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SHIFT FROM BILIARY TO URINARY ELIMINATION OF ACETAMINOPHEN GLUCURONIDE IN ACETAMINOPHEN PRETREATED RATS

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Abbreviations:
APAP - acetaminophen, APAP-glu - acetaminophen-glucuronide, Mrp1, Mrp2 and Mrp3 - multidrug resistance-associated proteins 1, 2 and 3, NAPQI - N-acetyl-p-benzoquinone imine, AST - aspartate aminotransferase, ALT - alanine aminotransferase, GST - glutathione-S-transferase, GSH - glutathione, GSSG - oxidized glutathione, TC – taurocholate, UGT – UDP-glucuronosyltransferase.

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ABSTRACT

Despite its toxicity, acetaminophen (APAP) is used increasingly as an analgesic, antipyretic and anti-inflammatory agent. We examined the effect of prior exposure to APAP on its biliary and urinary elimination. The biliary and urinary elimination of a test dose of APAP (150 mg/kg, i.v.) was determined in male Wistar rats 24 hr after pretreatment with vehicle, a single dose (1.0 g/kg, i.p.), or increasing daily doses (0.2, 0.3, 0.6, and 1.0 g/kg/day, i.p.) of APAP. Whereas elimination of the parent APAP was minimally affected, biliary excretion of APAP glucuronide was significantly decreased 70 and 80%, while urinary excretion was significantly increased 90 and 100% in the groups pretreated with single and repeated doses of APAP, respectively, relative to vehicle controls. Western analysis and confocal immunofluorescent microscopy indicated a marked increase in hepatic expression of Mrp3 in both groups pretreated with APAP, relative to expression of Mrp2. ATP-dependent transport of ^{3}H-taurocholate, an Mrp3 substrate, was significantly increased in basolateral liver plasma membrane vesicles from rats pretreated with repeated doses of APAP relative to controls. Entero-hepatic recirculation of APAP glucuronide after administration of the same test dose of the drug was significantly decreased in rats pretreated with repeated doses of APAP. These data indicate that APAP pretreatment induced a shift from biliary to urinary elimination of APAP glucuronide, consistent with the increased expression of Mrp3 in the basolateral domain of the hepatocyte. We postulate that decreased entero-hepatic recirculation contributes to decreased APAP hepatotoxicity by reducing liver exposure.

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INTRODUCTION

The increasing popularity of Acetaminophen (N-acetyl-*p*-aminophenol, APAP) as a nonprescription analgesic and antipyretic has resulted in a significant increment in the incidence of accidental and intentional poisonings with this drug (Kaplowitz, 2004). It is generally accepted as a safe drug when administered within the therapeutic range (Rumack, 2004). However, in large-single-dose ingestion it causes massive centrilobular necrosis, inducing severe hepatotoxicity in human and experimental animals that may be lethal (Thomas, 1993; Bessems and Vermeulen, 2001).

APAP is metabolized in the liver mainly by glucuronidation and sulphation, thus generating the non-toxic metabolites, APAP-glucuronide (APAP-glu) and APAP-sulphate (Thomas, 1993). At high doses of APAP these metabolic paths are saturated, and a substantial portion of the drug is metabolized by the CYP450 system, generating a reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI). This compound reacts with reduced GSH, leading to GSH depletion, or it covalently binds to cellular proteins, as likely explanations for APAP hepatotoxicity (Mitchell et al., 1973;Linscheer et al., 1980). APAP-glu excretion in bile is mediated by the canalicular multidrug resistance-associated protein 2 (Mrp2; Abcc2) (Xiong et al., 2000) and represents about 7% of a single dose (100 mg/Kg) of APAP administered systemically to rats (Brouwer and Jones, 1990). Basolateral efflux of APAP-glu in liver may also occur, and has been linked to expression of Mrp3 (Abcc3) (Manautou et al., 2004), an ATP-dependent transporter expressed on the basolateral domain of the hepatocyte (Crocenzi et al., 2004). Increased expression of Mrp3 in rats pretreated with phenobarbital, trans-stilbene oxide, diallyl sulfide, or oltipraz correlated well with increased basolateral and decreased biliary clearance of APAP-glu (Xiong et al., 2002;Slitt et al., 2003). Thus, increased expression of the basolateral Mrp3 transporter relative to the canalicular Mrp2 transporter is associated with a shift from biliary to basolateral, and thereby, urinary excretion of APAP-glu.

Increased resistance to APAP hepatotoxicity in response to repetitive administration of the drug has been observed in humans and experimental animals, though the mechanisms are only partially understood. Studies in mice demonstrated that autoprotection was likely due to down-regulation of the CYP450s involved in formation of NAPQI and to a potentiated proliferative response (Shayiq et al., 1999). Increased tolerance to APAP intoxication was also observed in rats and was linked to increased GSH availability from regenerating hepatocytes, due to induction of the enzymes involved in GSH synthesis (Dalhoff et al., 2001). The evidence thus indicates that APAP pretreatment may influence its toxic effects by modifying key metabolic pathways. The possibility that APAP hepatotoxicity is also affected by changes in expression or activity of transporters involved in APAP metabolite disposition has never been explored. Aleksunes et al. (2005) demonstrated that APAP induced an increase in Mrp2 and Mrp3 mRNA expression in mice, though the effect on protein levels or activities was not evaluated. In a recent study, we found an increase in Mrp2 protein level in liver from rats treated with a single 1.0 g/Kg dose of APAP, together with increased biliary excretion of the Mrp2 substrate, oxidized glutathione (GSSG) (Ghanem et al., 2004). We proposed that Mrp2 induction by APAP may represent an adaptive mechanism to accelerate liver disposition of the non-toxic metabolite APAP-glu and to increase elimination of GSSG to maintain the redox equilibrium in the hepatocyte. To test the former possibility, we evaluated the biliary and urinary disposition of APAP-glu in rats pretreated with a single dose of APAP (1.0 g/Kg), or with repeated and increasing doses of the drug (0.2 to 1.0 g/Kg). The influence on expression of Mrp2 was also explored. Because of the potential impact of basolateral Mrps on liver to blood transport and subsequent urinary elimination of APAP-glu, we also evaluated the expression of Mrp1 and Mrp3 in the same animals.

