Microsomal Quercetin Glucuronidation in Rat Small Intestine Depends on Age and Segment^S

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ABSTRACT:

UDP-glucuronosyltransferase (UGT) activity toward the flavonoid quercetin and UGT protein were characterized in three equidistant small intestine (SI) segments from 4-, 12-, 18-, and 28-month-old male Fischer 344 rats ($n = 8/\text{age}$) using villin to control for enterocyte content. SI microsomal intrinsic clearance of quercetin was increased 3- to 9-fold from 4 months in the proximal and distal SI at 12 and 18 months. Likewise, at 30 μM quercetin, SI microsomal glucuronidation activity was increased with age: 4.8- and 3.9-fold greater at 18 months than at 4 months. Quercetin UGT regioselectivity was not changed by age. The distal SI preferentially catalyzed glucuronidation at the 7-position, whereas the proximal SI produced the greatest proportion of 4'- and 3'-conjugates. Enterocyte UGT content in different SI segments was not consistently

changed with age. In the proximal SI, UGT1A increased 64 and 150% at 12 and 18 months and UGT1A1, UGT1A7, and UGT1A8 were also increased at 12 and 18 months. However, age-related changes in expression were inconsistent in the medial and distal segments. Microsomal rates of quercetin glucuronidation and UGT expression were positively correlated with UGT1A1 content for all pooled samples ($r = 0.467$) and at each age ($r = 0.538\text{--}0.598$). UGT1A7 was positively correlated with total, 7-O- and 3-O-quercetin glucuronidation at 18 months. Thus, age-related differences in UGT quercetin glucuronidation depend on intestinal segment, are more pronounced in the proximal and distal segments and may be partially related to UGT1A1 and UGT1A7 content.

Introduction

Quercetin, a flavonol, displays protective effects against chronic diseases in animal models and clinical trials through multiple mechanisms, e.g., antioxidation and anti-inflammation (Bischoff, 2008;

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Boots et al., 2008). Dietary or supplemental quercetin has low oral bioavailability and is extensively glucuronidated, sulfated, and methylated in intestine and liver with large interindividual variations (Mullen et al., 2006; Moon et al., 2008). These phase II enzyme conjugations facilitate quercetin efflux back to the intestinal lumen before its systematic distribution and play a role in its limited bioavailability (van der Woude et al., 2004). After quercetin consumption, the aglycone is not detectable in rat or human plasma (Graf et al., 2006; Mullen et al., 2006). In non-gastrointestinal tissues of rats, quercetin is present mainly as glucuronide and sulfate conjugates, with less than 10% in the aglycone form (de Boer et al., 2005; Graf et al., 2006). Thus, flavonoid phase II metabolites appear to be critical to biological activities of flavonoids after their consumption.

In a previous study, we reported that in rats advanced age modifies hepatic microsomal quercetin glucuronidation regioselectivity and isoflavone metabolism kinetics (Bolling et al., 2010). Likewise, Handler and Brian (1997) also reported that K_m and V_{max} values for hepatic microsomal glucuronidation of 4-nitrocatechol were increased in senescent Sprague-Dawley rats. Changes to first-pass glucuronidation regioselectivity or capacity with advanced age could affect bioefficacy or bioavailability of flavonoids in elderly individuals. Because older populations are at higher risk for chronic diseases, more work is needed to characterize age-related changes in flavonoid metabolism because flavonoids are commonly consumed in the form of foods and supplements.

Intestinal abundance of UGT has been partially characterized in humans and rodents, but the effect of advanced age on UGT abun-

ABBREVIATIONS: UGT, UDP-glucuronosyltransferase; HPLC, high-performance liquid chromatography; TBST, Tris-buffered saline Tween 20; SI, small intestine; M1, uncharacterized quercetin glucuronide.

dance is unknown. *Ugt1A1*, *1A3*, *1A4*, *1A6*, *1A10*, *2B4*, *2B7*, *2B10*, and *2B15* mRNA have been detected in human small intestine (Strassburg et al., 2000). In Sprague-Dawley rats, the orthologs *Ugt1A1*, *1A2*, *1A3*, *1A5*, *1A6*, *1A7*, *1A8*, *2B6*, *2B8*, *2B12*, and *2B3* mRNA have also been reported and respond differentially to pregnane X receptor and nuclear factor erythroid 2-related factor 2 ligands (Shelby et al., 2003; Shelby and Klaassen, 2006). UGT protein expression is also dependent on small intestine segment. Rat UGT1A1 protein increased along the descent of the small intestine, whereas UGT1A7 protein decreased (Miles et al., 2006). Quercetin glucuronidation has been partially characterized using some recombinant human UGT isoforms (Boersma et al., 2002; Chen et al., 2008). However, the cumulative contribution of all individual UGT isoforms to glucuronidation activity and regioselectivity toward quercetin and other polyphenols has not been characterized. Therefore, the aim of the present study was to characterize the effect of age on glucuronidation activity and regioselectivity toward quercetin in the small intestine of Fischer 344 rats.

