetic values of genistein transport were significantly reduced by a combination of aglycones and glycosides in the transport experiment across Caco-2 cells, indicating that the transport of genistein across Caco-2 cell monolayers could be mediated by a carrier system. Glycosides were transported

across IEC-18 and HCEC monolayers, but not across the Caco-2 cell monolayer. The glycosides were metabolised in Caco-2 and IEC-18 cells to their respective aglycones, indicating that the glycosides might be hydrolysed at/or in the cellular monolayer. Genistein and daidzein were glucuronidated and sulfated in Caco-2 cells, to glucuronidated forms in IEC-18 cells and to sulfated conjugates in HCEC cells. *In vivo* studies and studies with perfusions of isolated rat intestinal segments confirmed that no or only small amounts of flavonoid glycosides appeared in the plasma compartment (Piskula et al., 1999; Setchell et al., 2001, Spencer et al., 1999; Andlauer et al., 2000a; Sesink et al., 2001). Furthermore, in these studies the plasma profiles of the glycosides showed similarities with the plasma profiles of their corresponding aglycones. Comparing these *in vivo* results to the results of our *in vitro* model systems, it can be concluded that the Caco-2 cell line appears to be a better cellular model for transport and metabolism studies than those systems based on the IEC-18 or HCEC cell line, because looking at characteristic parameters (i.e. the level of glycoside transport and formation of glucuronide and sulfate metabolites) they reflect the human *in vivo* situation to a better extent.

In Chapter 4, various segments of the intestinal tract of the rat were perfused with genistein and genistin to study the intestinal absorption and transport mechanism of these isoflavones in this intestinal rat model. Thereto, 10 cm of intestinal segments (i.e. proximal and distal jejunum, ileum and colon segments) were isolated from the rat and mounted in a perfusator and subsequently perfused for 2 hours with the various model compounds. Chapter 4 shows that the transport of genistein was higher compared to the transport of its glycoside in all perfused gut segments. The highest resorbate (i.e. serosal side) concentration of genistein was observed for the ileac segments, and amounted to approximately 2% of the initially applied genistein concentration. Whereas, the total recovery of genistein and its metabolites in the resorbate of jejunal segments after 2 hours perfusion accounted to 26 and 17% of the applied genistein recovered in the resorbate of jejunal proximal and distal segments, respectively. In the ileac and colonic intestinal segments, genistein was recovered after 2 hours for up to only 10%. For genistin, recovery in the resorbate was significantly lower. Less than 0.2% of the initial concentration of genistin appeared in the resorbate fluid of all isolated gut segments. The amount of genistin metabolites (i.e. genistein and glucuronides) in the resorbate was about 7% of the genistin administered for the jejunal segments. Metabolites of genistin did not occur in the resorbate of isolated ileum and colonic segments. However, in the perfusate fluid of the colonic segments approximately 50% of the applied genistin was recovered in the

form of metabolites (i.e. genistein and unknowns). This indicates that genistin is especially deglycosylated at the luminal side of these segments. The conclusion of this chapter was that the main site of transport and metabolism of genistein and its glycoside is located in the jejunal compartment of the rat gut. Furthermore, for the first time, it was shown that the metabolites of genistin did not occur in the resorbate of isolated and perfused colonic segments, but that genistin can be metabolised either by epithelial cells or by remaining microflora present in the isolated colonic segments. Therefore, it is concluded that there is no transport of genistin and/or its metabolites in the colonic gut wall tissue of the rat.

In chapter 5 detailed information was obtained on the transport and metabolism of genistein and its glycoside genistin, either as pure compounds or from soy protein isolate extract, in an *in vivo* model, with special emphasis on the possibilities for uptake of the flavonoid in its glycosylated form. To this end, rats with a permanent cannulation of the portal vein were used as a low stress condition model for studying the intestinal transport and metabolism of genistein and its glycoside. Permanent cannulation of the portal vein allowed for repeated blood sampling, while the rats were unanaesthetized and freely moving (Van Dongen et al., 1990). Using this model, time-dependent genistein- and genistin- derived portal vein plasma metabolite profiles could be obtained and compared to metabolite patterns in the small intestine, colon and faeces. Together this provides detailed insight in the bioavailability of genistein and its glycoside genistin. The results of chapter 5 reveal that genistein is readily bioavailable, already being observed in portal vein plasma at the first point of detection at 15 minutes after dosing, with a total maximum concentration of 16.5 µM. The $AUC_{(0\text{-}24\ hr)}$ for total genistein and its conjugates was 54, 24 and 13 µM h for genistein, genistin and an enriched protein soy extract, respectively. These results indicate that the bioavailability of genistein in the systemic circulation is higher for the aglycon than for its glycosidic form. The results also reveal that genistin is partly absorbed in its glycosylated form. This latter result is different from that obtained in other studies, where the authors analysed whole body plasma instead of portal vein plasma. From this it could be concluded that deglycosylation of the absorbed genistin occurs upon first pass through the liver.

Altogether the data of chapter 5 reveal that bioavailability experiments based on portal vein plasma levels contribute to a better insight in the role of the intestine and liver in deglycosylation and uptake characteristics of glycosylated isoflavones, the form in which these bioactive food ingredients generally occur in our diet.

In chapter 6, the focus was on the intestinal metabolism and mechanism of absorption of the genistin with special emphasis on the role of the sodium dependent glucose transporter SGLT1. Thereto, *in situ* rat intestines with an intact vascular circulation were perfused with genistin (or genistein for comparison) in the presence or absence of phloridzin, a competitive inhibitor of SGLT1. At the same time, a cannulation of the portal vein was carried out and this allowed to exclude the contribution of liver metabolism upon repeated blood sampling. The results of chapter 6 show that the inhibition of SGLT1 by using phloridzin as a competitive inhibitor resulted in significant higher plasma concentrations of genistein compared to those measured after perfusions in the absence of phloridzin. The total level of metabolites of genistein, glucuronides and sulfates in the portal vein plasma increased for both genistin and genistein exposed rats when phloridzin was simultaneously applied in the perfusate. In the perfusion fluid, the major metabolite was genistein in case of perfusion with genistin in the absence or presence of phloridzin pointing at an efficient deglycosylation. It is known that phloridzin rapidly inhibits not only SGLT1, but also indirectly that part of the GLUT2-mediated component controlled by SGLT1 through the glucose-induced activation and recruitment of GLUT2 to the brush border membrane (Helliwell and Kellett, 2002). Notwithstanding the complexity of the inhibitory effects of phloridzin in the enterocyte, it is postulated that phloridzin also inhibits efflux transporters located at the brush border membrane. This prevents genistein from being transported back to the lumen. Instead genistein is transported to the plasma, which explains the elevated genistein concentration found there. Unexpectedly, the concentration of genistin recovered in the perfusion fluid of experiments with genistin in the presence of phloridzin was not significantly increased compared to that of intestinal perfusions with genistin alone. The concentration of glucuronides in the perfusion fluid appeared to be somewhat lower both in case of rats perfused with genistin as well as in the perfusate of rats exposed in a similar way to genistein. These results of chapter 6 suggest that SGLT1 seems not to be involved in the absorption of genistin in the rat small intestinal tract and that phloridzin may even inhibit cellular glucuronidation to some extent. Furthermore, the large amount of deglycosylated genistin observed in the perfusate indirectly indicates that, instead, the transport of genistin is facilitated by a hydrolase in the brush border membrane of the intestine catalyzing the conversion of genistin to genistein before the actual absorption occurs.