The data indicate that both single and repeated doses of APAP led to a shift from biliary to urinary excretion of APAP-glu, correlating well with a marked induction of Mrp3 relative to Mrp2.

METHODS

Chemicals. APAP, APAP-glu, sucrose, sodium taurocholate (TC), creatine phosphate, creatine phosphokinase, NADPH, glutathione reductase, UDP-glucuronic acid, and bovine serum albumin were from Sigma Chemical Company (St. Louis, MO). [^{3}H] TC (2.1 Ci/mmol; 98% purity) was obtained from New England Nuclear (Boston, MA). All other chemicals were of analytical grade, and used as supplied.

Animals and experimental protocols. Male Wistar rats (250-290 g) were used throughout. The rats had free access to food and water and were maintained on a 12-hr automatically timed light and dark cycle. All procedures involving animals were conducted in accordance with NIH guidelines for the Care and Use of Laboratory Animals.

The following experimental groups were used:

(i) Single protocol. Animals were injected with a single dose of APAP**,** 1.0 g/kg b.w., i.p. APAP was administered as a solution of 100 mg/ml in 1:4 polyethylene glycol 400:saline. Control animals received the vehicle. Studies were performed 24 hr later. This group is termed Single APAP.

(ii) Repeated protocol. APAP was administered i.p. at increasing daily doses of 0.2 g/kg on day 1, 0.3 g/kg on day 2; 0.6 g/kg on day 3, and 1.0 g/kg on day 4. Administration of increasing non-toxic doses of APAP has been demonstrated to confer resistance to a subsequent lethal dose either in rats or mice (Shayiq et al., 1999; Dalhoff et al., 2001). Control animals received the same increasing volume of vehicle for 4 days. Studies were performed 24 hr after the last dose of APAP or vehicle. This group is termed Repeated APAP.

Different sets of animals were used in the different experimental designs according to:

i- Single APAP (N=4), Repeated APAP (N=4) and their respective controls (N=3 each) were used for assessment of biliary and urinary excretion of APAP and APAP-glu.

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ii- Repeated APAP (N=4) and their controls (N=3) with external biliary drainage vs. Repeated APAP (N=3) and their controls (N=3) with preserved entero-hepatic recirculation were used for assessment of entero-hepatic recirculation of APAP.

iii- Single APAP (N=6), Repeated APAP (N=4) and their respective controls (N=2 each) were used for assessment of renal function.

iv- A maximum number of 4 Single APAP animals, 4 Repeated APAP animals and 3 of their respective controls were used in assessment of serum marker activities, intrahepatic glutathione (GSH) levels, UDP-glucuronosyltransferase (UGT) activity and western blot and confocal microscopy studies.

v- Repeated APAP (N=3) and their controls (N=3) were used for assessment of Mrp3 activity.

vi- All these animals, together with those that did not survive the APAP treatment, were grouped for calculation of survival.

Assessment of biliary and urinary excretion of APAP and APAP-glu. The elimination of APAP and APAP-glu in urine and bile was determined following administration of a test dose of APAP (150 mg/kg, i.v.) to groups of animals that had been treated with APAP or vehicle according to the Single and Repeated protocols described above. Rats were anesthetized with sodium pentobarbital (50 mg/Kg, i.p.), and thus maintained throughout. Animals from APAP groups and controls were administered the same total doses of pentobarbital throughout the experiment in order to avoid any differential impact of the anesthetic on bile flow. Body temperature was measured with a rectal probe, and maintained at 37 ºC with a heating lamp. The jugular vein and the common bile duct were cannulated with polyethylene tubing (PE50 and PE10, respectively). The urinary bladder of the animals was exteriorized through a midline incision and cannulated with polyethylene tubing (PE75). Appropriate volumes of 5% bovine serum albumin in saline were administered intravenously throughout the experiment to replenish body fluids. After a 30-min stabilization period, two initial bile samples and one

urine sample were collected. APAP (150 mg/kg; 50 mg/ml in 1:3 propylene glycol:saline solution) was administered i.v. and bile (at 15-min intervals) and urine (at 30-min intervals) samples collected for 3 hr. Bile and urinary flow were determined gravimetrically. Samples of bile and urine were used for assessment of content of APAP and its glucuronide by HPLC. Analysis of APAP and APAP-glu content in basal bile and urine samples, prior to administration of the test dose, indicated no detectable levels in either Single or Repeated protocols.

Assessment of entero-hepatic recirculation of APAP. In a different set of animals we evaluated the effect of repeated APAP pretreatment on entero-hepatic recirculation of the drug. The rats were anesthetized and the jugular vein was cannulated as described above. A test dose of APAP (150 mg/Kg) was administered i.v. and 150 min later, the common bile duct was cannulated and bile collected for 30 min. Bile flow was determined gravimetrically. Samples of bile were used for determination of APAP-glu by HPLC. The data thus obtained were compared with those obtained after administration of the same test dose and during the equivalent period of bile collection, but under conditions of permanent biliary drainage, as described above. The 150-180 min time period was chosen based on the findings demonstrating no differences in APAP-glu biliary excretion between controls and Repeated APAP groups under conditions of external biliary drainage (see two last periods in Fig 1A). Higher excretion values in controls but not in the APAP group under conditions of preserved recirculation would indicate that Repeated APAP pretreatment interfered with the entero-hepatic recirculation.

Renal function. Functional parameters were assessed *in vivo* in a different set of APAP-treated animals. The rats were anesthetized and the urinary bladder was cannulated. After a 30-min stabilization period, urine was collected for 60 min. At the end of this period, blood was collected by cardiac puncture and serum immediately separated by centrifugation. Urinary flow was determined

gravimetrically. Samples of serum and urine were used for determination of renal functional parameters.

