The kinetic basis for age-associated changes in quercetin and genistein glucuronidation by rat liver microsomes[☆]

Bradley W. Bolling[a], Michael H. Court[b], Jeffrey B. Blumberg[a], C.-Y. Oliver Chen[a],*

[a]Antioxidants Research Laboratory, Jean Mayer USDA Human Nutrition Research Center on Aging, Tufts University, Boston, MA 02111, USA
[b]Department of Pharmacology, Tufts University, Boston, MA 02111, USA

Abstract

The dietary bioavailability of the isoflavone genistein is decreased in older rats compared to young adults. Since flavonoids are metabolized extensively by the UDP-glucuronosyltransferases (UGTs), we hypothesized that UGT flavonoid conjugating activity changes with age. The effect of age on flavonoid glucuronidation was determined using hepatic microsomes from male F344 rats. Kinetic models of UGT activity toward the flavonol quercetin and the isoflavone genistein were established using pooled hepatic microsomal fractions of rats at different ages, and glucuronidation rates were determined using individual samples. Intrinsic clearance (V_{max}/K_m) values in 4-, 18- and 28-month-old rats were 0.100, 0.078 and 0.087 ml/min/mg for quercetin-7-O-glucuronide; 0.138, 0.133 and 0.088 for quercetin-3′-O-glucuronide; and 0.075, 0.077 and 0.057 for quercetin-4′-O-glucuronide, respectively. While there were no differences in formation rates of total quercetin glucuronides in individual samples, the production of the primary metabolite, quercetin-7-O-glucuronide, at 30 μM quercetin concentration was increased from 3.4 and 3.1 nmol/min/mg at 4 and 18 months to 3.8 nmol/min/mg at 28 months, while quercetin-3′-O-glucuronide formation at 28 months declined by a similar degree ($P \leq .05$). At 30 and 300 μM quercetin concentration, the rate of quercetin-4′-O-glucuronide formation peaked at 18 months at 0.9 nmol/min/mg. Intrinsic clearance values of genistein 7-O-glucuronide increased with age, in contrast to quercetin glucuronidation. Thus, the capacity for flavonoid glucuronidation by rat liver microsomes is dependent on age, UGT isoenzymes and flavonoid structure.

1. Introduction

Flavonoids are dietary polyphenols found ubiquitously in plant foods. Prospective cohort studies have associated generous flavonoid intake with reduced risk of cardiovascular disease, stroke and some forms of cancer [1–3]. Flavonoids may confer protection against chronic diseases through an array of putative actions, including antioxidation, detoxification, anti-inflammation and anti-proliferation. Any health benefits derived from dietary flavonoid intake must depend upon their bioavailability, metabolism and distribution [4], parameters that may be affected by age-related changes in physiology. Limited flavonoid bioavailability and extensive metabolism, particularly when combined with a low intake of these phytochemicals [5], may decrease their putative contribution to health promotion and disease prevention in older adults.

Upon consumption, flavonoids are extensively transformed via Phase 2 enzymes through conjugation with glucuronide, sulfate and/ or methyl moieties, particularly in small intestine and liver [6]. This pre-systemic biotransformation of flavonoids within liver and small intestine affects their biological half-life and may decrease their efficacy. For example, glucuronidation of flavonoids limit their putative actions to protect cells against oxidative injury and inhibit lipoxygenase and xanthine oxidase activity [7,8]. Due to the diversity of the Phase 2 enzymes, metabolites of flavonoids produced through this pathway also depend on tissue type. For example, in rats fed quercetin, the liver contained a majority of sulfated monoglucuronide derivatives, whereas intestinal tissue had the greatest proportion of monoglucuronides [6].

Physiological and environmental factors such as age, genetics and dietary behavior affect the bioavailability of nutrients as well as drugs. However, information on the extent to which flavonoid metabolism is altered by these parameters is scarce. The Phase 2 detoxification pathway is essential to flavonoid metabolism and is partly influenced by aging [9]. van Bezooijen [10] has described how age-related changes in Phase 2 metabolism are dependent upon factors such as enzyme isoform, substrate, gender and diet. However, the effect of age-related changes in flavonoid metabolism mediated by Phase 2

enzymes responsible for glucuronidation, sulfation and methylation remains to be established. Characterizing this relationship is important to elucidating the impact of dietary flavonoids on the health of older adults.

Previously, we observed that fasted senescent rats fed genistein for 4 weeks had a lower steady-state concentration of this isoflavone in liver and plasma than adult rats [11]. The mechanisms underlying this difference remain to be determined. We hypothesized that UDP-glucuronosyltransferase (UGT) flavonoid activity changes with age. Therefore, the aim of this study was to determine whether changes in hepatic glucuronidation of selected flavonoids account for the previously observed age-related decline in their bioavailability in rats. Our objective was to determine the variation in glucuronidation kinetics of isoflavone genistein and the flavonol quercetin using pooled hepatic microsomes collected from 4-, 18- and 28-month-old rats. Acetaminophen glucuronidation was also determined in the same samples to validate enzyme kinetic modeling and as a negative control for age-related changes in glucuronidation, as hepatic microsomal rates of acetaminophen glucuronidation are reportedly unchanged with advanced age in rats and mice [12,13].

2. Materials and methods

2.1. Chemicals and supplies

HPLC-grade acetonitrile and methanol were obtained from Fisher Scientific (Thermo Fisher Scientific, Rockford, IL). Genistein was purchased from Cayman Chemical (Ann Arbor, MI), and rutin was obtained from Extrasynthese (Genay, France). Quercetin, UDP-glucuronic acid, alamethicin and all other chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO).

