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Ethynylestradiol increases expression and activity of rat liver Mrp3

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ABSTRACT

We evaluated the effect of EE administration (5 mg/kg b.w., s.c., for 5 consecutive days) on the expression and activity of Multidrug resistance-associated protein 3 (Mrp3) in rats. Western blotting analysis revealed decreased Mrp2 (-41%) and increased Mrp3 (+200%) expression by EE. To determine the functional impact of up-regulation of Mrp3 vs Mrp2, we measured the excretion of acetaminophen glucuronide (APAP-glu), a common substrate for both transporters, into bile and perfusate in the recirculating isolated perfused liver (IPL) model. APAP-glu was generated endogenously from APAP, which was administered as a tracer dose (2 µmol/ml) into the perfusate. Biliary excretion of APAP-glu after 60 min of perfusion was reduced in EE-treated rats (-80%). In contrast, excretion into the perfusate was increased by EE (+45%). Liver content of APAP-glu at the end of the experiment was reduced by 36% in EE group. The total amount of glucuronide remained the same in both groups. Taken together, these results indicate that up-regulation of Mrp3 led to an exacerbated basolateral vs canalicular excretion of conjugated APAP in IPL. We conclude that induced expression of basolateral Mrp3 by EE may represent a compensatory mechanism to prevent intracellular accumulation of common Mrp substrates, either endogenous or exogenous, due to reduced expression and activity of apical Mrp2.

INTRODUCTION

The pathogenic mechanisms underlying ethynylestradiol (EE)-induced cholestasis are still uncertain. More than one mechanism is likely involved in the development of cholestasis by this synthetic estrogen. Due to a decrease in bile salt-independent bile flow (BSIBF) observed in rats, several studies focused their attention on the regulation of the canalicular multidrug resistance-associated protein 2 (Mrp2 or Abcc2) by EE, and found a decrease in its expression (Trauner et al., 1997; Lee et al., 2000). However, Koopen et al. (1998) demonstrated that impairment in the activity of Mrp2 is not the main cause of EE-induced cholestasis in the rat, because the absence of Mrp2 in TR^- rats (hereditarily deficient in Mrp2) did not prevent cholestasis by this estrogen derivative. They concluded that reduction in Mrp2 expression and/or activity does not necessarily represent the key event in the etiology of EE-induced cholestasis.

Sinusoidal multidrug resistance-associated protein 3 (Mrp3 or Abcc3) expression is very low in normal rat and human liver. However, high levels are found in naturally Mrp2-deficient animals (Hirohashi et al., 1998; Johnson et al., 2005), in animals with obstructive cholestasis (Donner and Keppler, 2001; Soroka et al., 2001), and in Dubin-Johnson liver patients (König et al., 1999). Using *Mrp3*-null mice, Belinsky et al. (2005) verified that this pump acts as an alternative route for export of bile acids and glucuronides from cholestatic hepatocytes. Hepatic Mrp3 protein is inducible after treatment with different xenobiotics (Owaga et al., 2000; Cherrington et al., 2002; Xiong et al., 2002; Ghanem et al., 2005) leading to an augmented basolateral efflux of common Mrp substrates (Akita et al., 2002; Slitt et al., 2003; Xiong et al., 2002; Ghanem et al., 2005). Recently, Kamisako and Owaga (2005) reported that EE treatment increased hepatic Mrp3 mRNA expression. It is not known whether EE treatment

produces a concomitant increase in Mrp3 protein level, and more importantly, whether Mrp3 induction results in augmented basolateral vs biliary transport of substrates common to both Mrp2 and Mrp3, thus explaining the impairment in biliary secretion of organic anions in EE-cholestasis.

In the present study, we evaluated the effect of EE administration on expression and activity of Mrp3 in rat liver. The data strongly support a role for increased Mrp3 expression in effluxing Mrp substrates across the basolateral domain of the hepatocyte into blood.

MATERIALS AND METHODS

Chemicals. EE, leupeptin, phenylmethylsulfonyl fluoride (PMSF), pepstatin A, glutathione (GSH), glutathione reductase, 3α-hydroxysteroid dehydrogenase, β-NAD, NADPH, HEPES, palmitoyl lysophosphatidylcholine (PLPC), D-Saccharic acid 1,4-lactone, UDP-glucuronic acid, acetaminophen (APAP) and acetaminophen-glucuronide (APAP-glu) were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade purity.