Collection of liver samples. In a different set of animals, which were sacrificed by cardiac puncture under pentobarbital anesthesia, livers were rinsed with PBS and portions of the organ were gently frozen in liquid nitrogen and preserved at -80 ºC until used for crude plasma and microsomal membrane preparation, frozen in precooled isopentane for confocal microscopy, or used in assessment of total GSH content. Serum samples were separated for determination of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities. For preparation of basolateral plasma membranes (BLPM), livers were removed and immediately used for membrane preparation.

Preparation of liver membranes. Crude plasma membranes (Meier et al., 1984) and microsomal membranes (Luquita et al., 1994) were prepared from each experimental group and were used in western blot studies to determine content of Mrp1-3, and in assessment of UGT activity towards APAP, respectively. BLPM vesicles were prepared by continuous sucrose separation (Meier et al., 1984) in two days in total from control or Repeated APAP rats and were used in transport studies. Protein concentration was measured as described (Lowry et al., 1951). Na^+-K^+-ATPase (µmol Pi per hr per mg protein) activity in BLPM vesicles prepared from control rats was 24.6 ± 5.0 (mean ± SD, N=3) and was not different in the Repeated APAP group (data not shown). This activity represents an enrichment of 28 ± 9 fold with respect to homogenates. Activity of 5'-Nucleotidase (µmol per hr per mg protein) in BLPM membranes from control rats was 4.7 ± 1.5 (mean ± SD, N=3) and was not affected by APAP pretreatment (data not shown). This value represents a 3.7 ± 0.2 fold increase with respect to homogenates and indicated minimal contamination with canalicular membranes, as previously described (Meier et al., 1984). Analysis of activities of UDP-glucuronosyltransferase, acid phosphatase, and aspartate aminotransferase,

markers of microsomal, lysosomal and mitochondrial membranes, respectively, indicated no appreciable contamination with these fractions (data not shown).

Western blot studies. Immunoblotting was performed with crude plasma membranes as previously described (Mottino et al., 2002) using a rat monoclonal antibody to human MRP1 (Alexis Biochemicals, Carlsbad, CA), a mouse monoclonal antibody to human MRP2 (M_2 III-6, Alexis Biochemicals) and a rabbit polyclonal antibody to rat Mrp3 (Ogawa et al., 2000). Subsequent densitometry was performed using the Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD) software.

Immunofluorescence microscopy. Liver slices (5 µm) were prepared with a Zeiss Microm HM5000 microtome cryostat, air dried for 1 hr, and fixed for 10 min with cold acetone (-20 ºC). Tissue sections were incubated overnight with the monoclonal anti-human MRP2 (1:100) and rabbit anti-human MRP3 (1:100, Sigma Chemical Company) antibodies, followed by incubation with the appropriate fluorescent secondary antibodies and confocal microscope analysis as described (Mottino et al., 2002).

BLPM transport studies. BLPM were diluted with suspension buffer (10 mM Tris-HCl, 250 mM sucrose, pH: 7.4) to 5.0-5.5 mg/ml of protein, and vesiculated by 20 passages through a 25-gauge needle. ATP-dependent uptake of the Mrp3 substrate, TC, into liver BLPM, was determined as previously described for intestinal BLPM (Shoji et al., 2004). Some modifications were introduced. Briefly, 30 µl of transport medium (10 mM Tris-HCl, 250 mM sucrose, 10 mM $MgCl_2$, pH: 7.4) containing radiolabeled TC (1.5 µM) was preincubated at 37ºC for 3 min and then rapidly mixed with 15 µl of membrane vesicles (80 µg of protein). The reaction mixture contained 5 mM ATP and an ATP regenerating system (10 mM creatine phosphate and 100 µg/ml creatine phosphokinase) or 5 mM AMP. The transport reaction was stopped by addition of 1 ml of ice-cold buffer containing 250 mM sucrose, 0.1 M NaCl, and 10 mM Tris-HCl (pH: 7.4), at 20, 40, 60, and

120 sec. This mixture was filtered through a 0.45 µm membrane filter (Millipore, Bedford, MA) and washed twice with 3.5 ml of stop solution. Radioactivity retained on the filter was determined in a scintillation analyzer (RackBeta 1214, Pharmacia Wallac Oy, Turku, Finland).

Analytical methods. APAP and APAP-glu in bile and urine were assayed by HPLC (Howie et al., 1977). Retention times of APAP and APAP-glu were determined by authentic standards. Proteins from bile and urine samples were removed by precipitation with equal volumes of trichloroacetic acid solution (25% w/w) followed by centrifugation before HPLC analysis.

Liver toxicity was assessed by measuring AST and ALT activity in serum using commercial kits (Human, Wasebaden, Germany). For assessment of total GSH (reduced + oxidized) in liver samples, the tissue was homogenized (20% w/v in saline) and 2 volumes of the homogenate were mixed with 1 volume of 10% sulfosalicylic acid, centrifuged at 5000 g for 5 min, and the supernatant used in the GSH assay (Tietze, 1969). Survival was assessed by calculating the ratio between the number of animals surviving the treatment and the total number of animals treated with the drug.

Incubation conditions for assessment of UGT activity were as described (Kessler et al., 2002), except that Triton X-100 was incorporated to activate microsomes (Luquita et al., 1994). At the end of the incubation, mixtures were deproteinized as described above and the formed APAP-glu was detected in supernatants by HPLC (Howie et al., 1977).

To evaluate renal function, the concentration of sodium and creatinine was determined in serum and urine by ion-selective electrode methodology (Hitachi 911, Ibaraki, Japan) and with a commercial kit (Biosystem, Barcelona, Spain), respectively. Creatinine clearance and fractional sodium excretion were then calculated.

Statistical analysis. Data are presented as the means ± SD. Statistical analysis was performed using one-way ANOVA, followed by the Bonferroni test, unless otherwise stated. Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

Effect of APAP on liver toxicity, hepatic GSH content, and survival. Table 1 shows significantly increased values of AST and ALT in the Single APAP group, as previously reported for the same dosage of APAP (Ghanem et al., 2004), clearly indicating hepatotoxicity induced by the drug. Less pronounced changes in these parameters were obtained in the Repeated APAP group when compared with Single APAP group. GSH content in liver was not affected by the Single protocol but increased by 31% in the Repeated group. These data were consistent with previous reports indicating that GSH content returned to normal values 18-24 hr after injection of 0.5 g/Kg of APAP to rats (Noriega et al., 2000), and with increased capability for GSH synthesis by regenerating liver under conditions of chronic treatment (Dalhoff et al., 2001). Table 1 also shows that survival was significantly higher in the Repeated APAP treatment group, indicating development of resistance to drug toxicity in this group relative to rats in the Single APAP group.