In chapter 7 the role of SGLT1 in the mechanism of intestinal uptake of genistin was further investigated *in vitro*. To this end Caco-2 cells grown on semi-permeable filters

were again used as a preferred model to quantify the transport of genistin (and genistein for comparison) in the presence of phloridzin across the intestinal epithelium. Phloridzin added at the apical side of a Caco-2 monolayer significantly increased instead of decreased the concentration of the aglycon genistein and of the glucuronide metabolites at the basolateral side both for experiments with genistin or genistein added at the apical side. It is suggested that phloridzin inhibits not only SGLT1, but also (indirectly) efflux-transporters, such as MRP2 (multidrug resistance protein transporter) at the apical membrane of the enterocyte, which would normally facilitate the transport of genistein and its metabolites back to the apical side of the Caco-2 cells (Walle and Walle, 2003). Upon inhibition of this process by phloridzin, it can be explained that the concentration of genistein at the basolateral side of the Caco-2 monolayer is increased. Also unexpectedly, the concentration of genistin recovered at the basolateral side of the Caco-2 monolayer with genistin added at the apical side was not significantly different compared to that of results of transport experiments in which genistin was added in the presence of phloridzin at the apical side. This indicates that the results of the *in vitro* Caco-2 cell model are in line with the results observed in chapter 6 in the *in vivo* model and corroborate the conclusion that SGLT1, but also that part of the GLUT2-mediated component controlled by SGLT1, is not involved in intestinal uptake of genistin.

Conclusion and future perspectives

Gastrointestinal models

This thesis examined various *in vitro*, *in situ* and *in vivo* gastrointestinal models for studying the bioavailability of the isoflavones using the model compounds genistein and its glycoside genistin. The conclusion of chapter 2 and 3 is that the Caco-2 cells grown on semi-permeable filters provide a good predictive model for quantifying the transport of the glycoside genistin. Although, the Caco-2 cell line express SGLT1, it displays a low expression of LPH (Day et al., 2003), and therefore this cellular model cannot be used to study the contribution of LPH to the intestinal uptake of glycosylated (iso)flavonoids. On the other hand, this makes the model optimal for studying specifically the role of other deglycosylation pathways possibly involved, including studies on the role of SGLT1 (chapter 7). Furthermore, in chapter 3 is concluded that the IEC-18 cell line can be used to obtain an insight in the metabolism of genistein and genistin, but it does not facilitate the paracellular or transcellular transport of these compounds. In addition, the HCEC cell line is not a good tool for

quantifying the transport and metabolism of these compounds, due to the fact that the transport and metabolic activity of the cells do not match the characteristics reputed in human volunteer and rat studies.

In chapter 4 isolated perfused segments were used as a model for studying the location of transport and metabolism of the compounds. Chapter 4 concludes that this model allows a more detailed study of the separate segments of the small intestine. A disadvantage of this model is the interruption of the blood supply of the intestine. This high stress perfusions (i.e. mechanical stress as segments become blown up and distended) may likely to be a factor that affects, for instance, the paracellular route of absorption (Helliwell and Kellett, 2002).Therefore, the data obtained in this model should always be verified with *in vivo* data.

The *in vivo* model of the perfused intestines with an intact vascular circulation in chapter 6 appeared to be an excellent single-pass, low mechanical stress perfusion model for studying the mechanisms underlying transport and bioavailability of the isoflavones. On the other hand, the rats must be kept under anaesthesia in this model and this limited in representing the actual features of the small intestine. Therefore, it is advocated that the model used in chapter 5 with freely moving unanaesthetized cannulated rats is the best model to be used, because it allowed time dependent genistein- and genistin-derived portal vein plasma profiles to be obtained that were comparable to the metabolite pattern recovered in human volunteers studies. Although the latter model provided a detailed insight in the bioavailability of genistein and its glycoside genistin, there are some serious drawbacks: the *in vivo* models use laboratory animals, are expensive and time-consuming. The use of laboratory animals does not match with the present focus on reduction, refinement and replacement (3Rs) of laboratory animal studies. Therefore, it is still essential to develop usefully predictive *in vitro* models. For example, based on the results of the present thesis it is recommended to improve the hydrolase activity of cell lines by, for example, genetic modification of the Caco-2 cell line, with an accent on higher expression of LPH making therefore the Caco-2 cell line an even better *in vitro* model for human bioavailability studies. Finally, table 8.1 summarises the major results and conclusions with respect to the suitability of the various gastrointestinal models compared in this thesis.

***Table 8.1**: Summary of studies with various gastrointestinal models performed in this thesis including the major conclusions with respect to their suitability to study bioavailability, metabolism and mechanisms of intestinal uptake of (glycosylated) isoflavonoids. The table also indicates the extent to which the models support the reduction, refinement and replacement (3Rs) of laboratory animals.*

	Bioavailability	Metabolism	Mechanism	3Rs*	Chapter
Caco-2	+	-	++/-	++	2,3,7
IEC-18	-	+	n.d.	++	3
HCEC	-	-	n.d.	++	3
Segment Perfusion	±	±	±	±	4
Cannulated Rat	++	++	+	-	5
In situ perfusion	+	+	++	-	6

- bad, ± possible, + good, ++ excellent, n.d. not detected *-3Rs= supporting the Reduction, Refinement and Replacement of laboratory animals

Bioavailability of genistein and genistin in various in vitro, in situ and in vivo gut models compared to human in vivo studies

Table 8.2 summarizes the results of bioavailability studies for genistein and genistin and conjugated genistein (i.e. glucuronides and sulfates) obtained in this thesis. The conclusion is that in all intestinal models studied genistein is more bioavailable than its glycoside genistin. The maximum concentration of genistein in the medium of exposed intestinal cell lines was comparable to the concentrations in the perfusates of the *in situ* and *in vivo* studies performed, whereas the maximum concentration of metabolites generated in about 24 hours was lower in the cellular *in vitro* models. Furthermore, the amount of the cells grown on the transwell monolayers is not comparable to the number of enterocytes present in the intestinal tract models. Therefore, it can be postulated that this is the reason for a lower concentration of metabolites found in the intestinal cell models. The studies reported in the literature usually give only information on the total metabolites, i.e. free genistein combined with conjugated genistein (Manach et al., 2005; King et al., 1996; Coldham et al., 2000; Mallis et al., 2003). Our rat studies with genistein are well in line with other studies reported (King et al., 1996; Coldham et al., 2000; Mallis et al., 2003). However, the concentration of genistein measured in the plasma of human volunteers is usually lower than the plasma concentration observed in rat studies (reviewed by Manach et al., 2005). One of the reasons for this discrepancy could be the incorporation of genistein into various food matrices for application to human