2.2. Tissues and preparation

Whole rat liver of tumor-free male Fischer F344 rats aged 4, 18 and 28 months ($n=5$/group) was obtained from the Aging Rodent Tissue Bank of the NIA/NIH (Bethesda, MD). Male rats were chosen to limit any influence of hormone fluctuation on detoxification pathways. Body weights (mean±S.D.) were 290±12 g (4 months), 441±14 g (18 months) and 406±19 g (28 months). Liver weights increased with advancing age at 12±1, 17±1 and 17±1 g for 4-, 18- and 28-month-old rats, respectively.

Hepatic microsomal fractions were prepared as previously described [14] with slight modifications. Whole liver was pulverized under liquid nitrogen and stored at −80°C until homogenization. Liver aliquots (~0.5 g) were homogenized in 10 vol sucrose buffer (50 mM Tris–HCl and 0.25 M sucrose, pH 7.5) in an ice bath, using a Tekmar Tissumizer, SDT-1810 (Spokane, WA), at 85% power for 2 min. Homogenate was centrifuged at 1000×g for 15 min at 4°C. Supernatant kept at 4°C was successively centrifuged at 10,000×g for 20 min to pellet nuclei and mitochondria, and the resulting supernatant was spun at 100,000×g for 1 h to produce the microsomal pellet. After decanting the cytosol fraction, the microsomal pellet was rinsed with 400 µl suspension buffer (0.1 M potassium phosphate buffer, pH 7.5, containing 20% glycerol) and then resuspended in the same volume. Microsomal protein concentration was determined by a Pierce BCA kit (Thermo Fisher Scientific) and adjusted to 5 mg/ml with phosphate buffer (0.1 M, pH 7.5). Aliquots of the microsomal fraction were stored at −80°C until use.

2.3. Kinetics and individual rate of glucuronidation

The kinetics of quercetin, genistein and acetaminophen glucuronidation by rat hepatic microsomes was determined as previously described [15,16]. Acetaminophen glucuronidation was used as a positive control [12]. Briefly, microsomal protein (0–1 mg/ml, final concentration) was preincubated with alamethicin (0.25 µg/ml) at 37°C for 5 min in microcentrifuge tubes containing previously dried substrate. A cofactor solution of UDP-glucuronic acid (5 mM, final concentration), magnesium chloride (5 mM, final concentration) and potassium phosphate buffer, pH 7.5 (0.05 mM, final concentration), was added to initiate the reaction in a final assay volume of 0.1 ml. After incubation for selected durations at 37°C, the reaction was terminated with 0.1 ml ice-cold methanol containing either 33 µM rutin or daidzein or 0.24 mM 2-acetamido-phenol as internal standards. After centrifugation at 14,000×g for 5 min, 180 µl of supernatant was dried under a stream of purified nitrogen gas at room temperature and stored at −20°C until HPLC analysis.

The enzyme kinetics of the glucuronide formation for each age was determined in duplicate using pooled microsomes. With 0.2 mg/ml microsomal protein and 30 min incubation, formation of quercetin and genistein metabolites was linear with

concentrations of 9–600 µM. Similarly, with 0.5 mg/ml protein and 30 min incubation, production of acetaminophen glucuronide was linear between 0.9 and 30 mM. At concentrations of 30 and 300 µM, linearity of quercetin and genistein glucuronidation was established with 0.05–5 mg/ml microsomal protein and incubation for 30 min, as well as with 0.2 mg/ml protein and incubation for up to 60 min. For acetaminophen at 0.5 and 30 mM, the glucuronidation rate was linear with 0.1–1.0 mg/ml protein for 30 min and with 0.5 mg/ml protein for up to 60 min.

The glucuronidation rate of quercetin, genistein and acetaminophen was determined in triplicate using individual microsomal samples. Concentrations of quercetin and genistein at 30 and 300 µM, which are near the K_m value and 10-fold higher, respectively, were employed. Acetaminophen glucuronidation was assayed at 0.5 and 10 mM substrate concentration, representing values 20-fold below and near its K_m value, respectively.

2.4. HPLC analyses of glucuronide metabolites

The products of the glucuronidation reaction were reconstituted in 90 µl of 50% methanol in water and analyzed using HPLC according to previously established methods. A Thermo-Finnigan Surveyor HPLC (Thermo Fisher Scientific, San Jose, CA) equipped with an autosampler, UV detector and Phenomenex Synergi 10 µm Hydro-RP80 250×4.6 mm column (Torrance, CA), with mobile phase A consisting of 20 mM phosphate buffer, pH 2.0, and mobile phase B consisting of acetonitrile, was employed. After normalization with the respective internal standard, glucuronide metabolites were quantified using a standard curve established in the absence of UDPGA. The method of Boersma et al. [15] was used to analyze quercetin and genistein and their metabolites. Following a 20-µl injection, at 1 ml/min flow rate, mobile phase B was held at 17% for 2 min and then increased to 25% in 5 min, to 35% in 8 min and to 50% in 5 min. After 3 min at 50%, mobile phase B was decreased to 17% in 4 min and held for 5 min to equilibrate the column for subsequent injections.

