Hepatic and Renal Conjugation (Phase II) Enzyme Activities in Young Adult, Middle-Aged, and Senescent Male Sprague-Dawley Rats (43259)

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Abstract. **Acetaminophen (APAP)-induced nephrotoxicity is age dependent in male Sprague-Dawley rats: nephrotoxicity occurs at lower dosages of APAP in 12- to 14-month olds compared with 2- to 3-month olds. The mechanisms responsible for enhanced nephrotoxicity in 12-month-old Sprague-Dawley rats are not entirely clear, but may be related to age-dependent differences in APAP metabolism in liver and/or kidney. Major pathways of hepatic APAP metabolism include sulfation and glucuronidation; glutathione conjugation represents a pathway for detoxification of reactive oxidative APAP metabolites. The present studies were designed to quantify *in vitro* activity of three Phase II enzyme activities: glutathione S-transferase using 1-chloro-2,4-dinitrobenzene as substrate, UDP-glucuronyl transferase using APAP as substrate, and sulfotransferase using APAP as substrate, in subcellular fractions of liver and kidney of 3-, 12-, 18-, and 30-month-old naive male Sprague-Dawley rats. In liver, glutathione S-transferase, UDP glucuronyl transferase, and sulfotransferase activities were not significantly different in rats from 3 through 30 months of age. Renal UDP glucuronyl transferase and sulfotransferase activities were similar in rats from 3 through 30 months of age. In contrast, renal glutathione S-transferase activity was characterized by a lower K_m in 12- and 30-month olds when compared with 3-month olds. These data suggest that the reduced total systemic clearance of APAP in 12-month-old male Sprague-Dawley rats previously observed cannot be attributed to age-dependent differences in hepatic APAP metabolism. In addition, it is unlikely that differences in renal APAP metabolism contribute to age-dependent APAP nephrotoxicity.** [P.S.E.B.M. 1991, Vol 197]

Middle-aged and senescent male rats are more susceptible than young adult rats to kidney damage due to several compounds (1–3). For example, acetaminophen (APAP)-induced nephrotoxicity is exacerbated in middle-aged Sprague-Dawley (SD) and middle-aged and senescent Fischer-344 (F-344) rats (4–8). The mechanisms contributing to enhanced susceptibility to APAP-induced nephrotoxicity in aging rats are not entirely clear. Previous studies identified age-dependent differences in APAP pharmacokinetics between 3- and 12-month-old male SD rats (5). Specifically, APAP volume of distribution and total systemic clearance were significantly reduced in 12-month-old compared with 3-month-old male SD rats, resulting in higher plasma APAP concentrations in the middle-aged rats (5). However, at equivalent plasma APAP concentrations in 3- and 12-month olds, nephrotoxicity was exacerbated in middle-aged compared with young adult male SD rats (5), suggesting that factors other than differences in plasma APAP concentrations were contributing to age-dependent nephrotoxicity.

APAP pharmacokinetics are nonlinear due to the presence of multiple, saturating pathways: half-life is prolonged and total systemic clearance is reduced as APAP plasma concentrations are increased (9). The observed reduction in APAP total systemic clearance in middle-aged rats (5) may also be related, at least in part, to age-dependent differences in hepatic glucuron-

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idation and/or sulfation, the two principle metabolic pathways involved in systemic clearance of APAP. Thus, the study presented here was designed to evaluate age-dependent effects on hepatic glucuronidation and sulfation of APAP, and, in addition, glutathione conjugation, as it represents an important pathway for detoxification of reactive APAP metabolites generated by oxidative metabolism (9). Age-dependent effects on renal Phase II enzyme activities important in APAP metabolism were also evaluated to test the hypothesis that intrarenal detoxification capacity (via conjugation) was altered in aging male SD rats.

Materials and Methods

Animals. Male SD rats (200–250 g body weight, approximately 2 months old) were obtained at various times from Charles River Breeding Laboratories (Wilmington, DE) and maintained at SmithKline & French Laboratories until 3, 12, 18, and 30 months of age. Orders were staggered so that tissue was obtained from rats of all four ages on each experimental day. Rats were housed in stainless steel or plastic cages in ventilated rooms with controlled humidity (50 ± 10%), temperature (21 ± 3°C), and a 12-hr light/dark cycle and were allowed free access to food (Purina Rodent Chow 5002; Purina, St. Louis, MO) and water. Rats were examined periodically by veterinarians at SmithKline & French for general good health and appearance. Rats used in the present experiments were in apparent good health before the study.