***Table 8.2**: Bioavailability studies of this thesis and in literature of genistein and its glycoside genistin*

Gut model	Dose	Tmax	Genistein applied			Genistin applied				Chapter
		(hour)	Cmax of free genistein (µM)	Cmax of conjugated genistein (µM)	Total metabolites (%)	Cmax of genistin (µM)	Cmax of free genistein (µM)	Cmax of conjugated genistein (µM)	Total Metabolites (%)	
Caco-2	50 µM	6	18.6	1.56	**39.2**	0.84	0.43	0.78	**4.08**	3, 7
IEC-18	50 µM	6	30.1	0.43	**61.1**	33.1	0.71	0.12	**67.9**	3
HCEC	50 µM	6	25.3	1.11	**52.9**	25.4	n.d.	0.15	**51.1**	3
Isolated segment: Jejunum A	50 µM	2	0.82	12.1	**25.7**	0.10	0.44	2.55	**6.16**	4
Isolated segment: Jejunum B	50 µM	2	0.47	7.90	**16.7**	0.09	0.28	3.02	**6.77**	4
Isolated segment: Ileum	50 µM	2	1.09	3.44	**9.05**	0.04	0.01	0.01	**0.09**	4
Isolated segment: Colon	50 µM	2	0.52	0.18	**1.40**	0.06	0.06	0.20	**0.63**	4
In vivo cannulated rat	15 mg/kg bw	0.15	5.49	11.0	**16.5 µM**	0.15	0.91	3.79	**4.85 µM**	5
Perfused intestine	50 µM	0.25	3.72	9.41	**26.2**	0.63	2.56	4.23	**14.8**	6
In vivo studies (Rat)	4-20 mg/kg bw	1			**8.3-35.5 µM**					1
In vivo studies (Human)	1 mg/kg bw	4.1			**1.3-4.5 µM**				**0.5-4.0 µM**	1, Manach et al.,

volunteers. For example, chapter 5 showed that genistein in a soy protein enriched extract had a lower bioavailability than when it was administered as a pure compound.

The concentration of genistin observed either in the resorbate or plasma compartment of all the gastrointestinal models studied in this thesis appeared to be rather low but significant. In *in vivo* studies with genistin and genistein exposed rats, the AUC for genistin was about 0.9 μmolh/l and approximately 10 times lower than the AUC for genistein. Furthermore, it appeared that deglycosylation of genistin to genistein and its subsequent intestinal metabolism to genistein glucuronides and sulfates is part of the major route of absorption of genistin.

The originally proposed model of glycoside absorption suggested that the flavonoid glycosides are too polar (i.e. hydrophilic) to be absorbed by the enterocytes of the small intestine, and therefore, the absorption was claimed to occur in the large intestine after bacterial deconjugation (Griffiths and Barrow, 1993). Chapter 4 concludes that there is no absorption of genistin and its metabolites through colonic gut wall tissue. However, *in vivo* rat studies show that the maximum peak concentration of metabolites of dietary flavonoid glycosides occur between 0-2 hours, which was also demonstrated by the experiments summarized in chapter 5, corroborating that the genistin absorption occurs in the first part of the intestine as also reported by Walle et al. (2000), Piskula et al. (2000), Sesink et al. (2001) and Coldham et al. (2000, 2002).

This thesis demonstrated that SGLT1 is not involved in the absorption of genistin, but genistin is hydrolysed by a hydrolase in the brush border membrane of the small intestine catalyzing the conversion of genistin to genistein before actual absorption occurs (Chapter 6 and 7). Genistein enters the intestinal cell by a carried mediated mechanism (Chapter 3) and or by passive diffusion and in the enterocyte it can be conjugated by uridine-5'-diphosphate-glucuronosyl transferases (UDPGT), sulfotransferases or catechol-*O*-methyltransferases (COMT) to glucuronide, sulfate or methyl conjugates. Upon conjugation genistein and its conjugated metabolites can be transported to the blood or could also be transported back to the lumen via efflux-transporters in the apical membrane of the enterocyte (i.e. BCRP1 or MRP2). In this thesis, genistin was also found as an intact molecule in the plasma or basolateral media of the models of the intestinal cells, which suggests by taking into account the previous finding that SGLT1 has no role in the transport, that genistin may be partly transported via the tight junctions of the enterocyte monolayer. Figure 8.1 shows the

possible routes of absorption and transport of genistin and genistein taking literature data but also the results of the present thesis into account.