Three principal quercetin glucuronide metabolites were identified as previously described [15], with the following retention times (Rts): 11.5 min for 7-*O*-glucuronide, 14.3 min for 4′-*O*-glucuronide and 14.8 min for 3′-*O*-glucuronide. Quercetin and its metabolites and rutin (Rt, 10.8 min) were monitored at 370 nm. Consistent with Doerge et al. [17], microsomal glucuronidation incubations with genistein yielded primarily the 7-*O*-glucuronide of genistein (Rt, 15.2 min), although with longer incubation times, the 4′-*O*-glucuronide was observed. Genistein and its metabolites and daidzein (Rt, 19.3 min) were monitored at 255 nm.

Acetaminophen and its glucuronide metabolite were analyzed according to the method of Court and Greenblatt [18]. Briefly, a 20-µl injection was eluted at 1.4 ml/min in an isocratic mode with 3% mobile phase B. Acetaminophen glucuronide (Rt, 5.0 min) was the sole glucuronide metabolite formed by microsomal incubations of aceta-minophen. The acetaminophen glucuronide metabolite and the internal standard 2-acetamidophenol (Rt, 13.7 min) were monitored at 254 nm.

2.5. Data analysis

Visual inspection of Michaelis–Menten, Lineweaver–Burk and Eadie–Hofstee plots and comparison of sum of squared residuals using GraphPad Prism v3.00 (GraphPad Software, San Diego, CA) were employed to select the best-fit kinetic models, as previously described by Court et al. [17,18]. This includes the Michaelis–Menten model [Eq. (1)] and the uncompetitive substrate inhibition model [Eq. (2)]:

$$V = V_{max} \times S/(K_m + S) \tag{1}$$

$$V = V_{max} \times S/(K_m + S \times (1 + S/K_s)) \tag{2}$$

where V is the velocity, S is the substrate concentration, V_{max} is the maximum velocity, K_m is the substrate concentration at 50% V_{max} and K_s is the substrate inhibition constant. Kinetic parameters are expressed as the estimate and 95% confidence interval.

The effect of age on glucuronidation rates of individual hepatic microsomes was assessed using one-way ANOVA. When *P* values were ≤.05, post hoc analysis was performed using Tukey's honestly significant difference test. Statistical analyses were performed using JMP IN (SAS Inc., Cary, NC). GraphPad Prism 5.01 (GraphPad Software) was used to determine Pearson's correlation values and significance (*P*≤.05) for microsomal glucuronidation and age.

3. Results

3.1. Acetaminophen glucuronidation

The enzyme kinetics of acetaminophen glucuronidation was best fit using the Michaelis–Menten model. Although V_{max} values of pooled microsomes increased with advanced age, K_m and intrinsic

Table 1
Enzyme kinetic parameters for glucuronidation of acetaminophen (AAP), genistein (G) and quercetin (Q) by hepatic microsomes of different aged male F344 rat

Metabolite	Age (months)	V_{max} (nmol/min/mg)	K_m (μM)	K_s (μM)	CL_{int}[a] (ml/min/mg)	Trend CL_{int}
AAP-4-O-glucuronide	4	4.6±0.6	9600±3800	–	0.000479	r=.02
	18	4.8±0.2	1800±200	–	0.000261	P=.99
	28	5.3±0.2	1300±100	–	0.000510	
G-7-O-glucuronide	4	3.0±0.3	23±6	–	0.130	r=.99
	18	3.0±0.3	21±4	–	0.138	P=.10
	28	4.4±0.8	30±5	–	0.148	
Q-7-O-glucuronide	4	9.7±2.4	97±38	469±237	0.100	r=−.66
	18	10±3	133±55	440±233	0.078	P=.54
	28	19±7	219±114	370±238	0.087	
Q-4′-O-glucuronide	4	0.63±0.08	4.6±2.6	456±177	0.138	r=−.86
	18	0.96±0.09	7.2±2.5	633±202	0.133	P=.34
	28	0.72±0.07	8.2±2.8	473±145	0.088	
Q-3′-O-glucuronide	4	4.9±0.9	66±22	695±322	0.075	r=−.76
	18	6.3±1.0	81±23	672±268	0.077	P=.45
	28	4.3±0.6	75±21	1017±472	0.057	

AAP glucuronidation and G glucuronidation were modeled by Michaelis–Menten kinetics, whereas Q glucuronidation was modeled by uncompetitive substrate inhibition kinetics. Data are the average of duplicate determinations. Data are model estimate with standard error.

[a] CL_{int} is defined as V_{max}/K_m.

clearance (V_{max}/K_m) did not follow this trend (Table 1). Age did not affect individual rates of acetaminophen glucuronidation measured at substrate concentrations of 0.5 and 10 mM (Table 2).

3.2. Genistein glucuronidation

Genistein 7-O-glucuronidation kinetics was best modeled by the Michaelis–Menten equation (Fig. 1). Age did not affect the V_{max} or K_m of genistein, with their values ranging from 3.0 to 4.4 nmol/min/mg and from 21 to 30 μM, respectively (Table 1). However, the intrinsic clearance (V_{max}/K_m) of genistein was increased modestly by age (r=.99, P=.10), with a 14% larger clearance at 28 months than at 4 months (Table 1). However, these increments were not confirmed in individual samples, as the rate of 7-O-glucuronide–genistein formation was unaltered overall by age (Table 2). Nevertheless, glucuronidation rates of the 28-month livers were 29% and 34% higher than those of the 4-month livers at genistein concentrations of 30 and 300 μM, respectively.