***In Vitro* Hepatic and Renal Phase II Reactions.** Animals were killed by cervical dislocation and decapitation. Kidneys and livers were excised quickly and placed in ice-cold 1.15% KCl. Renal inner medulla and papilla were discarded. Renal cortex and liver were minced and homogenized in 3 vol of 100 m*M* phosphate buffer (pH 7.4) containing 250 m*M* sucrose. Kidney and liver homogenates were centrifuged at 10,000*g* for 20 min. The resulting supernatant was centrifuged at 105,000*g* for 60 min. The 105,000*g* supernatant (cytosol) was used for the determination of glutathione *S*-transferase and sulfotransferase activity (see below). The microsomal fraction (pellet) was resuspended in phosphate-buffered sucrose (pH 7.4) and centrifuged at 105,000*g* for 60 min. The resulting microsomal fraction was resuspended in 0.5 vol (kidney) or 1 vol (liver) phosphate-buffered sucrose (pH 7.4) to a final concentration of 10–20 mg protein/ml. The microsomal fraction was used for the determination of UDP-glucuronyl transferase activity (see below). Protein was determined according to the method of Lowry *et al.* (10) using bovine serum albumin as a standard.

Glutathione *S*-transferase (GST) activity was determined in cytosol from liver and kidney (11). Reaction mixtures contained 5 m*M* reduced glutathione and 0.01–1 m*M* 1-chloro-2,4-dinitrobenzene (CDNB) as cosubstrates. The reaction was initiated by adding 10–20 μg of hepatic protein or 25–75 μg of renal protein. Changes in absorbance were monitored at 25°C using a Beckman DU6 spectrophotometer at a wavelength of 340 nm. Reactions were run in duplicate at each CDNB concentration. Nonenzymatic conjugation of CDNB was determined by monitoring changes in absorbance of the reaction mixture without cytosol. A molar extinction coefficient of 8.5 mM^{-1} cm^{-1} for the glutathione conjugate of CDNB was used to convert absorbance to enzyme activity units (11). Preliminary experiments indicated that the rate of CDNB conjugation was linear with respect to both time and protein concentration in cytosol prepared from kidneys and livers of rats of all four ages.

UDP-glucuronyl transferase activity was determined in microsomal preparations as described by Rush and Hook (12) using APAP as substrate. Reaction mixtures contained final concentrations of 5 m*M* UDP-glucuronic acid, 10 m*M* $MgCl_2$, 0.05% Triton X-100, and 1–25 m*M* APAP in a total volume of 500 μl of phosphate-buffered sucrose (pH 7.4). The reaction was started by addition of microsomes (1–2 mg of protein). Samples were incubated at 37°C under air in a Dubnoff metabolic incubator for 60 min. Reactions were stopped by addition of 125 μl of 70% perchloric acid. Samples were centrifuged and APAP and APAP-glucuronide analyzed by high-performance liquid chromatography (HPLC) (see below). Reactions were run in duplicate at each APAP concentration. Tissue blanks contained the complete reaction mixture with 25 m*M* APAP and were stopped with 70% perchloric acid before incubation with microsomes. In preliminary experiments, APAP glucuronidation was determined to be linear with respect to both time and protein concentration in renal and hepatic microsomes prepared from rats of all four ages.

Sulfotransferase (ST) activity was determined in cytosol from liver and kidney (13). The reaction mixture contained 100 μ*M* phosphoadenosine-phosphosulfate, 0.5 m*M* mercaptoethanol, and 0.1–5 m*M* [ring-^{14}C]APAP (7.3 mCi/mmol; Pathfinder Laboratories, St. Louis, MO); the reaction was initiated by addition of 1–2 mg of hepatic or renal protein. Samples were incubated at 37°C under air in a Dubnoff metabolic incubator for 30 min. Reactions were stopped by addition of 25 μl of 2 *M* acetic acid and protein precipitated by boiling the samples for 30 sec. Sulfation of APAP was determined by radiochemical detection of the sulfate conjugate (APAP-SO_3) after HPLC separation (see below). Tissue blanks contained the complete reaction mixture with 5 m*M* APAP and were stopped before incubation with cytosol. In preliminary experiments, APAP sulfation was determined to be linear with respect to both time and protein concentration in

renal and hepatic cytosol prepared from rats of all four ages.