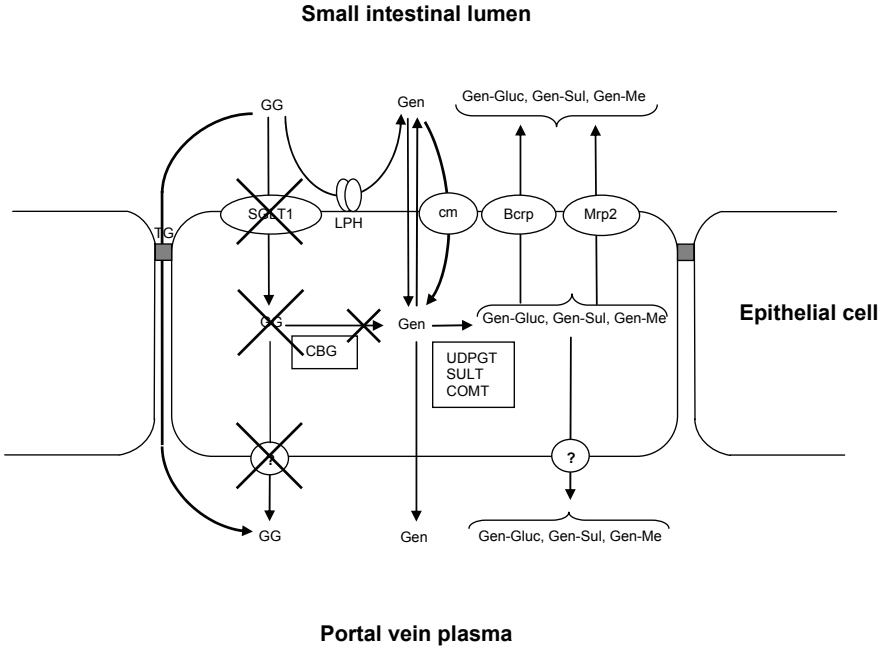

Figure 8.1*: Potential scheme for absorption of genistin and genistein by enterocytes of the small intestinal cells. Genistin (GG) is hydrolysed by lactase phloridzin hydrolase (LPH) and free genistein (Gen) enters the cell by a carrier mediated (cm) mechanism and/or by passive diffusion. SGLT1 has no role in the transport of genistin and subsequently genistin does not enter the epithelial cell. Once in the cell genistein is transported to the blood or conjugated, for example, with glucuronic acid (Gen-Gluc) by uridine-5'-diphosphate-glucuronosyltransferases (UDPGT,) sulfate (Gen-Sul) by sulfotransferases (SULT) or methyl groups (Gen-Me) by catechol-O-methyltransferases (COMT). These conjugates are partly transported to the basolateral side by a yet unknown transporter at the basolateral membrane. Genistein and/or its conjugates can also be transported back to the lumen via multidrug resistance-associated protein 2 (Mrp2) or breast cancer resistance protein BCRP1/ABCG2(Bcrp). Genistin is also partly transported via the tight junctions (TG) and enters the plasma as an intact molecule.*

Overall conclusion

The bioavailability of genistein and its glycoside genistin in various gastrointestinal models is low. Therefore, it is important to study the mechanism of absorption of genistein and its glycoside. Mechanistic studies can be performed *in vivo*, but animal experiments are expensive and time-consuming and should be reduced, refined or replaced whenever feasible also for ethical reasons. The Caco-2 cells provide a good alternative for studying the mechanism of isoflavones absorption, however the hydrolase activity in these cell lines should be improved to mimic the human situation to a better extent. Our mechanistic studies show that the bioavailability of genistin could be improved by the naturally occurring compound phloridzin, present in apples, because phloridzin inhibits the efflux-transporters located at the brush border (apical) membrane of the enterocyte. Genistin can be absorbed, but it is also efficiently deglycosylated before and during intestinal uptake. This thesis provides an insight in the mechanism of bioavailability of this important class of health beneficial soy based food ingredients and even points at inhibition of apical transporters as a way to improve the bioavailability of genistin and genistein.

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Abbreviations

ALP	Alkaline phosphatase
Bcrp	Breast cancer resistance protein 1 BCRP1/ABCG2
Caco-2	Human colon adenocarcinoma cell line
CBG	cytosolic ß-glucosidase
COMT	Catechol-*O*-methyltransferase
cm	Carrier mediated
Dai	Daidzein
DG	Daidzin
DMEM	Dulbecco's Minimum Essential Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene-diamine-tetra-acetic acid
Gen	Genistein
Gen-Gluc	Genistein conjugated with glucuronic acid
Gen-Me	Genistein conjugated with a methyl group
Gen-Sul	Genistein conjugated with a sulphate group
GG	Genistin
EMEM	Eagle's Minimum Essential Medium
FDA	Food and Drug Administration
FCS	Fetal Calf Serum
FOSHU	Food of Special Health Use

HBSS	Hank's Balance Salt Solution
HCEC	Immortalized Human Colonic Epithelial Cell line
HPLC	High Pressure Liquid Chromatography
IEC-18	Ileum epithelial cell line of the rat (nontransformed)
Jejunum A	Proximal part of the jejunum
Jejunum B	Distal part of the jejunum
LDH	Lactate Dehydrogenase
LPH	Lactase Phloridzin Hydrolase
MRP2	Multidrug Resistance-associated Protein 2
MW	Molecular Weight
NVT	Nederlandse Vereniging voor Toxicologie
ODMA	*O*-desmethylangolensin
PEG4000	[^{14}C] polyethyleneglycol (MW 4000)
PET	Postgraduated Education of Toxicology
Pd	Passive diffusion
TEER	Transepithelial electrical resistance
TEM	Transmission electron microscopy
TG	Tight junctions
TGFß	transforming growth factor-ß
SGLT1	Sodium-dependent Glucose Transporter 1
UDPGT	Uridine-5'-diphosphate-glucuronyltransferase
UV	Ultra-Violet

Nederlandse samenvatting en slotopmerkingen

Samenvatting

Genisteïne is een stof die behoort tot de groep van de isoflavonen. Isoflavonen zijn aanwezig in soja en in sojaproducten. De belangstelling voor de isoflavonen is ontstaan omdat ze een mogelijk gezondheidsbevorderend effect hebben. Ze zouden vooral beschermen tegen "hormoonachtige kankersoorten" zoals borst- en prostaatkanker, hart- en vaatziekten en osteoporose. In de natuur komen isoflavonen voornamelijk voor als suikerderivaten (glycosiden) zoals genistin, de geglycosileerde vorm van genisteïne. Genisteïne, de vorm zonder de suikergroep, wordt ook wel het aglycon genoemd.

Doel van het promotieonderzoek is om inzicht te krijgen in de biologische beschikbaarheid en opnamemechanismen van genisteïne en van genistin. Een tweede doelstelling van het onderzoek is een antwoord te vinden op de vraag welke *in vitro*- en *in vivo*-modellen voor biologische beschikbaarheid het beste een beeld geven van de opnameprocessen en het werkingsmechanisme van deze isoflavonen in het menselijk lichaam.

In hoofdstuk 2 wordt een experiment beschreven waarbij Caco-2 cellen worden gekweekt op semi-permeabele filters om zodoende het transport van genisteïne, daidzeïne en hun glycosiden door deze cellaag te onderzoeken. De Caco-2 cellen zijn oorspronkelijk afkomstig van een menselijke dikkedarmtumor, geschikt voor dit soort experimenten. Na kweken differentiëren de cellen namelijk spontaan tot cellen met morfologische en functionele karakteristieken zoals die ook aanwezig zijn in de menselijke dunnedarmcel. De resultaten van hoofdstuk 2 laten zien dat de integriteit van de Caco-2 cellaag goed is, gemeten met transepitheel elektrische weerstand en bij de bepaling van de permeabiliteit van de monolaag voor de radioactieve marker polyethyleenglycol (PEG 4000). De aglyconen genisteïne en daidzeïne toegevoegd aan de apicale kant van de cellaag, worden voor 30-40% getransporteerd naar de basolaterale kant. De glycosiden worden door de Caco-2 cellen echter nauwelijks getransporteerd. Ook is er in dit modelsysteem geen significant metabolisme van de stoffen genisteïne en daidzeïne. Daarentegen worden de glycosiden gemetaboliseerd tot hun respectievelijke aglyconen, die vervolgens vooral worden waargenomen aan de basolaterale kant en niet aan de apicale kant van de cellen. Dit betekent dat de

gedeglycosyleerde metabolieten, gevonden aan de basolaterale kant van de cellaag, gevormd konden worden omdat de Caco-2 cel een endogene glycosidase-activiteit bezit.