Materials and Methods

Chemicals and Supplies. Acetonitrile and methanol were HPLC grade from Thermo Fisher Scientific (Waltham, MA). Immunoblotting, loading, running, Tris-buffered saline Tween 20 (TBST), and transfer buffers were from Boston Bioproducts (Boston, MA). Precast SDS-acrylamide gels, polyvinylidene difluoride membrane, and transfer paper were from Bio-Rad Laboratories (Hercules, CA). The primary antibodies toward rodent UGT1A (C-19), UGT1A1 (V-19), UGT1A5 (F-19), UGT1A6 (D-20), UGT1A7 (E-15), UGT1A8 (T-17), and villin (H-60), secondary antibodies, and luminol reagent were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Human UGT1A1 was from BD Biosciences (San Jose, CA). Quercetin, daidzein, UDP-glucuronic acid, alamethicin, and all other chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Tissue Preparation. Male Fischer 344 rats aged 4, 12, 18, and 28 months ($n = 8/\text{group}$) were obtained from the Aging Rodent Colony of the National Institutes of Health National Institute on Aging (Bethesda, MD). Animals were acclimated at the Jean Mayer USDA Human Nutrition Research Center on Aging (Boston, MA) for 2 weeks and handled according to an approved institutional animal care and use committee protocol. During the acclimation period, rats were housed with a 12-h light/dark cycle and fed standard chow ad libitum. After anesthesia using isoflurane (Aerrane, Baxter, Deerfield, IL), animals were sacrificed by terminal exsanguinations. The gastrointestinal system was removed and rinsed in ice-cold saline. The small intestine (SI) length was measured, and it was divided into three equidistant segments. Small intestine segments were cut lengthwise and rinsed with saline, and mucosa was collected by scraping with a glass slide on an ice-cold surface. Mucosa samples were snap-frozen in liquid nitrogen and stored at -80°C until analyses.

Animals had body masses of 317 ± 28 g (4 months), 360 ± 22 g (12 months), 367 ± 37 g (18 months), and 342 ± 50 g (28 months). SI lengths were 99.1 ± 8.6 cm (4 months), 103.4 ± 8.8 cm (12 months), 105.7 ± 2.8 cm (18 months), and 104.1 ± 9.8 cm (28 months).

Mucosa samples were homogenized in 10 volumes of sucrose buffer (50 mM Tris-HCl, 0.25 M sucrose, pH 7.5) in an ice bath, using a Tekmar Tisumizer (SDT-1810; Tekmar Co., Cincinnati, OH) at 85% power for 2 min. Microsomal and cytosolic fractions in the resulting homogenate were prepared as described previously (Bolling et al., 2010). Microsomes were resuspended in 0.1 M potassium phosphate buffer, pH 7.5, containing 20% glycerol. Protein concentrations in microsomal and cytosolic fractions were determined by a Pierce BCA kit (Thermo Fisher Scientific) and then adjusted to 5 and 1 mg/ml, respectively. Aliquots were stored at -80°C until use.

Kinetics and Individual Rate of Glucuronidation. The glucuronidation kinetics of rat SI microsomes was determined as described previously with slight modifications (Bolling et al., 2010). Microsomal protein (final concentration, 0.2 mg/ml) was preincubated with alamethicin (0.25 $\mu\text{g}/\text{ml}$) at 37°C for 5 min in microcentrifuge tubes containing quercetin previously dried under nitrogen gas. To initiate the reaction, a cofactor solution of UDP-glucuronic acid (final concentration, 5 mM), magnesium chloride (final concentration, 5 mM), and potassium phosphate buffer, pH 7.5 (final concentration, 0.05 mM)

was added 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 of ice-cold methanol containing 33 μM daidzein as an internal standard. After centrifugation at $14,000g$ 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.

SI mucosa microsomes were pooled by age and segment for linearity and kinetic analysis. When microsomes were incubated with 30 μM quercetin for 30 min, total glucuronide formation was linear from 0.05 to 0.4 mg of microsomal protein/ml in 4- and 28-month pooled samples, and with 300 μM quercetin, total glucuronide formation was linear from 0.05 to 0.5 mg of microsomal protein/ml. Likewise, at 0.25 mg of microsomal protein/ml, total glucuronide formation was linear from 0 to 60 min at both quercetin concentrations. The kinetics of quercetin glucuronide formation for each age and SI segment was determined in duplicate using pooled microsomes at 0.2 mg/ml and a 30-min incubation period with concentrations ranging from 4.7 to 300 μM quercetin. We attempted to achieve linearity with shorter incubation periods and less protein content to assay within the direct range of K_m values. However, assay conditions were not linear or resulted in quercetin glucuronide peaks below the limit of quantification.

After kinetic analysis, the quercetin glucuronidation rate of individual microsomal samples from each age and SI segments was determined in triplicate, using 30 and 300 μM quercetin, 0.2 mg of microsomal protein/ml, and a 30-min incubation.

HPLC Analyses of Quercetin Glucuronides. The quercetin glucuronidation products were analyzed using a HPLC method as described previously (Boersma et al., 2002; Bolling et al., 2010). Reconstituted products were injected into a Thermo Finnigan Surveyor HPLC system (Thermo Fisher Scientific) equipped with an autosampler, a UV detector, and a Synergi 10- μm Hydro-RP80 250×4.6 mm column (Phenomenex, Torrance, CA). A gradient of 20 mM phosphate buffer at pH 2.0 and acetonitrile was used to separate quercetin glucuronides (Bolling et al., 2010). Quercetin glucuronides were quantified by integrating the area under the curve of peaks at 340 nm and normalizing to the concentration of the internal standard daidzein that was integrated at 250 nm. Concentrations were determined using standard curves of quercetin, and daidzein subjected to the glucuronidation protocol with the absence of UDP-glucuronic acid. Quercetin was used for quantification of quercetin glucuronides on the basis of the assumption that quercetin glucuronides display the same UV extinction coefficient as quercetin aglycone. The limit of quantification for quercetin was 1 μM .

The four principal quercetin glucuronides produced by rat intestine microsomes were identified as described previously by Boersma et al. (2002) and van der Woude et al. (2004) with retention times of 7-*O*-glucuronide at 13.4 min, 3-*O*-glucuronide at 13.8 min, 4'-*O*-glucuronide at 16.6 min, and 3'-*O*-glucuronide at 17.1 min. An additional quercetin glucuronide product was observed at 12.4 min and was designated M1.