Enzyme kinetics in kidney and liver were analyzed according to the method of Lineweaver and Burk (14). Double-reciprocal plots were constructed using 10–12 individual reaction rates (six substrate concentrations run in duplicate) for each rat and tissue. The Michaelis constant (K_m) was calculated from the abscissa intercept and the maximal reaction rate (V_{max}) from the ordinate intercept of Lineweaver-Burk plots of least-squares regression equations.

Analytical Methods. APAP and APAP-glucuronide were determined by HPLC by modification of the method of Jung (15) as described previously (5). The mobile phase consisted of 50 m*M* sodium sulfate (pH 2.2)/4% acetonitrile at a flow rate of 1.5 ml/min. Retention times for APAP-glucuronide and APAP were 6 and 11 min, respectively. APAP and APAP-glucuronide were quantified by extrapolation from peak area calibration curves of synthesized standards. No interfering peaks were detected in reaction mixtures stopped before incubation.

APAP and APAP-SO_3 were determined according to the method of Corcoran (16). The mobile phase consisted of 50 m*M* sodium acetate (pH 4.4):methanol in a ratio of 92:7 at a flow rate of 1.3 ml/min. Retention times for APAP-SO_3 and APAP by HPLC were 8 and 12 min, respectively. Radiochemical detection after HPLC separation was necessary to quantify the small amounts of APAP-SO_3 formed by hepatic and renal cytosol. Column effluent was collected using an automatic fraction collector (ISCO, Inc., Lincoln, NE). Radioactivity was determined by liquid scintillation techniques after addition of 1 min effluent fractions (1.3 ml) to 10 ml of Aquasol-2. Radioactivity was determined in each fraction (15 total) of at least one sample injection to verify the exact elution times of APAP and APAP-SO_3. For subsequent injections, three or four fractions including and surrounding the APAP-SO_3 peak were counted for radioactivity. Radioactivity in fractions collected after HPLC separation of tissue blank mixtures (reaction stopped before incubation) was subtracted from corresponding fractions of reaction mixtures to correct for background radioactivity.

Statistics. All data are expressed as mean ± SE. Data were analyzed by the General Linear Models procedure followed by least-squares means test (17). The 0.05 level of probability was used as the criterion of significance.

Results

Body weights at 12 and 18 months of age were significantly greater than at 3 months of age (Table I). Absolute liver weight followed a pattern similar to body weight: liver weight was significantly increased at 12 and 18 months of age compared with 3-month olds (Table I). Liver weight as a percentage of body weight was significantly reduced compared with 3-month olds only at 18 months of age (Table I). Absolute kidney weight was significantly increased at 18 and 30 months of age compared with 3-month olds (Table I). Kidney weight as a percentage of body weight was not significantly different between 3-month olds and other ages (Table I). Despite differences in absolute organ weights, protein concentrations (per g wet weight) of kidney and liver cytosol and microsomal preparations were similar in rats of all ages (data not shown).

There were no age-dependent differences in hepatic GST activity (Fig. 1A). K_m and V_{max} for *in vitro* hepatic CDNB conjugation were similar in male SD rats from 3 through 30 months of age (Table II). Rates of renal CDNB conjugation were similar in male SD rats at 3 and 12 months of age but tended to be reduced in 30-month olds compared with 3-month olds (Fig. 1B). Age-dependent differences in K_m for renal GST activity toward CDNB were observed: K_m was significantly decreased in 12- and 30-, compared with 3-month olds (Table II). V_{max} for renal GST activity was significantly reduced in 30- compared with 3-month olds (Table II). Age-dependent differences in V_{max} for renal GST activity were not observed in 3- through 18-month olds (Table II). Renal K_m was significantly greater than hepatic K_m at 3, 18, and 30 months of age (Table II). V_{max} for renal GST activity ranged from 34% (at 30 months) to 56% (at 12 months) of hepatic GST activity (Table II).

No consistent age-dependent differences were observed in APAP glucuronidation *in vitro* by liver or kidney microsomes (Fig. 2). Rates of UDP glucuronyl transferase activity in liver and kidney did not appear to saturate at APAP concentrations up to 25 m*M* (Fig. 2). Higher concentrations of APAP could not be tested due to limited solubility of APAP under the conditions of the assay. Since the reactions did not conform to Michaelis-Menton assumptions, kinetic values are not reported.