3.3. Quercetin glucuronidation

In contrast to genistein and acetaminophen glucuronidation, the enzyme kinetics of quercetin glucuronidation was described best by the uncompetitive substrate inhibition model (Fig. 2). Intrinsic clearance values declined from 4 to 28 months by 13%, 36% and 24% for the 7-O-, 3′-O- and 4′-O-glucuronides, respectively (Table 1). This age-related decline of intrinsic clearance appears principally due to the disproportionate increases in V_{max} and K_m. For example, for the most abundant quercetin metabolite, quercetin-7-O-glucuronide, the V_{max} and K_m increased by 125% and 96%, respectively, from 4 to 28 months.

While there were no differences in the rate of total quercetin glucuronidation from individual hepatic microsomes, the production of quercetin-7-O-glucuronide at 30 μM quercetin concentration increased 12% from 4 to 28 months, while 3′-glucuronide formation at 28 months declined by a similar degree (P≤.05) (Fig. 3). The rate of quercetin-4′-glucuronide formation peaked at 18 months. The magnitude and pattern of age-related changes in glucuronidation rates were similar between quercetin concentration at 30 and 300 μM. However, at 300 μM, changes in 7-O-glucuronide rates were insignificant (Fig. 3).

4. Discussion

A wide variation in flavonoid bioavailability has been observed in clinical trials [19], which can be attributed to environmental, physiological, dietary and genetic factors. Age may be a contributing factor to this variation of flavonoid bioavailability, since we have previously observed that older rats had lower steady state of genistein disposition than younger rats [11]. Although the mechanisms responsible for this difference have not been explored, physiological changes associated with advancing age may complicate or distort patterns of metabolism that could affect the bioavailability and bioactivity of dietary polyphenols. Such age-related changes could include decreased liver size and volume of distribution and increased intestinal transit time [20]. Furthermore, age-related changes in metabolic enzymatic activity may alter the absorption and biotransformation of both nutrients and drugs [21]. Thus, in this study of hepatic microsomes in vitro, we examined the impact of age on the activity of UGT activity toward genistein and quercetin.

The maximum plasma concentrations following ingestion of flavonoid-containing foods or supplements in humans range from 0.1 to 8 μM for flavonol metabolites and from 0.3 to 25 μM for isoflavone metabolites [19]. We utilized a range of flavonoid concentrations up to 600 μM for kinetic determinations of glucuronidation and tested individual microsomal preparations from different ages at 30 and 300 μM on the basis of kinetic determinations, approximating K_m values and 10 times the K_m value. In a previous study, rats fed 4.45 g/kg quercetin for 6 weeks resulted in metabolite concentrations of 14 μM in plasma and 2.6 nmol/g in liver tissue [6]. Thus, while 300 μM flavonoid may be achieved in the stomach or gastrointestinal lumen after supplementation, concentrations of ≤30 μM flavonoid are commonly reported in plasma and tissue.

Table 2
Rates of acetaminophen (AAP) and genistein (G) glucuronidation by hepatic microsomes of different aged male F344 rat

Metabolite	Age (months)	Rate (nmol/min/mg)	
		0.05 mM AAP[a]	10 mM AAP
AAP-4-O-glucuronide	4	0.34±0.08	2.4±0.6
	18	0.24±0.18	2.1±1.4
	28	0.39±0.10	3.0±0.8
		30 μM G[b]	300 μM G
G-7-O-glucuronide	4	1.7±0.2	3.6±0.3
	18	1.8±0.4	3.8±1.0
	28	2.2±0.5	4.9±1.4

Values are expressed as mean±S.D., n=5.
Changes in rates are not significantly correlated with age (P<.05).
[a] Concentrations of AAP employed in microsomal incubations.
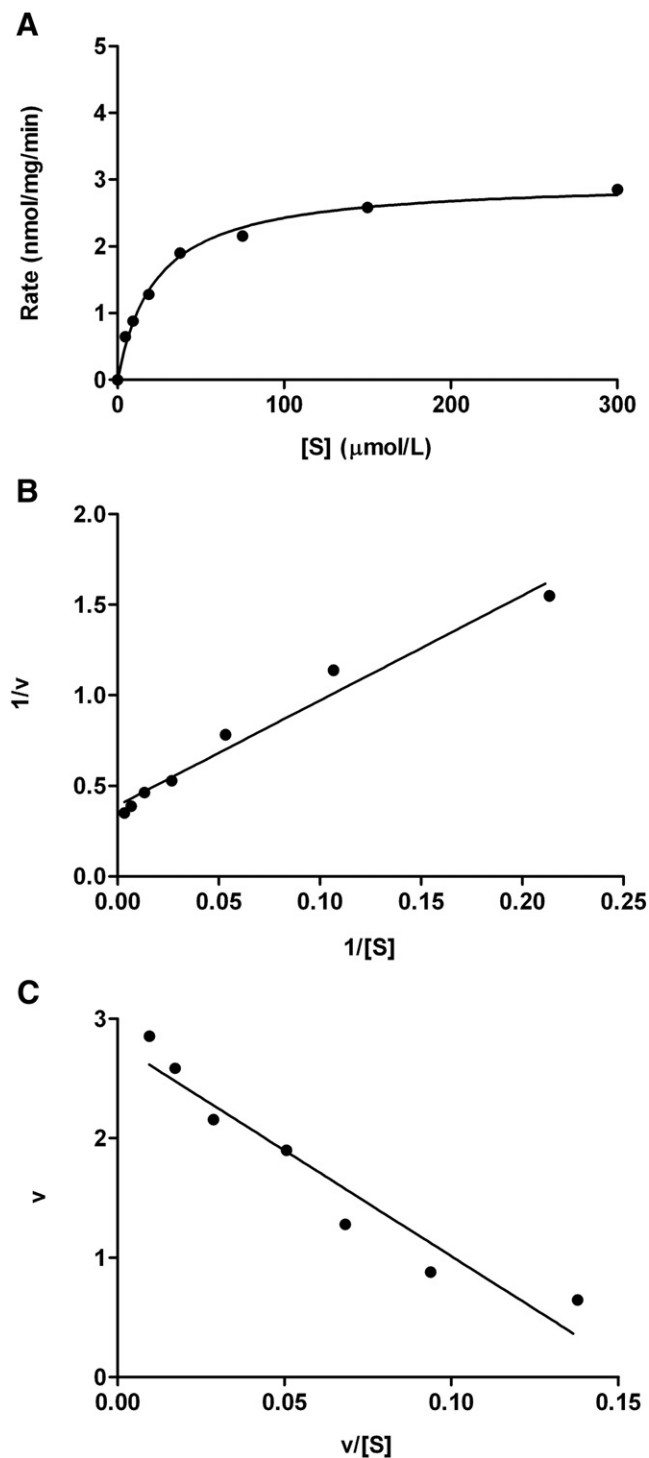[b] Concentrations of G employed in microsomal incubations.