Western Blotting. To further investigate the observed age-related differences in UGT activity toward quercetin, UGT protein content in microsomal samples from 4 and 28 months were measured using Western blotting. Aliquots of microsomal and cytosolic protein were separated on a 4 to 20% SDS-acrylamide gel using electrophoresis at 0.06 A in Tris-glycine-SDS running buffer. Gels were transferred to polyvinylidene difluoride membranes using semidry transfer (Bio-Rad Laboratories). Membranes were then blocked for 1 h at room temperature using 5% nonfat dried milk in TBST buffer, rinsed with TBST, and then incubated at 4°C overnight with primary antibody in 5% nonfat dried milk in TBST buffer. UGT1A, 1A1, 1A5, 1A6, 1A7, and 1A8 antibodies were incubated at 1:200 dilutions for microsomal protein, and villin antibody was incubated at 1:150 dilutions for cytosolic protein. After incubation with primary antibodies, membranes were allowed to return to room temperature, rinsed with TBST, and incubated with secondary anti-goat (UGT) and anti-rabbit (villin) IgG-horseradish peroxidase antibodies at a 1:2500 dilution at room temperature for 1 h. Membranes were rinsed with TBST, incubated with luminol reagent for 3 min, and then exposed to film (X-OMAT Blue XB; Kodak, Rochester, NY) for 3 min (UGT1A), 35 min (UGT1A1, UGT1A8 and villin), or overnight (UGT 1A5, UGT1A6, and UGT1A7) and developed in a darkroom. Densitometry analysis was performed using a GS-710 scanner (Bio-Rad Laboratories) and Quantity One version 4.1.0 software (Bio-Rad Laboratories).

Human UGT1A1 (for UGT1A) or samples with abundant UGT or villin content were used to create standard curves for immunoblotting. Villin is indicative of the enterocyte content of tissue preparations, and UGT protein content was expressed as UGT density/villin density.

Data Analysis. All results are expressed as the mean $\pm$ S.E. The best-fit kinetic models were selected by examining Michaelis-Menten, Lineweaver-Burk, and Eadie-Hofstee plots and by an *F*-test and comparison of Akaike information criterion values, a measure of the goodness of fit of an estimated statistical model, using GraphPad Prism (version 5.01; GraphPad Software Inc., San Diego, CA). The Michaelis-Menten model (eq. 1) and the uncompetitive substrate inhibition model (eq. 2) were used to model data, where *V* is the velocity, *S* is 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 S.E. of the model.

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

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

Logarithmic transformation of data was applied before statistical analysis to normalize unequal variance. Two-way ANOVA was used to test the effect of age and SI segment on quercetin glucuronidation, UGT immunoblotting, and villin concentration, and one-way ANOVA was used for analysis of regioselectivity. When *P* $\leq$ 0.05, post hoc analysis was performed using Tukey's honestly significant difference test. Pearson's correlation analysis was used to determine relationships between rates of glucuronidation and UGT protein expression. Statistical analyses were performed using GraphPad Prism (version 5.01).

Results

Determination of Villin. Villin is a cytoskeleton protein and was used to control for enterocyte content of tissue preparations. Villin was present in the cytosol (Supplemental Fig. 1), but not in the microsomal fraction. Villin was unchanged with age in the proximal and distal SI, but at 28 months was 115% greater than at 4 months in the medial SI (*P* = 0.0003) (Fig. 1). Similar to a previous study (Iizumi et al., 2007), the villin content of mucosa declined along the length of the SI in 4- and 28-month-old rats (*P* = 0.0003). Because villin content apparently varied by preparation, we subsequently normalized UGT activity and immunoblots to villin content. Data that were not normalized to villin are presented in Supplemental Table 1 and Supplemental Figs. 2 and 3.

Microsomal Quercetin Glucuronidation Kinetics. *Production of quercetin glucuronide isoforms.* Quercetin was glucuronidated by intestinal microsomes at the 7-, 3-, 4'-, and 3'-hydroxyl position and to a lesser extent to an uncharacterized glucuronide specified as M1.

Kinetic modeling of quercetin glucuronidation. Pooled SI microsomal UGT activity toward quercetin, as the sum of quercetin metabolites, was better modeled by Michaelis-Menten rather than by uncompetitive substrate inhibition kinetics in most SI segments and ages

FIG. 1. Relative cytosolic villin content of tissue preparations at different ages and rat SI segment. Data are means $\pm$ S.E.M.; *n* = 8/age. Log-transformed data were analyzed by two-way ANOVA. *P* < 0.0003 for section and age.

A Proximal SI

B Medial SI

C Distal SI

FIG. 2. Age-related changes in villin-adjusted kinetics of quercetin glucuronidation, as the sum of all metabolites, by pooled microsomal protein fractions from proximal (A), medial (B), and distal (C) SI segments of male F344 rats. Solid lines indicate that a Michaelis-Menten model and dashed lines indicate that an uncompetitive inhibition model were best fits for data as specified under *Materials and Methods*.

(Fig. 2) on the basis of inspection of Lineweaver-Burk and Eadie-Hofstee plots. Total quercetin glucuronidation by the medial SI at 4 and 28 months and distal SI at 28 months was better modeled by uncompetitive substrate inhibition than by Michaelis-Menten kinetics on the basis of Akaike information criterion values (Supplemental Table 2). Likewise, kinetics of quercetin glucuronide isomer production in the medial and distal SI at 28 months were also better modeled by uncompetitive substrate inhibition. Visual inspections of the plots revealed only slight differences between fitted curves, so for comparative purposes, both kinetic models are presented in Supplemental Tables 2 and 3. *K*_m values for the 7-*O*-glucuronide, 3-*O*-glucuronide, and 4'-*O*-glucuronide at 4 and 12 months in the proximal small

intestine were below the lowest concentration used in the glucuronidation assay, so these results should be interpreted with caution.