Hepatic ST activity toward APAP *in vitro* was similar in rats from 3 through 30 months of age (Fig. 3). In addition, K_m and V_{max} values for hepatic ST activity were similar in all age groups (Table III). In contrast, Lineweaver-Burk transformation and regression analysis of renal ST rates for each rat yielded lines with slopes that were not significant, and Michaelis-Menton values are not reported. However, the rates of renal APAP-SO_3 formation did not differ significantly with age at each substrate concentration (Table IV), suggesting that renal ST activity did not differ with age.

Discussion

APAP nephrotoxicity is age dependent in male SD and F-344 rats: middle-aged (12- to 14-month-old) male rats exhibit significant increases in blood urea nitrogen

Table I. Body, Liver, and Kidney Weights in Male Sprague-Dawley Rats of Different Ages

Age (months)	Body weight (g)	Liver weight (g)	$\frac{\text{Liver weight}}{\text{Body weight}} \times 100$	Kidney Weight (g)	$\frac{\text{Kidney weight}}{\text{Body weight}} \times 100$
3	406 ± 43[a]	17.0 ± 0.7	4.4 ± 0.6	3.5 ± 0.2	0.90 ± 0.09
12	775 ± 78	25.0 ± 1.6[b]	3.3 ± 0.3	4.9 ± 0.2	0.65 ± 0.05
18	887 ± 99[b]	26.1 ± 2.1[b]	3.1 ± 0.4[b]	5.6 ± 0.3[b]	0.67 ± 0.08
30	571 ± 46	22.0 ± 2.3	3.9 ± 0.5	6.0 ± 1.0[b]	1.06 ± 0.18

[a] Values represent mean ± SE of four observations.
[b] Significantly different from 3-month-old.

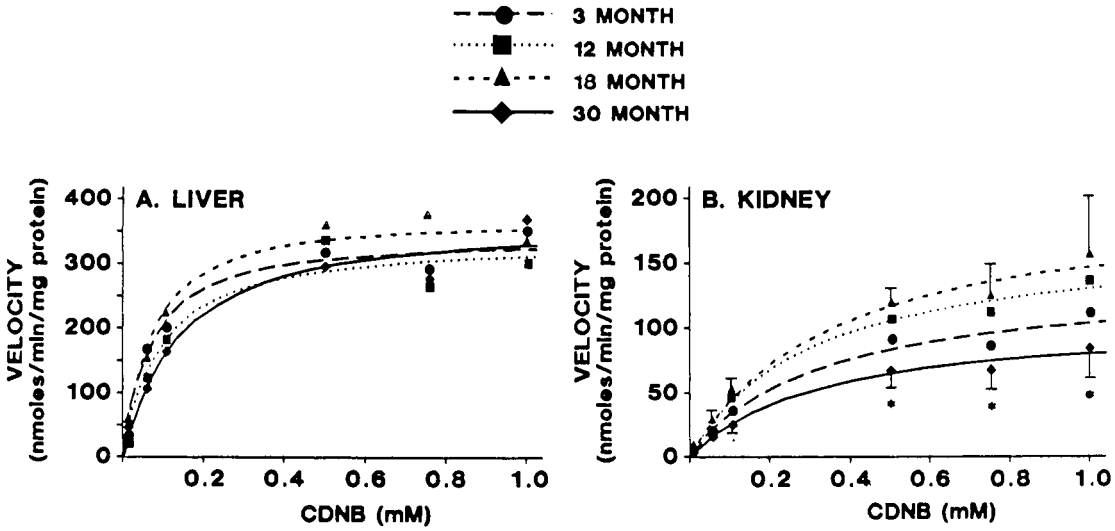

Figure 1. Rates of CDNB conjugation with GSH *in vitro* as a function of substrate concentration by hepatic (A) and renal (B) cytosol from male Sprague-Dawley rats of different ages. Lines were drawn using the calculated K_m and V_{max} for each age group. Values represent means of three to four observations. For liver, bars representing SE have been omitted for the sake of clarity. For kidney, SE bars have been omitted from reaction rates in 3- and 12-month old rats. Asterisks indicate points that are significantly different from reaction rates in 3-month-old rats.