Fig. 1. Kinetic plots of genistein glucuronidation using pooled hepatic microsomes from five 4-month-old male F344 rats. (A) Michaelis–Menten plot, with estimate best modeled by Michaelis–Menten kinetics. (B) Lineweaver–Burk plot. (C) Eadie–Hofstee plot. The kinetic parameters are summarized in Table 1.

Weiner [13] did find that acetaminophen glucuronidation by cultured hepatocytes was increased in old compared to young and adult rats. Therefore, the effect of advanced age on UGT activity toward acetaminophen may be influenced by the species and strain of the animal model.
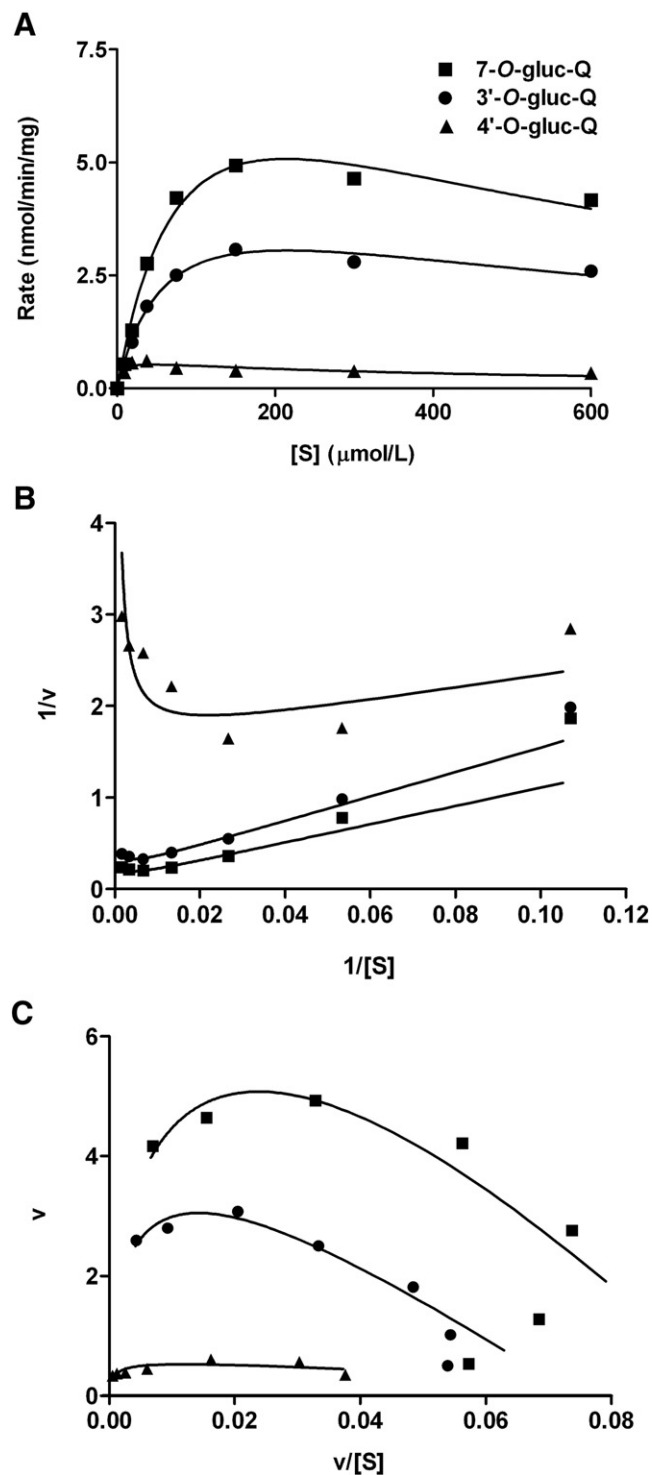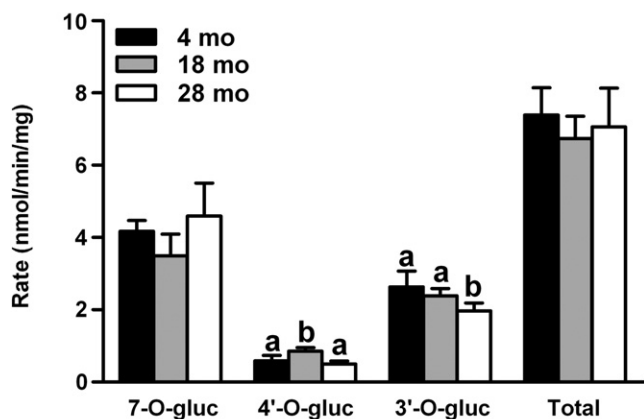