Proximal SI quercetin glucuronidation kinetics. In the proximal SI, age-related changes in microsomal quercetin glucuronidation kinetics as the sum of glucuronide isoforms were apparent beginning at 12 months but were most profound at 18 months, with a 13-fold increase in $V_{\max}$ from 4 months (Fig. 2; Supplemental Table 2). In the proximal SI, intrinsic clearance (CL_{int}) increased by 3- and 9-fold at 12 and 18 months compared with 4 months (Supplemental Table 2). K_m values increased by 50% gradually from 4 to 28 months, but were offset by larger increases in $V_{\max}$ values at 12 and 18 months. Increases in CL_{int} at 12 and 18 months followed a similar trend for the 7-, 3-, 4'-, and 3'-O-quercetin glucuronides (Table 1). K_m values peaked at 28 months for 7-glucuronide and at 18 months for the 3- and 4'-glucuronides and at 12 months for the

3'-glucuronide. $V_{\max}$ values for 7-, 3-, 4'-, and 3'-glucuronides declined from 18 to 28 months, but were still 1.7- to 2.8-fold higher than at 4 months.

Medial SI quercetin glucuronidation kinetics. Age-related changes in the medial SI $V_{\max}$ values for UGT activity, as a sum of all quercetin glucuronides, were not as pronounced as in the proximal and distal SI segments (Fig. 2). In the medial SI, total quercetin CL_{int} values increased 1.4-fold at 12 months, were similar at 18 months, but declined at 28 months (Supplemental Table 2). The decline in total quercetin CL_{int} from 18 to 28 months was mainly due to a 68% decrease in $V_{\max}$. $V_{\max}$ values for the 7-, 3-, 4'-, and 3'-glucuronides declined by a magnitude similar to that for total quercetin glucuronides (Table 1). Total quercetin K_m values at 12 months were 50% of those at 4 months but were restored at 28 months. For quercetin glucuronide isomers, similar changes in K_m values were observed (Table 1).

TABLE 1

Villin-adjusted kinetics of pooled microsomal quercetin glucuronidation in the small intestine of male F344 rats

Data are modeled by Michaelis-Menten kinetics and are expressed as the estimate $\pm$ S.E. of duplicate determinations.

Isomer and Segment	Age	$V_{\max}$	K_m	CL_{int}	
	mo	$\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}/\text{villin}$	μM	$\text{ml} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}/\text{villin}$	
7- <i>O</i> -Glucuronide	Proximal	4	0.086 ± 0.007	2.67 ± 0.97	0.0321
		12	0.496 ± 0.038	3.13 ± 1.10	0.159
		18	1.41 ± 0.15	3.16 ± 1.45	0.446
		28	0.330 ± 0.044	3.77 ± 2.15	0.0873
	Medial	4	2.53 ± 0.13	5.34 ± 1.71	0.473
		12	2.99 ± 0.17	2.35 ± 1.19	1.27
		18	4.24 ± 0.23	3.81 ± 1.48	1.11
		28	1.37 ± 0.07	5.10 ± 1.56	0.268
	Distal	4	4.29 ± 0.14	6.06 ± 1.14	0.707
		12	19.3 ± 0.7	13.7 ± 2.3	1.41
		18	20.4 ± 0.5	14.0 ± 1.7	1.46
		28	5.81 ± 0.18	9.45 ± 1.48	0.615
3- <i>O</i> -Glucuronide	Proximal	4	0.039 ± 0.001	0.45 ± 0.12	0.0862
		12	0.202 ± 0.012	0.83 ± 0.30	0.244
		18	0.569 ± 0.062	1.62 ± 0.90	0.352
		28	0.130 ± 0.014	1.58 ± 0.89	0.0825
	Medial	4	0.884 ± 0.053	3.85 ± 1.64	0.229
		12	1.06 ± 0.07	1.51 ± 1.10	0.701
		18	1.46 ± 0.10	2.37 ± 1.40	0.615
		28	0.498 ± 0.024	3.92 ± 1.30	0.127
	Distal	4	1.42 ± 0.06	4.92 ± 1.26	0.288
		12	5.88 ± 0.22	10.8 ± 1.9	0.547
		18	6.08 ± 0.24	10.7 ± 2.0	0.571
		28	1.78 ± 0.08	7.04 ± 1.84	0.253
4'- <i>O</i> -Glucuronide	Proximal	4	0.134 ± 0.007	2.97 ± 0.70	0.0452
		12	0.723 ± 0.080	3.70 ± 1.76	0.1954
		18	1.78 ± 0.15	5.65 ± 1.80	0.3154
		28	0.358 ± 0.041	5.14 ± 2.34	0.0697
	Medial	4	1.56 ± 0.09	15.2 ± 3.8	0.102
		12	1.90 ± 0.11	6.81 ± 2.30	0.279
		18	2.47 ± 0.14	9.12 ± 2.66	0.271
		28	0.819 ± 0.067	15.1 ± 5.3	0.054
	Distal	4	1.65 ± 0.06	13.2 ± 2.2	0.125
		12	4.50 ± 0.27	15.7 ± 4.0	0.286
		18	5.94 ± 0.17	14.0 ± 1.8	0.424
		28	1.92 ± 0.05	14.0 ± 1.5	0.137
3'- <i>O</i> -Glucuronide	Proximal	4	0.146 ± 0.007	8.08 ± 1.36	0.0180
		12	0.763 ± 0.054	12.4 ± 2.8	0.0617
		18	2.01 ± 0.17	10.0 ± 2.9	0.2008
		28	0.426 ± 0.038	10.9 ± 3.2	0.0390
	Medial	4	2.13 ± 0.05	27.6 ± 2.3	0.077
		12	2.75 ± 0.08	15.0 ± 1.9	0.183
		18	3.55 ± 0.12	15.9 ± 2.2	0.223
		28	1.08 ± 0.07	19.3 ± 4.8	0.056
	Distal	4	2.59 ± 0.11	23.1 ± 3.5	0.112
		12	9.76 ± 0.80	37.3 ± 9.6	0.262
		18	12.3 ± 0.5	37.8 ± 5.2	0.326
		28	4.00 ± 0.17	39.9 ± 5.3	0.100