In de experimenten van hoofdstuk 3 worden drie cellijnen (Caco-2, IEC-18 en HCEC) gebruikt voor transportonderzoek met als doel om uiteindelijk de beste cellijn voor de experimenten te kiezen. IEC-18 cellen zijn afkomstig van dunnedarm-(ileum) crypt epitheelcellen van de rat. De IEC-18 celmonolagen hebben tight junctions (stevige verbindingen tussen de cellen) die erg veel lijken op die van de menselijke dunne darm. Deze tight junctions vertonen bijvoorbeeld een vergelijkbaar lekken en/of sluiten van de paracellulaire route als wordt waargenomen in de menselijke dunne darm. Het nadeel van de Caco-2 cellen is echter dat dit celtype een verschillend cytochroom P450-patroon heeft in vergelijking met normale menselijke dunnedarmcellen. Daarom zijn ook geïmmortaliseerde menselijke dikke darm epitheelcellen, de HCEC cellijn, gebruikt waarvan de cytochromen P450 op hetzelfde niveau zijn als menselijke dikkedarmcellen. Deze drie cellijnen groeien op semi-permeabele filters. De membraanintegriteit, het cytotoxische effect van de stoffen en de differentiatiefase worden gecontroleerd door de transepitheelelektrische weerstand, de LDH lekkage en de alkalinefosfataseactiviteit voor elke cellijn en elke stof te meten. Er wordt geen negatief effect op de membraanintegriteit waargenomen, noch zijn er cytotoxische effecten van de te onderzoeken stoffen gevonden. Het transport en het metabolisme van genisteïne, daidzeïne en hun glycosiden zijn geanalyseerd en vergeleken met deze drie cellijnen. De resultaten uit het onderzoek van dit hoofdstuk zijn dat genisteïne en daidzeïne op ongeveer dezelfde manier en snelheid getransporteerd worden door Caco-2, IEC-18 en HCEC cellen. Een experiment met een combinatie van genisteïne, daidzeïne en hun glycosiden toegevoegd aan de apicale kant van een Caco-2 cellaag laat zien dat de kinetische waarden van het genisteïnetransport significant gereduceerd worden in aanwezigheid van de andere stoffen. Dit duidt erop dat transport van genisteïne mogelijk via een carrier-gemedieerd systeem zou kunnen plaatsvinden. De glycosiden worden getransporteerd over IEC-18 en HCEC celmonolagen, maar nauwelijks over de Caco-2 celmonolaag. Daarnaast worden de glycosiden in Caco-2 en IEC-18 cellen gemetaboliseerd naar hun aglyconen, hetgeen duidt op een hydrolyseactiviteit in of op de cellulaire monolaag. Ook worden genisteïne en daidzeïne in Caco-2-cellen geglucuronideerd en gesulfateerd, in IEC-18 cellen geglucuronideerd en in HCEC cellen gesulfateerd. Gepubliceerd *in vivo* onderzoek en onderzoek met perfusies van geïsoleerde dunnedarmsegmenten van de rat bevestigen dat geen of geringe hoeveelheden

flavonoïde glycosiden (bijvoorbeeld quercetin-3-glucoside) in plasma aanwezig zijn. In deze onderzoeken vertonen de plasmaprofielen gelijkenissen met de plasmaprofielen van de overeenkomstige aglyconen. Als deze *in vivo* resultaten vergeleken worden met onze *in vitro* resultaten dan is de conclusie dat de Caco-2 cellijn een beter cellulair model voor transport en metabolisme onderzoek is dan de modellen gebaseerd op een IEC-18 of HCEC cellijn. De gemeten karakteristieke parameters, zoals het niveau van glycosiden transport en de vorming van o.a. glucuronide- en sulfaatmetabolieten, tonen aan dat met Caco-2 celmonolagen de menselijke situatie het beste ingeschat kan worden.

In de experimenten van hoofdstuk 4 worden verschillende segmenten van de dunne en dikke darm geperfuseerd met de stoffen genisteïne en genistin. Dit om de darmabsorptie en het transportmechanisme van deze isoflavonen in een ‘meer complex’ model te kunnen bestuderen. De methode die wordt gebruikt is 10 cm van verschillende delen van de darm van de rat (proximaal- en distaaldeel van het jejunum, het gehele ileum en een stuk dikke darm) te isoleren en op te hangen in een perfusator, waarna de segmenten gedurende 2 uur worden geperfuseerd met radioactief gelabelde genisteïne, genistin of radioactief gelabelde polyethyleenglycol (als markeerstof voor het transport). De experimenten van hoofdstuk 4 laten zien dat het transport van genisteïne hoger is dan het transport van genistin in alle geperfuseerde darmsegmenten. De hoogste resorbaat (serosale zijde van het darmsegment) genisteïneconcentratie wordt gevonden in het ileac segment, dit was ongeveer 2 % van de beginconcentratie. Daarentegen is de totale terugwinning van genisteïne plus de genisteïne metabolieten in het resorbaat van de jejunale darmsegmenten na 2 uur ongeveer 26% en 17% van de beginconcentratie gevonden respectievelijk in het proximale en distale geïsoleerde segment. In het ileacale- en dikkedarmsegment is de gevonden hoeveelheid genisteïne na 2 uur maximaal 10%. Voor genistin is de gevonden hoeveelheid in het resorbaat significant lager. De hoeveelheid genistinmetabolieten (o.a. genisteïne en genisteïne glucuroniden) in het resorbaat van de jejunum segmenten is ongeveer 7 % van de beginconcentratie. Er worden geen genistinmetabolieten gevonden in het ileacale- en dikkedarmsegment. Echter, in de perfusaatvloeistof van dikkedarmsegmenten wordt ongeveer 50% van de beginconcentratie genistin gevonden in de vorm van metabolieten (genisteïne en genisteïne glucuroniden). Dit duidt erop dat genistin is gedeglycosyleerd aan de luminale kant van de darmsegmenten. De conclusie van dit hoofdstuk is dat het transport en metabolisme van genisteïne en zijn glycoside hoofdzakelijk plaatsvindt in het jejunale compartiment van de rattendarm. Er worden geen metabolieten van

genistin in het resorbaat van geïsoleerde dikkedarmsegmenten gevonden, maar wel in de perfusievloeistof. Dit wordt waarschijnlijk veroorzaakt doordat genistin kan worden gemetaboliseerd hetzij door enzymen van de epitheelcellen of door overgebleven microflora. Dit laatste wordt onwaarschijnlijk geacht. De conclusie is daarom dat er geen transport van genistin en/of metabolieten in de dikke darm van de rat plaats vindt.