Effect of APAP pretreatment on excretion of APAP and APAP-glu. Basal bile flow was not affected by APAP pretreatment (1.9 ± 0.4, 2.5 ± 0.7, and 2.4 ± 0.8 µl/min/g liver in control, Single APAP and Repeated APAP groups, respectively, N=4-6). The test dose of APAP increased this measure by up to 40%, coincident with the peak of excretion of APAP-glu in all groups (data not shown). Both Single APAP and Repeated APAP protocols markedly decreased biliary excretion of APAP-glu with respect to controls, whereas no substantial differences were observed in the rate of excretion of APAP-glu between the two APAP pretreatment protocols (Fig 1A). Cumulative biliary excretion of APAP-glu was decreased by 70% and 80% in response to Single APAP and Repeated APAP treatment, respectively (Fig 1E). In contrast, the rate of urinary excretion of APAP-glu was increased significantly by both protocols (Fig 1B). As occurred with biliary excretion, no differences in the rate of urinary excretion of APAP-glu were detected between Single APAP and Repeated APAP groups. Cumulative

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renal excretion of the glucuronide was increased by 90 and 100% in Single APAP and Repeated APAP, respectively (Fig 1F). Comparison of the data in Figs 1E and 1F indicates that diminution of the percent contribution of biliary APAP-glu to overall elimination of the drug was compensated by increased renal excretion. Excretion rates of parent APAP are depicted in Fig 1C and 1D. In control animals, the parent drug contributed minimally to total APAP biliary elimination and moderately to its urinary elimination. Though statistically significant differences in biliary or urinary excretion of APAP were observed in some periods in response to both protocols of administration (Fig 1C and D), these differences had little impact on the cumulative measures (Fig 1E and F). Specifically, the decrease in cumulative urinary excretion of APAP in the Single group (Fig 1F) could be tentatively associated with the impairment in creatinine clearance (see Table 2), since intact APAP is not extensively bound to plasma proteins and it is likely to undergo considerable glomerular filtration (Forrest et al., 1982). Urinary excretion peak of both APAP-glu and intact APAP exhibited a delay from 60 to 120-150 min in APAP pretreated animals (Fig 1B and 1D).

Effect of Repeated APAP protocol on entero-hepatic recirculation of the drug. Following its biliary excretion, APAP-glu undergoes intestinal hydrolysis and the released intact APAP is partially reabsorbed, thus indicating entero-hepatic recirculation (Watari et al., 1983). Biliary excretion rates of APAP-glu in animals with preserved entero-hepatic recirculation are depicted in Fig 2, together with the values obtained for the same periods under non-recirculating conditions. Under conditions of preserved recirculation, this measure was decreased by 70% in the Repeated APAP group vs controls. In contrast, there was no difference between these two groups under conditions of permanent biliary drainage. The difference in biliary excretion of the glucuronide in control animals with preserved vs non-preserved recirculation, confirmed APAP entero-hepatic recirculation in our model. This difference was not observed in rats pretreated with APAP, suggesting minimal contribution of a preserved entero-

hepatic circuit to biliary excretion of APAP-glu at 150-180 min after injection of the APAP test dose.

Effect of APAP on UGT activity. Microsomal UGT activity towards APAP in control rats was 3.64 ± 1.50 nmol/min/mg protein (N=6, which includes measures in animals receiving both single and repeated doses of solvent). Pretreatment with APAP either as Single (4.22 ± 1.32 nmol/min/mg protein, N=4) or Repeated (5.54 ± 2.01 nmol/min/mg protein, N=4) protocol did not significantly modify enzyme activity.

Effect of APAP on renal function. Animals receiving a single dose of APAP exhibited a significant decrease in urinary flow rate and creatinine clearance when compared with controls (Table 2). The Repeated APAP group showed a similar decrease in the urinary flow rate, but without alteration in the creatinine clearance. All groups showed a fractional excretion of sodium lower than 1%, indicating preservation of concentrative tubular function in response to APAP. Decreased urinary flow rate in the Repeated APAP group, in spite of preserved creatinine clearance, likely resulted from increased tubular reabsorption, as indicated by the values of fractional excretion of sodium.

Effect of APAP on expression of Mrp1, Mrp2, and Mrp3. Expression of Mrp1, as detected by western blotting, was not affected by either Single APAP or Repeated APAP (Fig 3). In contrast, both protocols induced a similar increase in Mrp2 content of about 65%. The most striking finding was the marked increase in Mrp3 levels (greater than 400%), which was clearly higher in magnitude than that for Mrp2. While the Mrp1 signal was of similar level as Mrp2 in spite of its usual low expression in normal rat liver (Crocenzi et al., 2004), this was likely due to differences in sensitivity of the respective antibodies in addition to different times of exposure of the films to chemiluminescence reagents. The marked induction of Mrp3 relative to Mrp2 in response to APAP was confirmed by immunofluorescence. Fig 4 shows a very low expression of Mrp3 restricted to the

basolateral membrane of perivenous hepatocytes surrounding the central vein (CV) in control rats (green fluorescence), as previously described (Soroka et al., 2001). The inset in the upper panel shows a typical pattern of immunofluorescence of Mrp2 in the canaliculus (red fluorescence) and of Mrp3 (green fluorescence) in the basolateral domain (see white arrow). The yellow fluorescence seen occasionally in canalicular or pericanalicular regions may reflect some degree of cross-reactivity between anti-MRP2 and -MRP3 commercial antibodies. APAP treatment markedly increased immunodetection of Mrp3, which was extended to periportal hepatocytes in both Single and Repeated APAP treatment protocols. Though no quantitative analysis was performed in these studies, simultaneous immunodetection of Mrp2 and Mrp3 in the perivenous region clearly demonstrates a significant increase in expression of basolateral Mrp3 relative to canalicular Mrp2 in APAP livers (see insets).