Distal SI quercetin glucuronidation kinetics. In the distal SI, total quercetin $V_{\max}$ values increased by 3- and 3.5-fold at 12 and 18 months compared with values at 4 months, whereas K_m values were increased at least 42% from 12 to 28 months (Supplemental Table 2). The K_m values of 7-, 3-, and 4'-glucuronides peaked at 12 months but declined to 28 months, whereas the 3'-O-glucuronide peaked at 28 months (Table 1). In contrast, $V_{\max}$ values for 7-, 3-, 4'-, and 3'-glucuronides peaked at 18 months, with increases from 2.6- to 3.8-fold from 4 months.

Comparative SI segment quercetin glucuronidation kinetics. Villin-adjusted total quercetin CL_{int} values increased from the proximal to distal SI at each age as a result of increases in $V_{\max}$ values (Supplemental Table 2). Likewise, total quercetin K_m values increased stepwise from the proximal to distal SI in 12-, 18-, and 28-month-old rats but declined by 7% from the medial to distal segment at 4 months. The pattern of proximal > medial > distal SI in $V_{\max}$, K_m , and CL_{int} values was apparent for most quercetin glucuronide isoforms at each age (Table 1). However, K_m values peaked in the medial SI for the 4'-glucuronide at 4 and 28 months and for the 3'-glucuronide at 4 months. The K_m value for the 7-glucuronide was also lowest in the medial SI at 12 months only. Thus, age-related changes in quercetin glucuronidation kinetics in SI were dependent on segment and were most pronounced at 12 and 18 months.

Quercetin Glucuronidation at 30 and 300 μM . After characterization of pooled microsomal kinetics, rates of quercetin glucuronidation were determined in SI segments from each rat at 30 and 300 μM quercetin. These substrate concentrations were in the range of K_m values and 10 times K_m values, respectively, and were linear with respect to time and protein concentration for rate of quercetin glucuronidation. Using this approach, significant age- and segment-related differences in villin-adjusted quercetin-UGT activity (as the sum of all metabolites) were observed at 30 μM quercetin ($P < 0.0001$) (Fig. 3). In all SI segments, rates of quercetin glucuronidation were increased at 12 and 18 months relative to those at 4 months. The greatest increases were observed in the proximal and distal SI at 18 months, in which total quercetin glucuronidation rates increased by 4.8- and 3.9-fold, respectively. The formation of 7-, 3-, 3'-, and 4'-glucuronide isomers increased with age in each SI segment and followed a nearly identical trend with total quercetin glucuronidation at 30 μM (Fig. 4). In the proximal SI, activity at 18 months was 4.3- to 5.5-fold greater than that at 4 months for each glucuronide isoform. In the medial and distal SI, increases were evident beginning at 12 months, 40 to 79% greater than at 4 months. In each segment, the changes in activity with age were of similar magnitude among isoforms, because there was not a significant interaction ($P = 1.0$).

FIG. 3. Villin-adjusted rates of microsomal glucuronidation of 30 μM quercetin by SI segment and age with 0.2 mg of microsomal protein/ml and a 30-min incubation. Data are means of the sum of metabolites $\pm$ S.E.M.; $n = 8/\text{age}$. Log-transformed data were analyzed by two-way ANOVA. $P < 0.0001$ for age and segment, and $P = 0.3050$ for interaction between age and segment.

A Proximal SI, 30 μM Quercetin

B Medial SI, 30 μM Quercetin

C Distal SI, 30 μM Quercetin

FIG. 4. Villin-adjusted rates of glucuronidation of 30 μM quercetin by microsomal fractions collected from mucosa of SI segment with 0.2 mg of microsomal protein/ml and a 30-min incubation. A, proximal; B, medial; C, distal. Data are means $\pm$ S.E.M. of $n = 8/\text{age}$. Log-transformed data were analyzed by two-way ANOVA. gluc, glucuronide.

Significant age-related increases in rates of total quercetin glucuronidation were also observed at 300 μM quercetin ($P = 0.0013$) (Supplemental Fig. 4). As at 30 μM , the formation of different quercetin glucuronide isoforms mirrored total quercetin UGT activity within SI segments (Supplemental Fig. 5).

At each age and quercetin concentration, rates of villin-adjusted glucuronidation increased by descending segment of the SI. For example, at 4 months and 30 μM quercetin, the ratio of UGT activity for proximal/medial/distal SI segments was 1:7:12, whereas at 12 months, this ratio was 1:7:23 and was 1:2:6 at 28 months. Thus, age had a significant impact on relative enterocyte quercetin glucuronidation rates between SI segments.