Table II. K_m and V_{max} for *In Vitro* Hepatic and Renal GST Activity from Male Sprague-Dawley Rats of Different Ages[a]

Age (months)	Liver		Kidney	
	K_m (m*M*)	V_{max} (nmol/min/mg protein)	K_m (m*M*)	V_{max} (nmol/min/mg protein)
3	0.074 ± 0.015[b]	353 ± 104	0.492 ± 0.091[c]	199 ± 19
12	0.129 ± 0.041	316 ± 24	0.278 ± 0.068[d]	177 ± 34
18	0.083 ± 0.010	400 ± 60	0.346 ± 0.101[c]	192 ± 28
30	0.065 ± 0.014	285 ± 93	0.242 ± 0.068[c,d]	96 ± 30[c,d]

[a] GST activity was determined by conjugation of CDNB as described in Materials and Methods. Kinetic values (K_m and V_{max}) were calculated for individual rats and tissues following Lineweaver-Burk transformation of the data.
[b] Values represent mean ± SE of three to four observations.
[c] Significantly different from liver.
[d] Significantly different from 3-month-old.

concentration and proximal tubular necrosis at lower dosages of APAP than do young adult (2- to 3-month-old) males (4–8). Differences in APAP pharmacokinetics, specifically reduced volume of distribution and total systemic clearance of APAP in middle-aged compared with young male SD rats, may contribute, at least in part, to enhanced APAP nephrotoxicity in middle-aged SD rats (5).

Major pathways of APAP metabolism include glucuronidation and sulfation (9). A minor pathway of APAP metabolism with major toxicologic impact involves oxidative metabolism via cytochrome P-450-dependent mixed function oxidases followed by conjugation of a reactive quinonimine intermediate with glutathione (9). In a previous study, total systemic clearance of APAP was significantly reduced and neph-

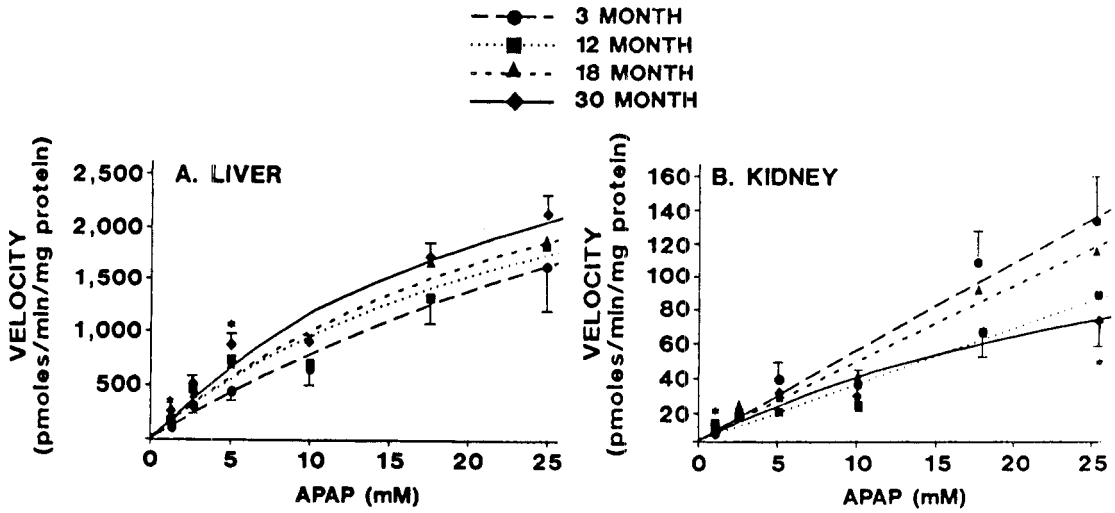

Figure 2. Rates of APAP conjugation by UDP glucuronyl transferase *in vitro* as a function of substrate concentration by hepatic (A) and renal (B) microsomes from male Sprague-Dawley rats of different ages. Lines were drawn using the calculated apparent K_m and V_{max} for each age group. Values represent means of three to four observations. Bars representing SE have been omitted from reaction rates for 12- and 18-month-old rats for the sake of clarity. Asterisks indicate points that are significantly different from reaction rates in 3-month-old rats.