Effect of APAP on TC uptake by liver BLPM vesicles. Consistent with increased Mrp3 expression by Western analysis, ATP-dependent transport of TC was significantly increased in the Repeated APAP group vs controls (Fig 5). Data on the purity of the BLPM in both groups do not support greater contamination of BLPM with canalicular membranes. Further, we have shown that a single 1g/Kg dose of APAP does not change *in vivo* biliary secretion of endogenous bile salts (Ghanem et al., 2004) or bile salt export pump levels as detected by western blotting in crude plasma membranes (unpublished results). Taken together, these data support an increase in transport activity due to the increased expression of Mrp3 in the basolateral domain of the plasma membrane.

DISCUSSION

APAP overdose represents the leading cause for calls to poison control centers in many countries and produces a considerable number of deaths due to acute liver failure each year (Lee, 2004). It was reported that chronic ingestion of an excess of APAP (several tablets per day, for weeks) leads to development of tolerance to the drug, usually delaying the onset of liver injury. Tolerance to the toxic effects of APAP in experimental animals has been linked to changes in activity of specific metabolic pathways involved in APAP bioactivation or GSH synthesis (Shayiq et al., 1999;Dalhoff et al., 2001). We here provide evidence that APAP pretreatment markedly induces liver basolateral Mrp3 vs canalicular Mrp2, leading to a shift from biliary to urinary excretion of its major metabolite, APAP-glu, and decreased entero-hepatic recirculation of APAP. These findings likely contribute to the decreased liver damage, as indicated by the decreased serum AST and ALT levels and decreased mortality following pretreatment with APAP in Repeated APAP protocol (Table 1).

We recently demonstrated that an acute toxic dose (1.0 g/Kg) of APAP increased the hepatic expression of Mrp2, and increased the biliary excretion of its model substrates dinitrophenyl-S-glutathione and GSSG (Ghanem et al., 2004). The current data indicate that pretreatment with APAP in the Repeated APAP protocol maintained, but did not further increase Mrp2 expression. Surprisingly, in spite of this increase in Mrp2 expression, the biliary excretion of APAP-glu was markedly decreased in APAP-pretreated groups, whereas its urinary excretion was increased. Although APAP-glu is a substrate for both canalicular Mrp2 and basolateral Mrp3 in rodents, constitutive expression of Mrp3 in normal rat liver is very low relative to canalicular Mrp2. Because the affinity of APAP-glu for Mrp3 is estimated to be several times higher than for Mrp2 (Xiong et al., 2002;Manautou et al., 2004), induction of Mrp3 leads to preferential efflux of APAP-glu across the basolateral membrane of liver into blood vs biliary excretion (Xiong et al., 2002;Slitt et al., 2003). Moreover, Mrp3-mediated excretion of APAP-glu critically modulates its intrahepatic levels (Manautou et al.,

2004). In our study, both APAP-pretreatment protocols induced Mrp3 expression to a much greater extent than Mrp2; this selective regulation almost certainly accounts for the vectorial change in disposition of APAP-glu. These findings agree well with studies by Xiong et al. (2002) and Slitt et al. (2003) that used different Mrp3 inducers and demonstrated increased urinary excretion of the glucuronide derivative.

Renal elimination of APAP-glu was markedly increased in APAP pretreated groups, irrespective of the protocol. A single 1g/Kg dose of APAP has been shown to induce impairment of the glomerular filtration rate, as estimated by creatinine clearance (Trumper et al., 1998). Our study also showed decreased creatinine clearance in rats pretreated with a single dose of APAP, whereas pretreatment in the Repeated APAP protocol did not affect this parameter. Despite this impairment in renal function, it is clear that the kidneys were able to increase the urinary excretion of APAP-glu. While APAP-glu urinary excretion has been postulated to result from both glomerular filtration and active tubular secretion under normal conditions (Forrest et al., 1982), it is not known whether APAP-glu is preferentially eliminated in urine by filtration or tubular secretion after APAP pretreatment.

Following intraduodenal administration of APAP-glu in rats *in vivo*, the glucuronide is hydrolyzed by the microflora and the liberated APAP is partially reabsorbed (Watari et al., 1983). Our data in Fig 1 showing decreased biliary excretion of APAP-glu thus constitutes a major finding, with potential consequences for the retention of the parent compound in the entero-hepatic circulation and consequent liver toxicity. To test this possibility, we performed experiments in animals with intact entero-hepatic recirculation, which was preserved until 150 min after injection with a test dose of APAP. The data clearly demonstrate that the Repeated APAP protocol decreased entero-hepatic recirculation of the drug. The shift from biliary to urinary excretion of only 10% of administered APAP as APAP-glu seems to be of minor relevance due to its low magnitude. However, such a 10% represented a two-thirds decrease with respect to the excretion in control rats (see Fig 1E). Because APAP-glu is the main

metabolite of APAP in rats and its biliary excretion constitutes the major determinant of APAP recirculation (Watari et al., 1983), our finding indicates that pretreatment with APAP may have a significant impact on liver exposure to the drug. We hypothesize that preferential induction of Mrp3 vs Mrp2, thus minimizing exposure of the liver to APAP and subsequent bioactivation of the drug, may contribute to the development of resistance under conditions of chronic treatment. In the Single protocol, survival following a large-acute-single dose of APAP could be associated with the ability of some animals to quickly induce basolateral vs canalicular Mrps. Interestingly, we observed more variation in the extent of Mrp3 induction in animals from the Single protocol than those from the Repeated protocol as detected by western blotting (see Fig 3). Unfortunatelly, because of technical limitations, it is not possible to evaluate whether animals that did not survive the Single protocol were unable to induce Mrp3.