Tissue Regioselectivity of Quercetin UGT Activity. In all SI segments, there was not a significant interaction between age and formation of quercetin glucuronide isoforms. Thus, data were pooled by age to examine the effect of SI segment on UGT regioselectivity. The relative microsomal rates of formation of the four identified quercetin glucuronide isomers changed along the length of the SI at 30

FIG. 5. Regioselectivity at 30 μ M quercetin in small intestine is independent of age. Data are means $\pm$ S.E.M.; $n = 32$. gluc, glucuronide. ^{a-c}, Means without sharing the same letter in the same metabolite group are different.

and 300 μ M quercetin, but those of M1 were unchanged (Fig. 5). From proximal to distal SI, UGT selectivity toward the 7- and 3-hydroxyl group of quercetin increased, whereas selectivity decreased toward the 4'- and 3'-hydroxyl group. Changes in UGT selectivity toward the 7- and 4'-hydroxyl groups of quercetin from proximal to distal SI were the most extreme, by an increase from 29 to 51% and decline of 11 to 27%, respectively.

Enterocyte UGT Expression. Upon observing age-related increases in enterocyte UGT activity, we further examined UGT protein expression in SI samples by semiquantitative Western blot. UGT1A5 and UGT1A6 proteins were not sufficiently abundant in SI micro-

somal samples for further screening. Standard curves of human UGT1A1 (for UGT1A) or samples with abundant UGT content were used to calibrate data (Fig. 6).

In contrast to quercetin glucuronidation kinetics and activities, enterocyte UGT protein content was not consistently affected by age (Fig. 7). For all antibodies, increases in proximal SI UGT content at 12 and 18 months from 4 months were evident but not in other segments. For UGT1A, there was a significant interaction between age and segment ($P = 0.0012$). Enterocyte UGT1A protein was increased by 64 and 150% at 12 and 18 months from 4 months in the proximal SI but decreased by 48 to 82% at the same time points in the medial and distal SI. At each age, enterocyte UGT1A protein was larger in the proximal SI than in the medial and distal segments. In 4-month-old rats, enterocyte UGT1A1 and UGT1A7 content increased from the proximal to distal SI segment, but not at other ages. These results notwithstanding, distal UGT1A1 and UGT1A7 content was greater than in the proximal or medial segments. UGT1A8 protein was significantly different by segment ($P = 0.0008$), with an interaction between age and segment ($P = 0.0034$), because medial SI content was the greatest at 4 months, whereas in the distal segment, rats at 28 months had 50% greater content relative to that at 4, 12, and 18 months.

Correlation of Microsomal UGT Content with Quercetin Glucuronidation. Correlation of microsomal UGT content with quercetin glucuronidation was performed to provide insight into age-related increases in UGT activity and changes in SI UGT regioselectivity (Supplemental Table 4). Independent of age or SI segment, UGT1A1 protein was positively correlated with total glucuronidation

A UGT1A

B UGT1A1

C UGT1A7

D UGT1A8

FIG. 6. Standard dilution curves and representative Western blots. A, UGT1A using recombinant human UGT1A1 for a standard curve and 37.5 μ g/well of rat proximal SI microsomal protein at different ages. B, UGT1A1 using mixtures of rat SI microsomal samples with high and low responses at 37.5 μ g/well and 37.5 μ g/well distal SI microsomes from 4- and 28-month-old rats. C, UGT1A7 using dilutions of rat SI microsomal samples with a high response and 37.5 μ g/well of proximal SI microsomal protein from 4- and 28-month-old rats. D, UGT1A8 using dilutions of a rat SI microsomal sample with a high response and 37.5 μ g/well of distal SI microsomal protein from 4- and 28-month-old rats.

FIG. 7. UGT1A (A), UGT1A1 (B), UGT1A7 (C), and UGT1A8 (D) enterocyte protein content [(UGT density)/(villin density)] assessed by Western blotting of equidistant small intestine mucosa from 4-, 12-, 18-, and 28-month-old rats. Data were analyzed by two-way ANOVA; $n = 8/\text{age}$. Log-transformed values were used. P values for UGT1A were 0.3064 for age, <0.0001 for segment, and 0.0012 for their interaction, for UGT1A1 were 0.0763 for age, <0.0001 for segment, and 0.0799 for their interaction, for UGT1A7 were <0.0001 for age, <0.0001 for segment, and 0.2668 for their interaction, and for UGT1A8 were 0.9585 for age, 0.0008 for segment, and 0.0034 for their interaction. Representative Western blots and semiquantitative standard curves for each antibody are presented in Fig. 6.

rates at 30 and 300 μM quercetin ($P < 0.001$) with r values of 0.467 and 0.447, respectively. In contrast, UGT1A and UGT1A8 content was significantly negatively correlated ($P < 0.001$) with total glucuronidation rates at both 30 and 300 μM quercetin with r values from -0.305 to -0.412 . Independent of age, UGT1A1 was positively correlated with 30 μM quercetin activity in the medial SI only with r values of 0.404 to 0.475. In contrast, at 300 μM quercetin, UGT1A1 was only correlated in distal SI for total quercetin glucuronidation and 7- and 3-glucuronide rates with r values of 0.398 to 0.467. Independent of SI segment, UGT1A1 was correlated with total quercetin glucuronidation rates with r values from 0.538 to 0.589 at each age. Because pooling SI segment data resulted in stronger correlations (r values of 0.385–0.614) than pooling ages (r values of 0.005–0.475), despite smaller sample size ($n = 24$ versus $n = 32$), UGT1A1 content may partly explain age-related differences in quercetin glucuronidation more so than SI segment differences.