Fig. 2. Kinetic plots of quercetin glucuronidation using pooled hepatic microsomes from five 4-month-old male F344 rats. (A) Michaelis–Menten plot, with estimate best modeled by uncompetitive substrate inhibition kinetics. (B) Lineweaver–Burk plot. (C) Eadie–Hofstee plot. The kinetic parameters are summarized in Table 1.

Acetaminophen has often been used as a model compound to evaluate glucuronidation activity. We did not find age-related changes in glucuronidation activity of hepatic microsomes toward this compound. This result is consistent with studies of hepatic microsomal acetaminophen glucuronidation in male and female Brown Norwegian rats at 4 and 34 months [12] and male C57BL/6NNia mice at 3–5, 11–13, 18–20 and 24–26 months [13]. However, Sweeny and

A 300 μM Quercetin
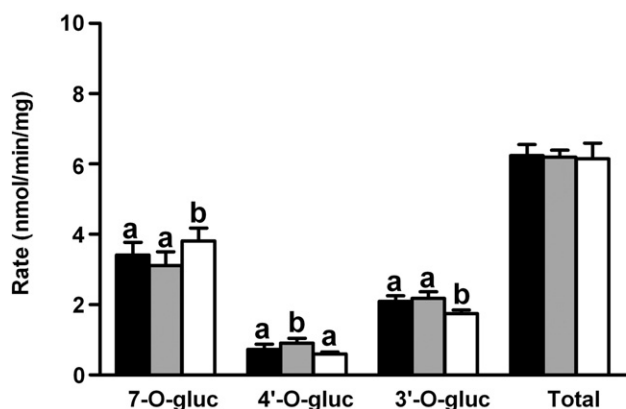

B 30 μM Quercetin

Fig. 3. Age-related changes in glucuronidation activities toward quercetin catalyzed by hepatic microsomal UGT of male F344 rats. Quercetin concentration at 30 μM was in range of the K_m value for total glucuronide metabolite formation. Data are expressed as mean±S.D. (n=5/group). P values of one-way ANOVA for 30 μM quercetin were .0381 for 7-O-glucuronide, .0034 for 4'-O-glucuronide, .00089 for 3'-O-glucuronide and .93 for total glucuronides. P values of one-way ANOVA for 300 μM were .058 for 7-O-glucuronide, .0009 for 4'-O-glucuronide, .016 for 3'-O-glucuronide and .50 for total glucuronides. [abc]Means within the same metabolite without the same letter differ, Tukey's HSD multiple comparison test, P≤.05.

Phase 2 conjugation reactions facilitate elimination of flavonoids from the body. Thus, increased microsomal rates of genistein glucuronidation may have contributed to the reported decrease in the steady-state concentration of genistein in older rats [11]. We found that the kinetic parameters of microsomal genistein glucuronidation were similar to those reported by Chen et al. [22] in adult male Sprague–Dawley rats. However, we did not observe an age-related change in glucuronidation activity toward genistein in vitro, although there was a tendency toward increased intrinsic clearance with age. Since the variability between individual rats increased with age, a larger sample size per age group or more time points may be necessary to better reveal an age-dependent effect on genistein glucuronidation. These in vitro results do not support our hypothesis that hepatic microsomal rates of glucuronidation significantly change with age. However, the effects of age on intestinal and renal glucuronidation or other Phase 2 conjugation enzymes warrant further investigation.

Although it was previously reported that age affects the steady-state concentration of genistein, evidence for age-related changes in isoflavone metabolism in humans is scarce and inconsistent. Cassidy et al. [23] found that bioavailability of daidzein was 30% higher in

postmenopausal women than in premenopausal women, while Faughnan et al. [24] reported that age did not affect urinary isoflavone excretion in women. Thus, the impact of advanced age on the bioavailability of flavonoids requires further study with careful consideration to hormonal status, sample size, dosage and magnitude of age difference.

Quercetin glucuronide metabolites have been characterized extensively while enzyme kinetic modeling of quercetin glucuronidation in rat hepatic microsomes has been lacking. We found that UGT-catalyzed quercetin glucuronidation exhibited uncompetitive substrate inhibition kinetics, consistent with a previous study using recombinant human UGT 1A3 and 1A9 [25]. Enzyme kinetic modeling suggested that hepatic quercetin glucuronidation decreased with age. The K_m and V_{max} values for 7-, 4'- and 3'-glucuronidation of quercetin in older rats were increased compared to those at 4 months of age but was associated with a decrease in CL_{int} values. However, quercetin glucuronidation rates of individual microsome preparations declined for the 3'- and 4'-metabolite and were constant when total glucuronidation activity (sum of the metabolites) was considered. Therefore, the preservation of total glucuronidation activity toward quercetin was due to differential age-related changes in the activity of different UGT isoenzymes. At 28 months, the increase in production of quercetin 7-O-glucuronide was offset by decreased production of the 3'-O-glucuronide. Since the bioactivity of metabolized flavonoids is dependent upon the location of hydroxyl moiety conjugation [8], quercetin glucuronides may have different actions at different ages.