It is known that saturation of UGT-mediated conjugation of APAP under conditions of drug overdose leads to increased production of NAPQI (Rumack, 2004). The relevance of the glucuronidation pathway was recently confirmed by Kostrubsky et al. (2005), who demonstrated that inhibition of APAP glucuronidation in cultured human hepatocytes leads to increased hepatotoxicity. In the current study, neither the Single nor the Repeated protocol affected UGT activity towards APAP. This was in agreement with preserved cumulative total (biliary + urinary) excretion of APAP-glu in both groups of APAP pretreated rats (see Fig 1E), and would indicate that decreased toxicity to APAP did not result from increased hepatic glucuronidation. We postulate that decreased CYP450-mediated production of NAPQI (Shayiq et al., 1999), increased synthesis of GSH by regenerating hepatocytes (Dalhoff et al., 2001), and induction of basolateral Mrp3, act concurrently in the development of tolerance to APAP given chronically.

Tredger et al. (1995) reported a case of a 58-year-old woman who consumed 15 to 20 g of APAP daily without developing liver damage. These authors found a delayed, but a two-fold increase in plasma levels and urinary

excretion of APAP-glu and APAP-sulphate in this patient relative to a group of normal volunteers. These authors concluded that resistance to APAP toxicity may have derived from a combination of factors, including delayed absorption of the drug, increased metabolism by conjugation, and reduced CYP450-mediated formation of cytotoxic metabolites. Our data demonstrating increased urinary excretion of the non-toxic glucuronide metabolite of APAP agrees well with those of Tredger et al., and further suggest a role for induction of Mrp3 in preferential secretion of APAP-glu from liver to blood in response to APAP pretreatment. Tredger's results also suggest a role for increased urinary excretion of APAP-sulphate in development of tolerance to APAP. Further studies would be necessary to confirm this possibility in experimental animals.

The mechanism by which APAP increases Mrp3 expression is not known. Our current and previous (Ghanem et al., 2004) data agree well with those from Aleksunes et al. (2005) showing increased expression of Mrp2 and Mrp3 mRNA 6 and 24 hr after a single (0.4 g/kg) dose of APAP in mice, which would suggest transcriptional regulation of *Mrp2* and *Mrp3* genes by APAP. Using *Mrp3*-null mice Belinsky et al. (2005) demonstrated that this pump functions as an alternative route for secretion of bile acids and endogenous glucuronides in cholestatic hepatocytes. Increased Mrp3 expression is seen in animals hereditarily deficient in Mrp2 or with experimental obstructive cholestasis, as well as in some forms of human cholestatic disease (Hirohashi et al., 1998;König et al., 1999;Shoda et al., 2001;Scheffer et al., 2002;Soroka et al., 2001). Because some bile salts are Mrp3 substrates, upregulation of the transporter is thought to limit hepatocellular injury resulting from accumulation of these compounds during cholestasis (Bohan et al., 2003). Interestingly, our study demonstrated an unusual coexistence of increased expression of both Mrp2 and Mrp3. Increased expression of Mrp3 in response to APAP does not seem to play a role in protection against bile salt accumulation since biliary secretion of bile salts is not affected by APAP (Ghanem et al., 2004). Because neither Mrp2 expression nor its activity were decreased, but rather increased by APAP, we also ruled out the

occurrence of harmful effects resulting from accumulation of Mrp2 endogenous substrates such as bilirubin.

In conclusion, we have demonstrated that pretreatment with APAP led to a marked increase in the hepatic expression and activity of Mrp3 that was correlated with a significant shift from canalicular to basolateral efflux of APAP-glu, and a decrease in its entero-hepatic recirculation. This decreased entero-hepatic recirculation is postulated to contribute to APAP decreased hepatotoxicity by minimizing exposure to the liver and APAP activation to the toxic reactive metabolite.

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LEGENDS FOR FIGURES

Fig 1. Biliary and urinary excretion of APAP and APAP-glu.

Panel A and B show elimination rates of APAP-glu, and panels C and D, elimination rates of intact APAP. Cumulative biliary and urinary excretion of both compounds are shown in panel E and F, respectively. Single and Repeated vehicle rats rendered similar values and were grouped. Data are means ± SD of 4 to 6 animals per group.

* Control different from Single APAP ($P < 0.05$).

† Control different from Repeated APAP ($P < 0.05$).

‡ Single APAP different from Repeated APAP ($P < 0.05$).

Fig 2. Biliary excretion of APAP-glu in rats with preserved entero-hepatic recirculation.

Data are means ± SD of 3 to 4 animals per group and correspond to the 150-180-min period of bile collection after administration of the test dose of APAP in conditions of preserved vs interrupted entero-hepatic circulation (0-150 min).

* The indicated groups differed significantly by Student t-test ($P < 0.05$).

Fig 3. Expression of Mrp1, Mrp2, and Mrp3 detected by western blotting.

Fifteen µg of protein per lane were loaded in all lanes. This amount of protein gave a densitometric signal in the linear range of the response curve for the different antibodies. Uniformity of loading and transfer from gel to nitrocellulose membrane was controlled with Ponceau S. Controls include 2 Single and 2 Repeated vehicle animals. Densitometry data are means ± SD.

* Different from Single and Repeated groups ($P < 0.05$).

Fig 4. Immunofluorescence detection of Mrp2 and Mrp3.

Mrp2 (red) and Mrp3 (green) were detected by confocal microscopy. Insets show double labelling for both proteins. The white arrow indicates a typical canalicular

and basolateral localization of Mrp2 and Mrp3, respectively. Similar pattern of staining was observed in 3 independent preparations per group. CV: central vein.

Fig 5. Sodium TC transport by basolateral plasma membrane vesicles.
Effect of time on transport of 1.5 μM ^{3}H-labeled TC by BLPM from control rats and from rats treated with the Repeated APAP protocol, in the absence or presence of ATP and an ATP regenerating system. Data are means ± SD of 3 individual experiments.
* Different from ATP-dependent transport in controls ($P < 0.05$).

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Table 1: Serum markers of liver damage, liver GSH, and survival.