Nog gedetailleerdere informatie over het transport en metabolisme van genisteïne en zijn glycoside genistin (als zuivere stof en/of geëxtraheerd van een soja-isoflavonenrijk eiwitisolaat) wordt verzameld in een *in vivo* model zoals beschreven in hoofdstuk 5. Daarbij is er speciale aandacht voor de mogelijke opname van de isoflavonen in de geglycosileerde vorm. Daartoe worden ratten voorzien van een permanente canule in de poortader, hetgeen een herhaalde bloedbemonstering toestaat, terwijl de ratten niet verdoofd zijn en zich vrij kunnen bewegen. Met het gebruiken van dit model is het mogelijk om uit plasma van de poortader een tijdsafhankelijke genisteïne- en genistinmetabolietenprofielen te verkrijgen en deze te vergelijken met metabolietenprofielen aanwezig in de inhoud van de dunne darm, dikke darm en faeces. Dit geeft een gedetailleerd inzicht in de biologische beschikbaarheid van genisteïne en zijn glycoside genistin. Uit de resultaten van hoofdstuk 5 komt naar voren dat genisteïne gemakkelijk biologisch beschikbaar is, omdat op het eerste meetpunt van 15 minuten wordt een totale maximum concentratie van 16.5 µM genisteïne waargenomen in plasma van de poortader. De oppervlakte onder de curve (AUC) voor totaal genisteïne en zijn conjugaten zijn respectievelijk 54, 24 en 13 µMh voor de geteste stoffen genisteïne, genistin en verrijkt sojaeiwitextract. Deze resultaten wijzen erop dat de biologische beschikbaarheid van genisteïne in de bloedcirculatie hoger is voor de aglycon dan voor zijn glycosidische vorm. De resultaten onthullen ook dat genistin gedeeltelijk is geabsorbeerd in zijn geglycosyleerde vorm. Dit laatste resultaat verschilt van de uitkomsten van andere gepubliceerde studies, maar deze studies analyseerden de gehaltes in totaal lichaamsplasma in plaats van poortaderplasma. Daarom kan geconcludeerd worden dat deglycosylering van geabsorbeerd genistin plaats vindt na eerste passage door de lever.

De gegevens van hoofdstuk 5 leiden tot de conclusie dat biologische beschikbaarheidsexperimenten gebaseerd op het bepalen van plasmaniveaus in de poortader bijdragen aan een beter inzicht in de rol van de darm en de lever bij de

deglycosylering en opname van geglycosyleerde isoflavonen, de vorm waarin deze bioactieve voedingsstoffen over het algemeen in onze voeding voorkomen.

In de experimenten van hoofdstuk 6 ligt de nadruk op intestinaal metabolisme en het mechanisme van de absorptie van genistin. Speciale aandacht is er voor de rol van de natrium afhankelijke glucosetransporter SGLT1. Als methode om dit te onderzoeken, worden *in situ* rattendarmen met een intacte vasculaire bloedsomloop geperfuseerd met genistin (of genisteïne ter vergelijking) in aan- of afwezigheid van de plantenstof phloridzin, een remmer van SGLT1. Op hetzelfde moment is de poortader voorzien van een canule zodat herhaalde bloedmonstering mogelijk is en de bijdrage van het metabolisme door de lever uit te sluiten is. De resultaten van hoofdstuk 6 tonen aan dat de remming van SGLT1 door phloridzin resulteert in significant hogere plasmaconcentraties van genisteïne. Het totale niveau van metabolieten van genisteïne, glucuroniden en sulfaten in plasma van de poortader is gestegen voor zowel genistin als aan genisteïne blootgestelde ratten op het moment dat phloridzin gelijktijdig aanwezig is in het perfusaat. In de perfusievloeistof komt de metaboliet genisteïne het meest voor in het geval van een perfusie met genistin in de afwezigheid of aanwezigheid van phloridzin, hetgeen duidt op een efficiënte deglycosylering. Er wordt verondersteld dat phloridzin niet alleen SGLT1 remt maar ook de effluxtransporters die zich bevinden op de brushbordermembraan van de enterocyt. Dit verhindert dat genisteïne teruggetransporteerd wordt naar het lumen. In plaats daarvan wordt genisteïne getransporteerd naar het plasma, hetgeen ook de hogere genisteïneconcentratie aldaar verklaart. De concentratie van genistin in de perfusievloeistof van experimenten met genistin in de aanwezigheid van phloridzin is onverwacht niet beduidend gestegen in vergelijking met die van darmperfusies met alleen genistin. De concentratie van glucuroniden in de perfusievloeistof is enigszins lager in het geval van perfusies met genistin dan in het perfusaat van ratten die op een gelijkwaardige manier worden blootgesteld aan genisteïne. Deze resultaten van hoofdstuk 6 duiden erop dat SGLT1 niet betrokken is bij absorptie van genistin in de dunne darm van de rat en dat phloridzin in enige mate de cellulaire glucuronidatie remt. De grote hoeveelheid gedeglycosyleerd genistin die in het perfusaat wordt waargenomen, wijst erop dat de absorptie van genistin wordt vergemakkelijkt door een hydrolase in de brushbordermembraan van de darmcel die de omzetting van genistin naar genisteïne katalyseert vóór de daadwerkelijke absorptie.

In hoofdstuk 7 wordt *in vitro* de rol van SGLT1 in het mechanisme van intestinale opname van genistin onderzocht. Daartoe worden Caco-2 cellen gekweekt op semi-

permeabele filters gebruikt voor het bestuderen van het transport van genistin (en genisteïne ter vergelijking) in aan- of afwezigheid van phloridzin. Phloridzin die aan de apicale kant wordt toegevoegd aan de monolaag van Caco-2 cellen verhoogt de concentratie van het aglycon genisteïne en van glucuronide-achtige metabolieten beduidend aan de basolaterale kant. Dit geldt bij zowel experimenten met genistin als bij experimenten met genisteïne. Verondersteld wordt dat phloridzin niet alleen SGLT1 remt, maar ook de effluxtransporters, zoals MRP2 gelokaliseerd in de apicale membraan van enterocyt. Deze zouden normaal het transport van genisteïne en zijn metabolieten terug naar de apicale kant van de Caco-2 cellen moeten vergemakkelijken. De remming van dit proces door phloridzin verklaart dat de concentratie van genisteïne aan de basolaterale kant van Caco-2 monolaag is gestegen. De concentratie van genistin aan de basolaterale kant van de Caco-2 monolaag is onverwacht niet beduidend verschillend in vergelijking met die van de concentratie van genistin in aanwezigheid van phloridzin. Dit wijst erop dat de resultaten van de *in vitro* Caco-2 als de modelcellijn overeenkomen met de *in vivo* resultaten die in hoofdstuk 6 zijn besproken. De resultaten bevestigen de conclusie dat SGLT1 niet betrokken is bij de intestinale opname van genistin.