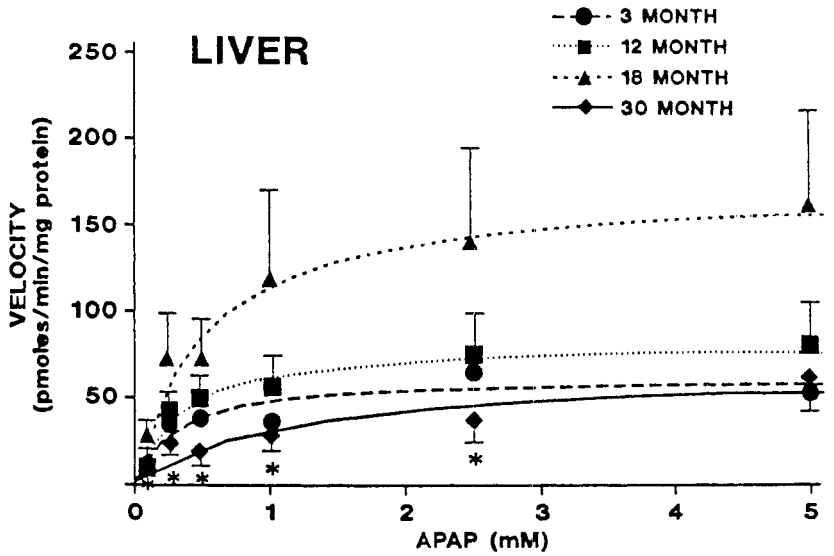

Figure 3. Rates of APAP conjugation by hepatic ST *in vitro* as a function of substrate concentration in male Sprague-Dawley rats of different ages. Lines were drawn using the calculated K_m and V_{max} for each age group. Values represent means of three to four observations. Bars representing SE have been omitted from reaction rates in 3-month-old rats for the sake of clarity. Asterisks indicate points that are significantly different from reaction rates in 3-month-old rats.

rotoxicity was exacerbated in 12- to 14-month-old compared with 3- to 4-month-old rats (5). Reduced total systemic clearance could contribute to age-dependent APAP nephrotoxicity by exposing the kidneys to proportionately higher intracellular concentrations of APAP in older rats. Since sulfation and glucuronidation represent the major pathways of hepatic APAP metabolism, the observed reductions in total systemic clearance (5) may be related to age-dependent reductions in hepatic Phase II enzyme activities.

In the present study, age-dependent differences for hepatic glucuronyl transferase activity toward APAP were not observed (Fig. 2). The inability to saturate glucuronidation (Fig. 2) was unexpected; previous investigators have reported an apparent K_m for APAP of 10 m*M* *in vitro* and 0.9 m*M* *in vivo* (18, 19). Strain differences in glucuronyl transferases or differences in methods of microsomal preparation may contribute to differences observed in the present study with regard to the apparent K_m of APAP with glucuronyl transferases. Even though saturation of glucuronyl transferases was not achieved, it is apparent that hepatic glucuronidation of APAP was unaltered with age (Fig. 2). Age-dependent changes in hepatic ST activity, with APAP as substrate, were not observed, and, in fact, activity tended to be enhanced in middle-aged (18-month-old) animals (Fig.

Table III. K_m and V_{max} for *In Vitro* Hepatic ST Activity from Male Sprague-Dawley Rats of Different Ages[a]

Age (months)	Liver	
	K_m (m*M*)	V_{max} (pmol/min/mg protein)
3	0.44 ± 0.05[b]	84.4 ± 57.8
12	0.39 ± 0.08	90.9 ± 51.3
18	0.46 ± 0.11	186.3 ± 118.3
30	0.63 ± 0.18	46.1 ± 12.1

[a] Sulfotransferase activity was determined by conjugation of APAP as described in Materials and Methods. K_m and V_{max} values were calculated for individual rats following Lineweaver-Burk transformation of the data.
[b] Values represent mean ± SE of three to four observations.

Table IV. Rates of *In Vitro* Renal APAP Sulfation in Male Sprague-Dawley Rats of Different Ages[a]

APAP (m*M*)	APAP-SO_3 formation (pmol/min/mg protein)			
	3-month	12-month	18-month	30-month
0.10	2.3 ± 0.6[b]	3.9 ± 0.8	4.0 ± 0.9	4.2 ± 2.1
0.25	9.2 ± 3.9	3.5 ± 1.1	9.9 ± 3.0	7.5 ± 5.6
0.50	8.8 ± 2.7	3.1 ± 0.9	6.3 ± 2.5	3.1 ± 9.2
1.00	14.0 ± 5.4	ND[c]	23.4 ± 10.3	ND
2.50	26.0 ± 8.9	6.3 ± 4.8	51.8 ± 24.3	ND
5.00	48.0 ± 15.5	31.9 ± 7.8	42.4 ± 21.6	37.9 ± 8.8

[a] Sulfotransferase activity was determined by conjugation of APAP as described in Materials and Methods.
[b] Values represent mean ± SE of three to four observations.
[c] ND = not detectable.