While age affected the hepatic glucuronidation of quercetin, both genistein and acetaminophen were not subject to the same influence. Previous animal studies have shown that age-related changes in glucuronidation capacity are substrate dependent. Substrates absent an influence of aging include acetaminophen, bilirubin, p-nitrophenol and estrone, while glucuronidation activity toward morphine, 1-naphthol and testosterone appears to be modified by advancing age [12,26,27]. The lack of a consistent age-related effect in flavonoid glucuronidation activity toward different substrates may arise from the diversity of UGT isoenzymes. Using human recombinant UGT, Boersma et al. [15] demonstrated that several UGT isoenzymes metabolized quercetin with different conversion rates, of which UGT1A9, UGT1A1, UGT1A3, UGT1A8 and UGT2B7 were the most efficient. Consistent with our results, tissue obtained from animals of different ages and with similar net glucuronidation rates but divergent UGT regioselectivity produced different metabolite profiles. For example, Boersma et al. [15] reported that half of the quercetin glucuronides formed by UGT1A3 were the 3'-O- form, while UGT1A9 produced the 7-O- form to the same extent. Liu et al. [28] found that UGT1A1 was primarily responsible for genistein glucuronidation, while the work of Bock et al. [29] indicated that UGT1A6 principally glucuronidated acetaminophen. This diversity of UGTs might also explain the observation by Brett et al. [30] that urinary excretion of hesperitin, but not naringenin, declined with advanced age in humans after drinking orange juice. Therefore, age-related effects on UGT activity toward flavonoid are likely dependent on isoenzyme-specific changes.

As reported in previous studies, incubations of rat liver microsomes yield the 7-, 3'- and 4'-glucuronides of quercetin. However, after quercetin consumption, glucuronides, diglucuronides, mixed sulfates and methyl conjugates are also present in rat tissues [6]. As reviewed by Mackenzie et al. [31], humans and rats have orthologous UGT1A1, UGT1A3, UGT1A5, UGT1A6, UGT1A7, UGT1A8 and UGT1A10. Humans have functional UGT1A4 and UGT1A9, whereas rats do not [31]. The human S9 liver fraction also forms the 3-glucuronide of quercetin, whereas in rat, it is produced in the intestine [32]. The equivalency of genistein or quercetin glucuronidation kinetics between recombinant human and rat UGT has not been established. Although the F344 rat is widely used as an animal model of aging, the

homology of its metabolite profile and UGT enzymes to humans does limit extrapolating our data to humans.

In conclusion, age-related changes in male rat hepatic microsomal glucuronidation of flavonoids were statistically significant but modest in their magnitude. The glucuronidation of genistein decreased with age, although the trend of increasing CL_{int} was small. Quercetin total glucuronidation capacity was constant with age, but young and old rats had different metabolite profiles. Further studies of the impact of age-related changes on specific UGT isoenzymes toward quercetin and other flavonoids are warranted, as more than 15 isoenzymes compose the UGT superfamily. In vivo studies are required to demonstrate the extent to which advancing age alters flavonoid bioavailability, metabolomics and bioactivity. As flavonoids are also metabolized extensively by Phase 2 enzymes in the intestine and kidney, research determining the potential for age-related changes in these organs is required.

Acknowledgments

References

[1] Cutler G, Nettleton J, Ross J, Harnack L, Jacobs Jr D, Scrafford C, et al. Dietary flavonoid intake and risk of cancer in postmenopausal women: the Iowa Women's Health Study. Int J Cancer 2008;123:664–71.

[2] Geleijnse J, Launer L, Hofman A, Pols H, Witteman J. Tea flavonoids may protect against atherosclerosis: the Rotterdam Study. Arch Intern Med 1999;159:2170–4.

[3] Keli S, Hertog M, Feskens E, Kromhout D. Dietary flavonoids, antioxidant vitamins, and incidence of stroke: the Zutphen study. Arch Intern Med 1996;156:637–42.

[4] Holst B, Williamson G. Nutrients and phytochemicals: from bioavailability to bioefficacy beyond antioxidants. Curr Opin Biotechnol 2008;19:73–82.

[5] Chun O, Chung S, Song W. Estimated dietary flavonoid intake and major food sources of U.S. adults. J Nutr 2007;137:1244–52.

[6] Graf B, Ameho C, Dolnikowski G, Milbury P, Chen CY, Blumberg J. Rat gastrointestinal tissues metabolize quercetin. J Nutr 2006;136:39–44.

[7] Day A, Bao Y, Morgan M, Williamson G. Conjugation position of quercetin glucuronides and effect on biological activity. Free Radic Biol Med 2000;29:1234–43.

[8] Stevenson D, Cooney J, Jensen D, Wibisono R, Adaim A, Skinner M, et al. Comparison of enzymically glucuronidated flavonoids with flavonoid aglycones in an in vitro cellular model of oxidative stress protection. In Vitro Cell Dev Biol—Animal 2008;44:73–80.

[9] McLean A, Le Couteur D. Aging biology and geriatric clinical pharmacology. Pharmacol Rev 2004;56:163–84.