Correlations of isoform-specific glucuronidation rates to UGT content were not largely different from the sum of glucuronide isoforms. However, for UGT1A1 activity, formation of 4'-glucuronide ($r = 0.320$) was more weakly correlated than that for other isoforms ($r > 0.424$). UGT1A7 was significantly correlated ($r = 0.431$) with the 3'-isoform production from 30 μM quercetin in the medial SI, whereas other isoforms were not. However, this relationship was not evident at 300 μM quercetin.

Discussion

Advanced age is known to affect intestinal function, including enterocyte structure and nutrient absorption (Drozdzowski and Thomson, 2006). Yet little is known about age-related differences in intestinal xenobiotic metabolism enzymes. Therefore, we used quercetin, a dietary flavonoid that is extensively glucuronidated upon absorption to evaluate SI microsomal enterocyte UGT activity and protein abundance in young adult to senescent male F344 rats.

Our observation that senescent rats had increased SI microsomal quercetin glucuronidation rates and intrinsic clearance values contrasts with the few animal studies of SI xenobiotic metabolism in advanced age. Phenolphthalein glucuronidation was unchanged with advanced age in intestinal tissues of male rats up to 30 months (Borghoff and Birnbaum, 1985). However, the authors noted that the low specific activity of phenolphthalein glucuronidation in tissues may have lacked the necessary sensitivity to the observed age-related changes. Warrington et al. (2004a,b) found that CYP3A and P-glycoprotein expression was unchanged in male F344 rat intestine, although NADPH reductase expression declined. To our knowledge, this study is the first report of age-related changes to enterocyte glucuronidation capacity or UGT content in rodents.

The use of villin as a control for enterocyte abundance in tissue preparation may have facilitated these observations. Villin is constitutively expressed in enterocytes and localized at the brush border (West et al., 1988). It has been used in other studies to normalize drug-metabolizing enzymes and transporters to enterocyte content of tissue preparations (Lown et al., 1994; Gibbs et al., 1999; Johnson et al., 2001). We could find no published studies to suggest that there are changes in villin content with age. Thus, it is not clear whether our observation is truly an age-dependent difference in enterocyte content of villin or the result of differences in villin-containing enterocytes relative to other cells in the preparations.

The changes in small intestine quercetin glucuronidation rates and kinetics were accompanied by changes in UGT expression. UGT1A1 and UGT1A7 were positively correlated to rates of SI quercetin glucuronidation, particularly when stratified with age. Of interest, Brand et al. (2010) concluded that UGT1A1 and UGT1A7 were the

predominant isoforms responsible for the microsomal glucuronidation of the flavanone hesperetin in rat liver and intestine. The significance of the negative correlations of UGT1A and UGT1A8 to quercetin glucuronidation rates is unclear, and they may suggest differential regulation of total UGT1A and UGT1A1 or UGT1A7. It should also be noted that substrate specificity of the primary antibodies toward UGT has not been extensively validated.

Previous studies of recombinant human UGT have characterized the differential activity of UGT isoforms toward quercetin. UGT1A3 and 1A9 from Sf9 cells have similar K_m values of $\sim 40 \mu\text{M}$, but $V_{\max}$ values of 2 and $10 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, respectively (Chen et al., 2008). Basu et al. (2004) also found that the specific activity for 16-h incubations of UGT from COS kidney cells with quercetin were greatest for UGT1A1, 1A6, 1A7, and 1A10 and lower for UGT1A8 and 1A9. In contrast, 30-min incubations with $100 \mu\text{M}$ quercetin demonstrated that UGT1A1, 1A3, 1A8, 1A9, and 2B7 had greater rates of glucuronidation than did UGT1A4, 1A6, 1A10, and 2B15 (Boersma et al., 2002). Differences in $V_{\max}$ and K_m values between UGT isoforms could lead to their increased or decreased glucuronidation activity toward substrates assessed at different test concentrations (Tang et al., 2009). In this study, correlations of UGT content to glucuronidation at 30 and $300 \mu\text{M}$ quercetin were generally similar. Of note, independent of age, UGT1A1 was positively correlated with $300 \mu\text{M}$ glucuronidation in the distal SI only, whereas $30 \mu\text{M}$ was correlated to the medial SI only. Comprehensive kinetic profiles of rat recombinant UGT isoforms toward quercetin are necessary to predict the contributions of UGT isoforms to age-related changes in quercetin glucuronidation.

The mechanism for the age-related changes in enterocyte UGT content and capacity needs further definition. It is plausible that increased oxidative stress or inflammation along with advance of aging might induce UGT expression. The intestine-specific transcription factor caudal-related homeodomain protein 2 and hepatocyte nuclear factor activate promoters for UGT1A8, 1A9, and 1A10 in human Caco-2 cells (Gregory et al., 2004). In *Drosophila*, both oxidative stress and age up-regulated intestinal caudal expression via the nuclear factor- κB pathway (Choi et al., 2008). In contrast, pharynx *ugt-9* gene expression is down-regulated in normal aging in *Caenorhabditis elegans* through erythroid-like transcription factor-3, but up-regulated through erythroid-like transcription factor-3 upon exposure to oxidative stress through paraquat (Budovskaya et al., 2008). Pregnane X receptor can modulate *Ugt1A2* mRNA expression in duodenum of rats (Shelby and Klaassen, 2006) but did not vary with age in the mouse liver (Echchgadda et al., 2004). Long-lived mice were found to have increased hepatic UGT1A9 mRNA expression regulated by bile acids through farnesoid X receptor (Amador-Noguez et al., 2007), but the impact of farnesoid X receptor on SI UGT expression has not been defined. Thus, whereas oxidative stress associated with aging may differentially regulate UGT isoforms, more work is needed to elucidate the pathways responsible for differences in enterocyte UGT in rats of older age.