	Control	Single APAP	Repeated APAP
AST (U/L)	69 ± 27 *	305 ± 72 †	200 ± 86
ALT (U/L)	47 ± 10 *	238 ± 16 †	160 ± 13
Hepatic GSH content (nmol/g liver)	7878 ± 699 †	6548 ± 1373 †	10341 ± 987
Percent survival at 24 hr	100 (25)	56 (25)	96 (23)

Single and Repeated vehicle rats rendered similar values and were grouped. Data on AST, ALT, and hepatic GSH content are means ± SD of 4 to 6 animals per group. All animals used in the different experimental models throughout the study were grouped for calculation of survival; these numbers are in parenthesis.

* Different from Single and Repeated APAP groups ($P < 0.01$).

† Different from Repeated APAP group ($P < 0.05$).

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Table 2: Renal function parameters.

	Control	Single APAP	Repeated APAP
Urinary flow (µl/min/g of kidney)	4.97 ± 1.43 *	2.95 ± 0.87	2.70 ± 1.59
Creatinine clearance (ml/min/g of kidney)	0.62 ± 0.12 †	0.23 ± 0.32	0.44 ± 0.17
Fractional excretion of sodium (%)	0.66 ± 0.12 *	0.26 ±0.14	0.06 ± 0.02

Single and Repeated vehicle rats rendered similar values and were grouped. Values are expressed as mean ± SD of 4 to 6 animals per group.

* Different from Single and Repeated APAP groups ($P < 0.05$).

† Different from Single APAP group ($P < 0.05$).

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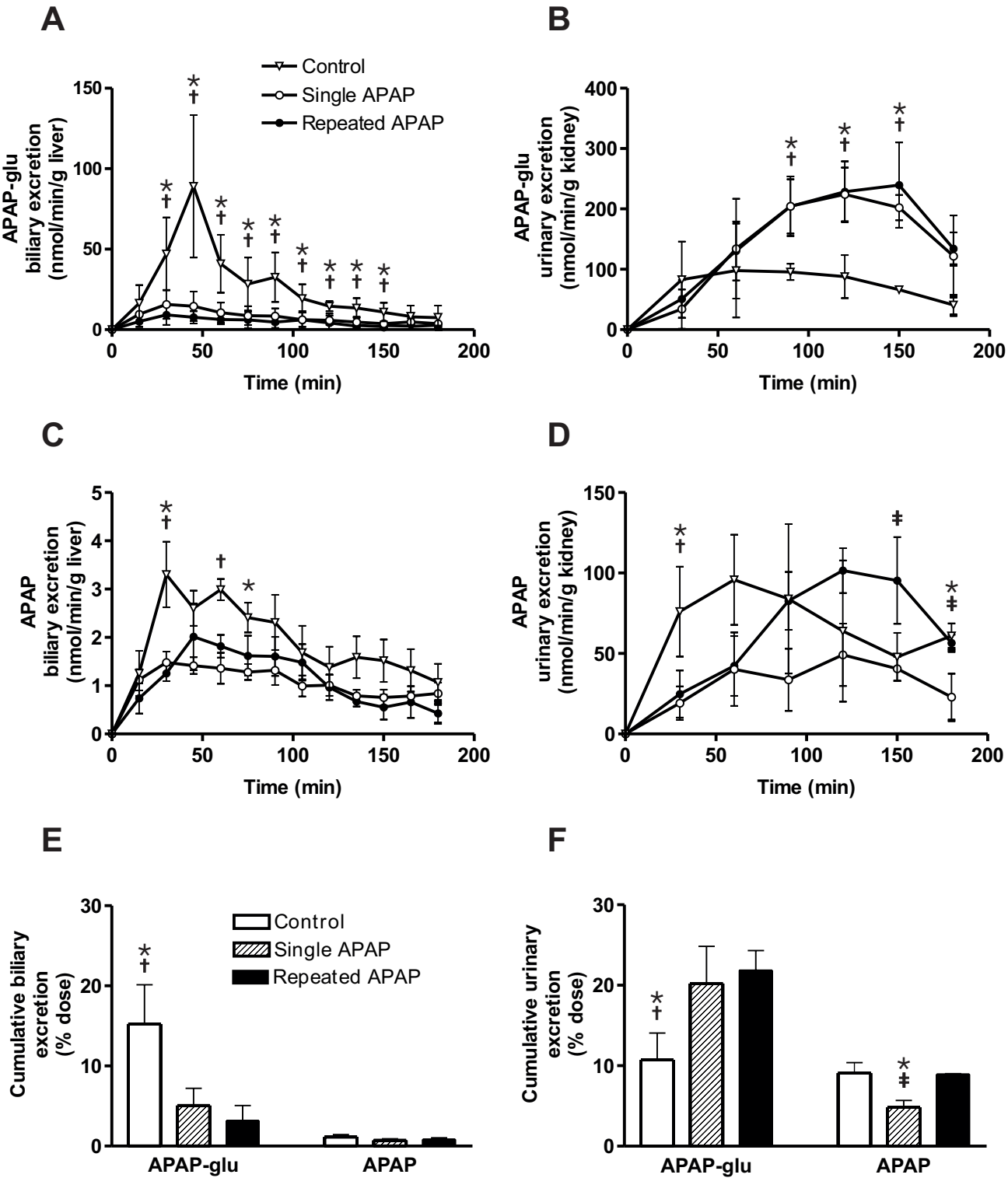