[10] van Bezooijen C. Influence of age-related changes in rodent liver morphology and physiology on drug metabolism—a review. Mech Ageing Dev 1984;25:1–22.

[11] Chen CY, Bakhiet R. Age decreased steady-state concentrations of genistein in plasma, liver, and skeletal muscle in Sprague–Dawley rats. Mech Ageing Dev 2006;127:344–8.

[12] Woodhouse K, Herd B. The effect of age and gender on glucuronidation and sulphation in rat liver: a study using paracetamol as a model substrate. Arch Gerontol Geriatr 1993;16:111–5.

[13] Sweeny D, Weiner M. Metabolism of acetaminophen in hepatocytes isolated from mice and rats of various ages. Drug Metab Dispos 1985;13:377–9.

[14] Court M, Duan S, Guillemette C, Journault K, Krishnaswamy S, Von Moltke L, et al. Stereoselective conjugation of oxazepam by human UDP-glucuronosyl-transferases (UGTs): S-oxazepam is glucuronidated by UGT2B15, while R-oxazepam is glucuronidated by UGT2B7 and UGT1A9. Drug Metab Dispos 2002;30:1257–65.

[15] Boersma M, van der Woude H, Bogaards J, Boeren S, Vervoort J, Cnubben N, et al. Regioselectivity of phase II metabolism of luteolin and quercetin by UDP-glucuronosyl transferases. Chem Res Toxicol 2002;15:662–70.

[16] Court M. Isoform-selective probe substrates for in vitro studies of human UDP-glucuronosyltransferases. Methods Enzymol 2005;400:104–16.

[17] Doerge D, Chang H, Churchwell M, Holder C. Analysis of soy isoflavone conjugation in vitro and in human blood using liquid chromatography–mass spectrometry. Drug Metab Dispos 2000;28:298–307.

[18] Court M, Greenblatt D. Molecular basis for deficient acetaminophen glucuronidation in cats. An interspecies comparison of enzyme kinetics in liver microsomes. Biochem Pharmacol 1997;53:1041–7.

[19] Manach C, Williamson G, Morand C, Scalbert A, Remesy C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. Am J Clin Nutr 2005;81:230S–42S.

[20] Le Couteur D, McLean A. The aging liver. Drug clearance and an oxygen diffusion barrier hypothesis. Clin Pharmacokinet 1998;34:359–73.

[21] Patki K, von Moltke L, Harmatz J, Hesse L, Court M, Greenblatt D. Effect of age on in vitro triazolam biotransformation in male human liver microsomes. J Pharmacol Exp Ther 2004;308:874–9.

[22] Chen J, Wang S, Jia X, Bajimaya S, Lin H, Tam V, et al. Disposition of flavonoids via recycling: comparison of intestinal versus hepatic disposition. Drug Metab Dispos 2005;33:1777–84.

[23] Cassidy A, Brown J, Hawdon A, Faughnan M, King L, Millward J, et al. Factors affecting the bioavailability of soy isoflavones in humans after ingestion of physiologically relevant levels from different soy foods. J Nutr 2006;136:45–51.

[24] Faughnan M, Hawdon A, Ah-Singh E, Brown J, Millward D, Cassidy A. Urinary isoflavone kinetics: the effect of age, gender, food matrix and chemical composition. Br J Nutr 2004;91:567–74.

[25] Chen Y, Xie S, Chen S, Zeng S. Glucuronidation of flavonoids by recombinant UGT1A3 and UGT1A9. Biochem Pharmacol 2008;76:416–25.

[26] Galinsky R, Manning B, Kimura R, Franklin M. Changes in conjugative enzyme activity and acetaminophen metabolism in young and senescent male F-344 rats following prolonged exposure to buthionine sulfoximine. Exp Gerontol 1992;27:221–32.

[27] Leakey J, Cunny H, Bazare J, Webb P, Lipscomb J, Slikker W, et al. Effects of aging and caloric restriction on hepatic drug metabolizing enzymes in the Fischer 344 rat. II. Effects on conjugating enzymes. Mech Ageing Dev 1989;48:157–66.

[28] Liu X, Tam V, Hu M. Disposition of flavonoids via enteric recycling: determination of the UDP-glucuronosyltransferase isoforms responsible for the metabolism of flavonoids in intact Caco-2 TC7 cells using siRNA. Mol Pharm 2007;4:873–82.

[29] Bock K, Forster A, Gschaidmeier H, Bruck M, Munzel P, Schareck W, et al. Paracetamol glucuronidation by recombinant rat and human phenol UDP-glucuronosyltransferases. Biochem Pharmacol 1993;45:1809–14.

[30] Brett G, Hollands W, Needs P, Teucher B, Davis B, Brodbelt J, et al. Absorption, metabolism and excretion of flavanones from single portions of orange fruit and juice and effects of anthropometric variables and contraceptive pill use on flavanone excretion. Br J Nutr 2009;101:664–75.

[31] Mackenzie P, Bock K, Burchell B, Guillemette C, Ikushiro S, Iyanagi T, et al. Nomenclature update for the mammalian UDP glycosyltransferase (UGT) gene superfamily. Pharmacogen Genomics 2005;15:677–85.

[32] van der Woude H, Boersma M, Vervoort J, Rietjens I. Identification of 14 quercetin phase II mono- and mixed conjugates and their formation by rat and human phase II in vitro model systems. Chem Res Toxicol 2004;17:1520–30.