Slotopmerkingen

Maag-Darmmodellen

Deze dissertatie onderzocht verschillende *in vitro*, *in situ* en *in vivo* maag-darmmodellen voor het bestuderen van het transport en intestinaal opname-mechanisme van genisteïne en zijn glycoside genistin. De conclusie van hoofdstuk 2 en 3 is dat Caco-2 cellen, gekweekt op semi-permeabele filters een goed model vormen voor het bestuderen van het transport van de glycoside genistin. Echter, de Caco-2 cellijn toont een lage expressie van LPH en daarom kan dit model niet worden gebruikt om de bijdrage van LPH te bestuderen in de intestinale opname van geglycosileerde (iso)flavonoïden. Anderzijds is dit model optimaal geschikt voor het specifiek bestuderen van de rol van andere mogelijke betrokken deglycosylatiewegen, met inbegrip van studies naar de rol van SGLT1 (hoofdstuk 7). Voorts wordt in hoofdstuk 3 geconcludeerd dat de IEC-18-cellijn kan worden gebruikt voor een inzicht in het metabolisme van genisteïne en genistin, maar in dit model kan het transport van deze stoffen niet worden bestudeerd, omdat er te veel transport plaatsvindt. Tevens is de HCEC-cellijn geen goed hulpmiddel voor het bestuderen van het transport en het metabolisme van deze stoffen vanwege het feit dat het

transport en de metabole activiteit van de cellen niet overeenkomen met kenmerken gevonden in het onderzoek met humane vrijwilligers en ratten.

In het experiment van hoofdstuk 4 worden geïsoleerde darmsegmenten gebruikt als model om de plaats van transport en metabolisme van genisteïne en genistin te bestuderen. De conclusie van dit hoofdstuk is dat afzonderlijke darmsegmenten het toelaten om het transport en de opname meer in detail te bestuderen. Een nadeel van dit model is de onderbroken bloedlevering aan de darmen en daarom moeten de gegevens, verkregen met dit model, altijd worden geverifieerd met *in vivo* gegevens.

Het *in vivo* model van perfusies van darmen met een intacte vasculaire omloop in hoofdstuk 6 blijkt een uitstekend model te zijn voor het bestuderen van mechanismen die ten grondslag liggen aan het transport en de biologische beschikbaarheid van de isoflavonen. In dit model waren de ratten wel onder anesthesie en dit beperkt de actuele eigenschappen van de dunne darm. Daarom wordt het model gebruikt in hoofdstuk 5 met vrij bewegende, niet verdoofde gecanuleerde ratten, als het best te gebruiken model aanbevolen, omdat met dit model tijdsafhankelijke genisteïne- en genistinprofielen afkomstig van poortaderplasma kunnen worden verkregen. Deze kunnen vergeleken worden met metabolietprofielen verkregen in menselijke vrijwilligersstudies. Hoewel dit model gedetailleerd inzicht verstrekt in de biologische beschikbaarheid van genisteïne en zijn glycoside genistin, zijn er ook ernstige nadelen. *In vivo* modellen gebruiken proefdieren, hetgeen duur en tijdrovend is. Tevens past het gebruik van proefdieren niet in het huidige beleid van vermindering, verbetering en vervanging (3Vs) van proefdierstudies. Daarom het is essentieel om *in vitro* modellen te blijven ontwikkelen. Gebaseerd op de resultaten van deze dissertatie wordt geadviseerd om de hydrolase-activiteit van de Caco-2 cellijn te verbeteren. Bijvoorbeeld door genetische wijziging van de Caco-2 cellijn, met accent op hogere expressie van LPH. Dit maakt de Caco-2 cellijn nog beter geschikt als *in vitro* model voor menselijke biologische beschikbaarheidsstudies met isoflavonen en hun glycosiden.

Biologische beschikbaarheid van genisteïne en genistin in verschillende in vitro, in situ and in vivo darmmodellen in vergelijking met menselijke studies

Uit de experimenten, beschreven in deze dissertatie, kan worden geconcludeerd dat genisteïne in hogere mate biologisch beschikbaar is dan zijn glycoside genistin. De maximum concentraties van genisteïne in de blootgestelde intestinale cellijnen waren vergelijkbaar met de concentraties gemeten in de perfusaten van de *in vivo*

uitgevoerde studies. Terwijl de maximum concentratie van metabolieten die in ongeveer 24 uur worden gevormd lager is in cellulaire *in vitro* modellen. De hoeveelheid cellen gekweekt op de semi-permeabele monolagen zijn echter niet vergelijkbaar met het aantal enterocyten in de darmen. Dit zou eventueel de reden zijn voor de lagere concentratie van metabolieten die in de intestinale celmodellen gevonden worden.

Onze dierproefonderzoeken met genisteïne vertonen overeenkomsten met andere gepubliceerde dierproefonderzoeken. Echter, de concentratie van genisteïne die in het plasma van menselijke vrijwilligers wordt gemeten, is gewoonlijk lager dan de plasmaconcentratie die in *in situ* rattenstudies wordt waargenomen (Manach et al., 2005). Eén van de redenen voor dit verschil zou de inbouw van genisteïne in voedselmatrixen kunnen zijn, zoals vaak toegepast wordt bij het onderzoek met menselijke vrijwilligers. Het experiment van hoofdstuk 5 toont bijvoorbeeld aan dat genisteïne in een soja-eiwitextract een lagere biologische beschikbaarheid heeft dan wanneer het als een zuivere stof wordt toegediend.

De waargenomen concentratie van genistin in het resorbaat en in het plasma van alle maag-darmmodellen uit deze dissertatie blijkt laag te zijn in vergelijking met genisteïne maar wel significant. In *in vivo* studies met aan genistin en genisteïne blootgestelde ratten is de AUC voor genistin ongeveer 0.9 µmolh/l en dus 10 keer lager dan AUC voor genisteïne. Tevens blijkt dat deglycosylering van genistin naar genisteïne en vervolgens intestinaal metabolisme naar genisteïne glucoronides en de sulfaten deel uitmaakt van de belangrijkste route van absorptie van genistin.

Het oorspronkelijk voorgestelde model van de glycosideabsorptie veronderstelde dat de flavonoïde glycosiden te polair zijn om te worden geabsorbeerd door de dunne darm, en daarom zou de absorptie plaatsvinden in de dikke darm na bacteriële deconjugatie door microflora (Griffits en Barrow, 1993). In hoofdstuk 4 wordt geconcludeerd dat er geen absorptie van genistin en zijn metabolieten is door het weefsel van de dikke darm. Dit spreekt de algemene veronderstelling tegen dat de dikke darm de flavonoïde glycoside absorbeert na een (bacteriële) deconjugatie. *In vivo* ratstudies tonen ook aan dat de maximum piekconcentratie van metabolieten van dieetflavonoïde glycosiden tussen 0-2 uur ligt, hetgeen wordt aangetoond in hoofdstuk 5. Dit bevestigt dat de genistinabsorptie in het eerste deel van de darmen plaatsvindt (Walle et al., 2000; Sesink et al., 2001, Piskula et al., 2000; Coldham, 2000 en 2002).