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Figure 2

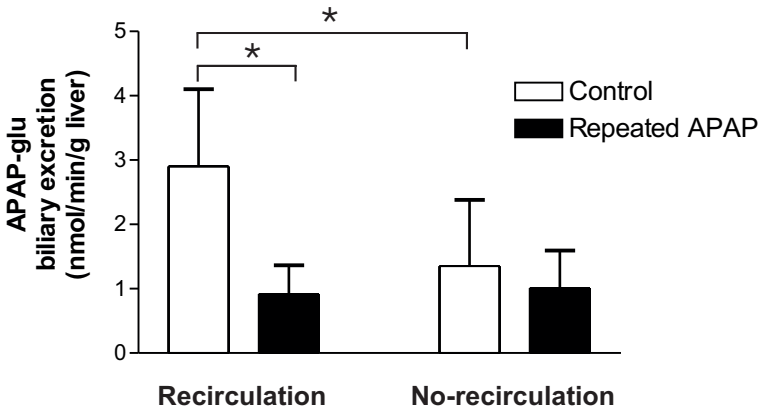

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Figure 3

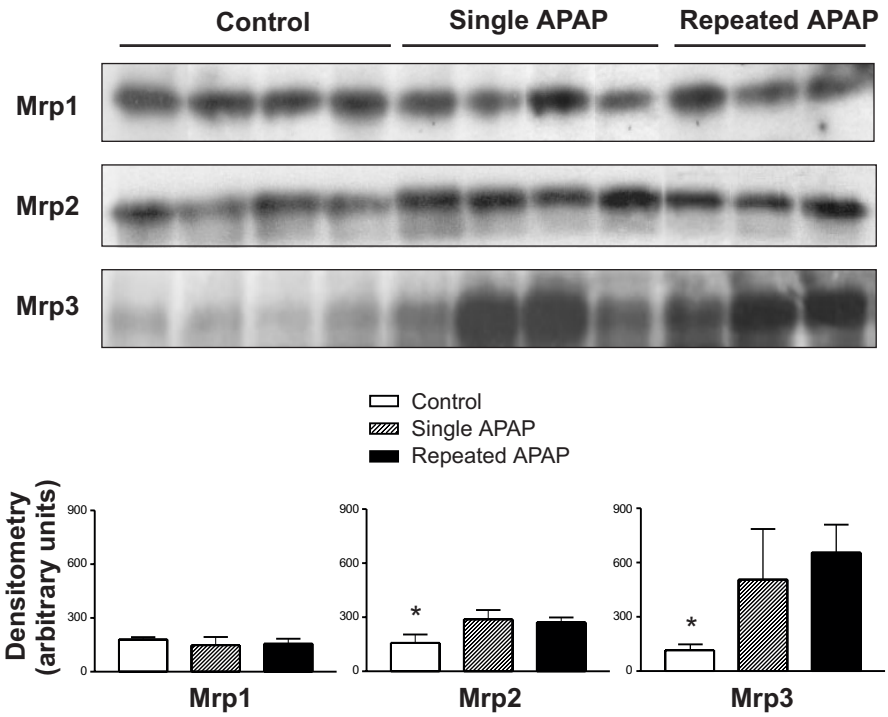

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Figure 4

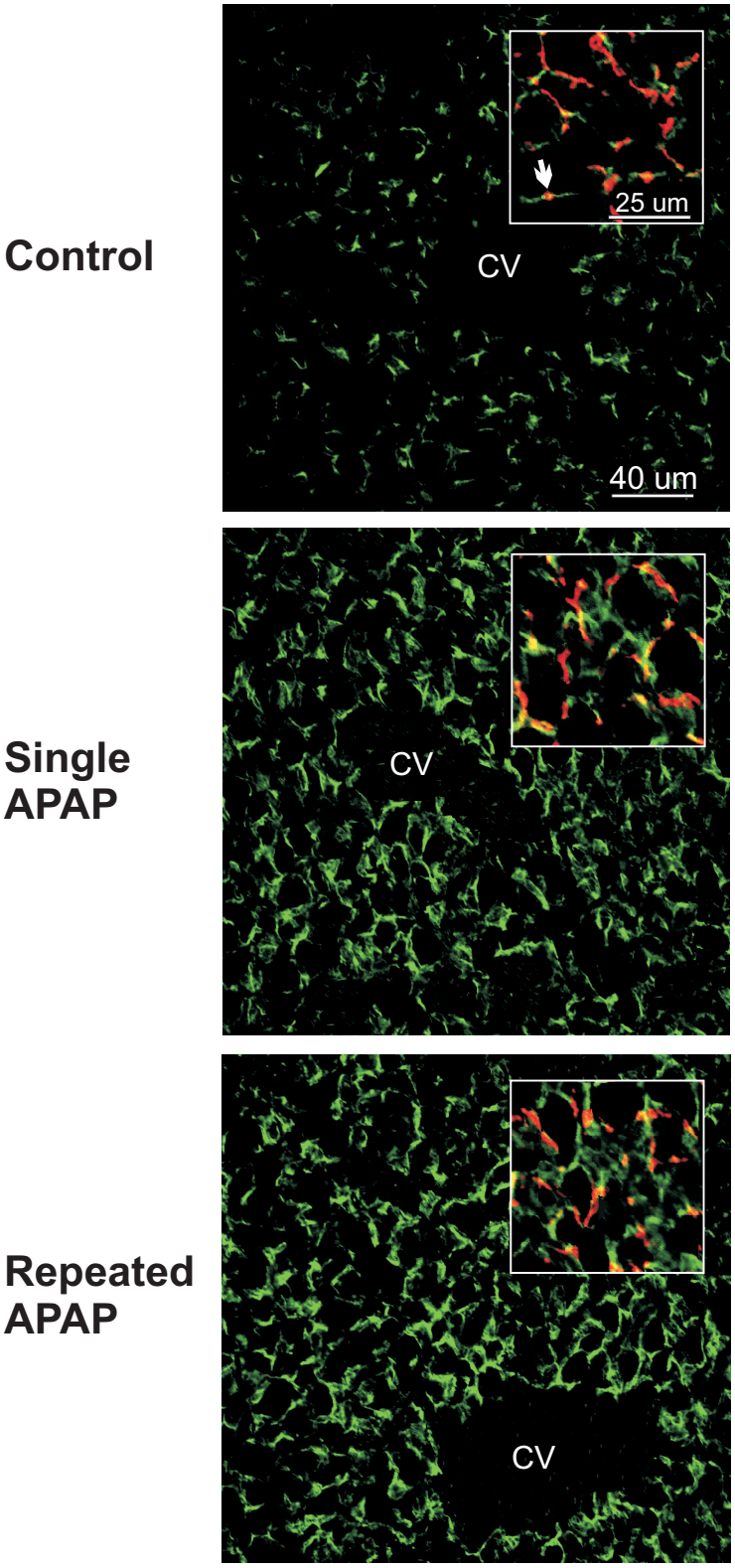

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Figure 5