3 and Table IV). This observation is in agreement with previous reports of modestly increased ST activity in middle-aged (12- through 24-month-old) male F-344 and SD rats toward several substrates (20–22). Age-dependent differences in hepatic GST activity, using CDNB as substrate, were not observed (Fig. 1 and Table II). Thus, the reduction in total systemic clearance of APAP observed previously (5) cannot be attributed to age-dependent differences in hepatic metabolism. Rather, the reduced total systemic clearance of APAP observed in 12- to 14-month-old rats (5) is more likely secondary to a reduction in APAP volume of distribution in 12- to 14-month-old rats, leading to elevated plasma APAP concentrations. Since major pathways of APAP metabolism are saturated at high plasma concentrations, APAP systemic clearance is reduced, as would be expected for any process involving saturation of metabolism.

In agreement with the present results, no age-dependent reductions in *in vitro* hepatic GST activity toward CDNB have been observed in male SD rats from 6 through 24 months of age (T. Leonard, personal communication). In contrast to SD rats, hepatic GST activity toward CDNB was significantly increased in 12-month-old male and female F-344 rats, largely due to increased V_{max} (23). In 24-month-old male F-344 rats, V_{max} remained elevated while K_m was also increased, indicating a shift toward lower affinity GST isozymes with senescence (23). Thus, strain differences between male SD and F-344 rats appear to exist for age-dependent differences in hepatic GST activity.

Age-dependent differences in renal Phase II activity and hence, in detoxification capacity, also may contribute to age-dependent APAP nephrotoxicity. In a previous study, at equivalent plasma APAP concentrations, APAP-induced nephrotoxicity remained exacerbated in middle-aged compared with young adult male SD rats (5), suggesting that intrarenal APAP metabolism may be altered in older rats.

In the present study, significant age-dependent differences were not observed in renal glucuronyl transferase or sulfotransferase activities toward APAP (Fig. 2 and Tables III and IV). Renal APAP glucuronidation tended to be reduced in 12- and 30-month-old rats only at high APAP concentrations (>15 m*M*) (Fig. 2). Nephrotoxicity occurs in 12-month-old SD rats at plasma and renal cortical APAP concentrations of about 5 m*M* (5). Thus, age-dependent differences in renal or hepatic glucuronidation were not observed at APAP concentrations that are relevant to *in vivo* observations. Renal ST activity has been less well-investigated than hepatic ST activity. In the present study, the ability to detect renal ST activity was extremely variable. However, the rates of APAP-SO_3 formation did not differ significantly with age at each substrate concentration tested (Table IV), suggesting that renal ST activity did not differ with age. In contrast to the lack of age-dependent differences in renal glucuronyl transferase or sulfotransferase activities, age-dependent differences in renal GST activity were observed. Specifically, K_m for CDNB conjugation was significantly reduced in 12- and 30-month olds compared with 3-month olds (Table II and Fig. 1). Shifts in K_m for renal GST activities may be explained by specific increases in high-affinity GST isozymes, so that saturation of renal GST enzymes would occur at lower concentrations of reactive APAP metabolites in 12- and 30-month-old rats than in 3-month olds. However, the rate of formation of reactive APAP metabolites is likely to be quite low, insufficient to saturate renal GST enzymes. Thus, although K_m for renal GST activity may be reduced in 12-month-old male SD rats, it is unlikely that this difference contributes to age-dependent APAP nephrotoxicity.

Age-dependent differences in *in vitro* conjugation reactions were not observed for hepatic enzyme activities in male SD rats. Thus, the previously observed age-dependent decline in total systemic clearance of APAP (5) cannot be attributed to age-dependent differences in hepatic APAP metabolism. Rather, reduced total systemic APAP clearance in 12-month-old male SD rats more likely is related to a smaller volume of distribution for APAP in older rats. Similarly, age-dependent

reductions in renal glucuronyl transferase and sulfotransferase activities were not observed and thus, do not contribute to age-dependent APAP nephrotoxicity. In contrast, age-dependent differences in renal GST activity, such as reduced K_m in 12- and 30-month-old rats, occur but are unlikely to contribute to age-dependent APAP nephrotoxicity.

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