Although human studies of intestinal glucuronidation in elderly individuals are lacking, in vitro results from liver bank studies indicate that advanced age makes little to no difference in the hepatic microsomal glucuronidation of valproate (Argikar and Remmel, 2009), *S*-oxazepam, trifluoperazine, serotonin, propofol, zidovudine (Court, 2010), and 4-methylumbelliferone (Parkinson et al., 2004). In rats, hepatic microsomal glucuronidation of acetaminophen was also unchanged with advanced age (Sweeny and Weiner, 1985; Woodhouse and Herd, 1993). However, we observed modest changes in quercetin and genistein microsomal glucuronidation in livers of F344 rats of advanced age (Bolling et al., 2010) and increased steady-state tissue

concentrations of genistein in older Sprague-Dawley rats (Chen and Bakhiet, 2006). Age-related diseases could also modify UGT activity, because arthritic rats had decreased hepatic microsomal rates of *p*-nitrophenol glucuronidation but not bilirubin and reduced $V_{\max}$ values of *R*- and *S*-ketoprofen glucuronidation (Meunier and Verbeeck, 1999).

We and others have reported that glucuronidation in the small intestine plays a key role in quercetin metabolism in rats (Graf et al., 2006; Mullen et al., 2008). Our results further confirm the importance of this organ for first-pass metabolism of flavonoids through glucuronidation. Prolonged quercetin feeding to F344 rats resulted in mainly mono-, di-, or mixed glucuronides or quercetin in intestinal tissue (Graf et al., 2006). In Sprague-Dawley rats, the primary metabolites of quercetin 4'-glucoside in intestinal tissue were also glucuronides (Mullen et al., 2008). We also found that SI microsomal quercetin CL_{int} was similar to that of the liver of F344 rats of our previous study (Bolling et al., 2010). However, the glucuronidation capacity of human intestinal microsomes could be more than 1-fold larger than that in rats (Boersma et al., 2002).

Species- and organ-specific UGT regioselectivity probably affects flavonoid bioefficacy, because the position of quercetin glucuronidation affects its bioactions, e.g., inhibition of lipoxygenase and xanthine oxidase (Day et al., 2000). Quercetin glucuronidation regioselectivity in vivo has not been quantitatively assessed, in part because of the lack of suitable standards. Our in vitro study showed that regioselectivity of microsomal quercetin glucuronidation in SI is segment-dependent, possibly due to differential expression of isoforms. For example, human recombinant UGT1A1 catalyzed mainly the 3'-hydroxyl quercetin glucuronidation, whereas UGT1A6 preferentially catalyzed the 4'- and 7-hydroxyl moieties (Boersma et al., 2002). In addition, such regioselectivity of UGT isoforms is dependent on flavonoid subfamilies, e.g., human recombinant UGT1A1 mainly catalyzed the 7-hydroxyl position of the flavanone hesperetin and UGT1A7 selectively glucuronidated the 3'-hydroxyl position (Brand et al., 2010). Furthermore, the regioselectivity of flavonoid glucuronidation in intestine is species-dependent, with 7% quercetin conjugated at the 7-hydroxy moiety in humans and 41% at this position in rats (Boersma et al., 2002). Differences between quercetin glucuronide profiles in rodents and humans probably derive from regulatory and nucleotide differences in UGT isoforms (Mackenzie et al., 2005). Of interest, 7-quercetin glucuronide isoforms were not found in human plasma (Day et al., 2001) despite production of 7-*O*-glucuronide in human liver microsomes (Boersma et al., 2002).

The potential impact of age-related changes on SI UGT expression and quercetin glucuronidation on in vivo bioavailability is unknown. Mizuma (2009) reported that extensive intestinal glucuronidation of raloxifene accounts for its low oral bioavailability. Quercetin likewise has low oral bioavailability (Moon et al., 2008), which may arise from its extensive intestinal glucuronidation. Wang et al. (2006) found that rat intestinal microsomal CL_{int} , $V_{\max}$, and K_m values for glucuronidation of the isoflavones were unrelated to rates of glucuronidation in an intestinal perfusion model. Data from clinical trials of flavonoid metabolism using a wide range of ages are lacking, but interestingly, in a study of 129 subjects consuming orange juice, urinary excretion of hesperetin was decreased with advanced age, equating to a 4% decrease between the ages of 20 and 80 years (Brett et al., 2009). Although the mechanism of the decrease in urinary excretion remains to be explored, age could be considered as a potential, albeit weak, modifier of flavonoid metabolism.

In conclusion, age-related differences in UGT quercetin glucuronidation activity in male F344 rats depend on intestinal segment. The rates of villin-adjusted SI microsomal quercetin glucuronidation were

increased from 4 to 12 to 28 months, accompanied by increases in K_m and V_{max} values. Age did not alter UGT regioselectivity toward quercetin in SI, whereas differential expression of UGT isoforms between segments could enable the formation of divergent glucuronide metabolite profiles. Given that only UGT1A1 and UGT1A7 content were positively correlated with age-specific quercetin glucuronidation, age could potentially affect expression of intestinal UGT isoforms. Because UGT isoforms displayed varied substrate specificity, our finding with quercetin is not readily extrapolatable to other UGT substrates. Thus, more work is needed to characterize the degree by which advanced age affects intestinal glucuronidation of other xenobiotics.

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Authorship Contributions

Participated in research design: Bolling, Court, Blumberg, and Chen.

Conducted experiments: Bolling and Chen.

Contributed new reagents or analytic tools: Court.

Performed data analysis: Bolling, Court, and Chen.

Wrote or contributed to the writing of the manuscript: Bolling, Court, Blumberg, and Chen.

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