Deze dissertatie toont aan dat SGLT1 niet betrokken is bij de absorptie van genistin, maar dat genistin wordt gehydrolyseerd door een hydroxylase in de brushbordermembraan van de dunne darm die de omzetting van genistin naar genisteïne katalyseert vóór de daadwerkelijke absorptie (hoofdstuk 6 en 7). Genisteïne gaat de intestinale cel in door een carrier-gemedieerd mechanisme (hoofdstuk 3) en/of door passieve diffusie. In de enterocyte kan genisteïne geconjugeerd worden door uridine-5'-difosfaat-glucuronosyl transferases (UDPGT), sulfotransferases of catechol-O-methyltransferases naar respectievelijk genisteïne glucuronides, sulfaten of methyl-conjugaten. Deze conjugaten kunnen enerzijds getransporteerd worden terug naar het lumen via effluxtransporters, gelokaliseerd in het apicale membraan van enterocyt (o.a. BCRP1 of MRP2), anderzijds kunnen ze getransporteerd worden naar het plasma door een nog onbekende transporter. In deze dissertatie wordt genistin als intacte molecuul in het plasma of in basolaterale media van de modellen van de intestinale cellen gevonden, hetgeen suggereert dat genistin gedeeltelijk via de tight junctions van de enterocyte getransporteerd kan worden gezien de afwezigheid van de rol van SGLT1.

Algemene conclusie

Het transport en de biologische beschikbaarheid van genisteïne en zijn glycoside genistin in verschillende maag-darmmodellen is laag. Daarom is het belangrijk om het mechanisme van absorptie van genisteïne en zijn glycoside te bestuderen. Deze mechanistische studies kunnen worden uitgevoerd *in vivo*, maar dierexperimenten zijn duur en tijdrovend en zouden moeten worden verminderd, verfijnd of vervangen. De Caco-2 cellen zijn een goed alternatief voor het bestuderen van het mechanisme van isoflavonenabsorptie, maar de hydrolaseactiviteit van deze cellijnen zou moeten worden verbeterd om de menselijke situatie beter na te kunnen bootsen. Onze mechanistische studies tonen aan dat de biologische beschikbaarheid van genistin kan worden verbeterd door phloridzin, omdat phloridzin effluxtransporters, gelocaliseerd in de apicale membraan van de enterocyt, remt. Genistin kan worden geabsorbeerd, maar het wordt ook efficiënt gedeglycosyleerd vóór en tijdens de intestinale opname. Deze dissertatie verstrekt inzicht in het mechanisme van biologische beschikbaarheid van deze belangrijke klasse van gezondheidsbevorderende, op soja gebaseerde, voedselingrediënten en benadrukt dat remming van apicale transporters de biologische beschikbaarheid van genistin en genisteïne kan verbeteren.

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Abstracts

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Steensma, A, Gros-van Hest, M. Hub Noteborn, H.P.J.M. and Kuiper, H.A. (2000) Bioavailability of soy food in Caco-2 cells. *AIO/OIO-dagen*, December 7-8, Kerkrade, The Netherlands

Curriculum Vitae

Aukje Steensma was born as one of a twin on April 6, 1966 in Leeuwarden, The Netherlands. In 1984 she completed secondary school (VWO) at the Stedelijke Scholen Gemeenschap in Leeuwarden, The Netherlands. She started with the education Food and Dietetics at the Hanzehogeschool Groningen and graduated in 1988 as dietician. At the same year she started to study Food Technology at the Wageningen Agricultural University (WAU), The Netherlands. As a part of this study, she conducted research projects at the Department Biochemistry and Toxicology of the WAU. Subsequently, the practical period was fulfilled at the British Industrial Biological Research Association (BIBRA) Toxicology International, Surrey, United Kingdom. In 1994 she graduated for her M.Sc. in Food Technology with specialisation toxicology and biochemistry. In 1995 she started as a researcher on the Post Doctoral Research Project at the Toxicology department of WAU. Subsequently, in 1996 she started as a Ph.D. student at the Toxicology Group of the WAU on the research presented in this thesis under supervision of Prof. Dr. J.H. Koeman. This research was conducted from 1996 till 2001 at Rikilt, Institute for Food Safety (Wageningen, The Netherlands) under supervision of Dr. H.A. Kuiper, Dr. H.P.M.J. Noteborn and Dr. M.J.B. Mengelers (first years of this thesis). In 2004, Prof. Dr. J.H. Koeman (retired in 2000) handed over the supervision to Prof. Dr. I.M.C.M. Rietjens, who supervised the last part of this thesis. Aukje worked as a Toxicologist at the department of Regulatory Affairs of DSM Food Specialties from 2002 till 2005.

Training and Supervision Plan

Overview of conferences and courses attended during PhD

Conferences: Food and Cancer Prevention II	1996
Conferences: Carotenoid	1996
Laboratory animal sciences (Postgraduate Education of Toxicology (PET))	1996
PhD Symposium (Nederlandse Vereniging voor Toxicologie (NVT))	1996
Working with radioactive labelled compounds and radiation sources (PET)	1996
International course on Ecophysiology of the Gastrointestinal Tract (VLAG)	1996
PhD Symposium (NVT)	1997
Conferences: Bioavailability (VLAG)	1997
COST 916 Workshop Phyto-oestrogens: exposure, bioavailability, health benefits and safety concerns	1998
Course: English for publishing in international scientific journals and effective presentation in English (Cressie Communication Services)	1998
Occupational Toxicology (PET)	1998
Euroconference The effects of endocrine disrupting compounds in animal feed on reproductive health in farm animals	1998
Pathobiology (PET)	1999
23rd International LOF-Symposium on Phyto-oestrogens, Gent, Belgium	1999
Conferences: Food and Cancer Prevention III, Norwich, United Kingdom	1999
Third International Symposium on the role of soy in preventing and treating chronic disease, Washington, DC, United States of America.	1999
Medical and Forensic Toxicology (PET)	1999
Genetic Toxicology and Carcinogenesis (PET)	2000
PhD Symposium (NVT)	2000
Ecotoxicology (PET)	2001
PhD symposium (NVT)	2003
Eurotox 41st congress of the European Society of Toxicology, Florence, Italy	2003
Languages training English (Talking Heads Communicatie & Taaltraining)	2004
PhD Symposium (NVT)	2004

The research described in this thesis was carried out at Rikilt, Institute of Food Safety (former State Institute for Quality Control of Agriculture Products), Wageningen, The Netherlands in cooperation with the Wageningen University, department of Agrotechnology and Food Sciences, division of Toxicology (former Department of Toxicology), Wageningen, The Netherlands.

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