Animals and experimental protocols. Adult male Wistar rats (300-350 g) were used throughout. They were maintained *ad libitum* on a standard laboratory pellet diet and were allowed free access to water during treatment. Animals were randomly divided in two experimental groups. EE-treated rats were administered EE dissolved in propylene glycol (33.7 mM), at a daily dose of 5 mg/kg body wt s.c., for 5 consecutive days (Crocenzi et al., 2001). Control rats were injected with vehicle (propylene glycol; 0.5 ml/kg body wt s.c.) according to the same schedule as described for EE. All procedures involving animals were conducted in accordance with NIH guidelines for the Care and Use of Laboratory Animals.

Eighteen hr after the last injection of EE or vehicle, bile was collected for determination of bile flow and biliary excretion of bile acids and glutathione as described (Ruiz et al., 2005). At the end of the collection period, the animals were sacrificed by exsanguination, and alkaline phosphatase (ALP; EC 3.1.3.1) determined in serum samples. Livers were perfused *in situ* with cold saline, removed, and the liver weight/body weight ratio calculated. Portions of hepatic tissue were used for assessment of total glutathione content (Ruiz et al., 2005), preparation of plasma and microsomal membranes, and immunoflourescence studies.

Preparation of membranes. Liver samples were homogenized in 0.3 mol/l sucrose containing 0.1 mmol/l PMSF, 25 μg/ml leupeptin and 5 μg/ml pepstatin A. Membrane fractions enriched in plasma membrane (mixed plasma membrane, MPM) were prepared from the homogenates by differential centrifugation as described (Carreras et al., 2003) and stored at –70°C until use in western analysis of transporters. Microsomal membranes were obtained from the homogenates as previously described (Catania et al., 1998) and stored at –70°C until used in assessment of activity and expression of UDP-glucuronosyltransferase (UGT). Protein concentration was measured using bovine serum albumin as standard (Lowry et al., 1951).

Western blot studies. Western analysis of Mrp2 was performed in MPM as described (Ruiz et al., 2005). Detection of Mrp3 was performed using a rabbit polyclonal antibody to rat Mrp3 (Ogawa et al., 2000), as previously described (Ghanem et al., 2005). Detection of UGTs was performed in microsomal preparations using a polyclonal anti-peptide antibody that specifically recognizes the 1A6 isoform belonging to group 1A as well as an antibody developed against a peptide common to all isoforms of this group (Ikushiro et al., 1995), as previously reported (Luquita et al., 2001). Immunoreactive bands were quantified using the Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD) software.

Immunofluorescence studies. *In situ* immunodetection of Mrp3 was performed as described (Ghanem et al., 2005) using a Zeiss Pascal LSM confocal system attached to a Zeiss Axioplan 2 imaging microscope.

Mrp3 transport activity in recirculating isolated perfused liver (IPL). To evaluate whether changes in expression of Mrp3 may affect liver secretory function, the

basolateral and apical excretion rates of a model substrate for both Mrp3 and Mrp2 were measured in a different set of animals. The livers were isolated and perfused for 60 min as described (Mottino et al., 2003). Briefly, the bile duct and portal vein were cannulated (PE10 and Abbocath-®T 14G, respectively) under sodium pentobarbital anesthesia (50 mg/kg body wt, i.p.) and the liver perfused *in situ via* the portal vein with oxygenated Krebs-Ringer bicarbonate buffer recirculating at a constant flow of 20 ml/min. Basal bile and perfusate samples were collected after a 10-min period of stabilization, then acetaminophen (APAP) was added to the perfusate reservoir (150 ml) in a tracer dose of 2 µmol/ml of perfusate. APAP is efficiently converted in liver to its glucuronide (APAP-glu), which is a substrate for both Mrp2 (Xiong et al., 2000) and Mrp3 (Manautou et al., 2004). Perfusate samples (100 µl) were collected at 5-min intervals for 60 min. Bile samples were collected for two 5-min initial periods and then at 10-min intervals for 60 min. At the end of the experiment, the liver was weighed and homogenized (20% w/v in saline). The homogenate (1 ml) was mixed with 70% perchloric acid (50 µl), centrifuged at 5000 x g for 5 min, and the supernatant used in APAP-glu detection. The content of APAP-glu in bile, perfusate and liver homogenate was determined by HPLC (Howie et al., 1977; Ghanem et al., 2005). Liver viability was controlled by monitoring bile flow (> 0.70 µl/min/g of liver and >0.30 µl/min/g of liver for control and EE-treated rats, respectively) and LDH leakage into the perfusate (with values < 10 IU/l between the start and the end of the experiment).

To rule out the possibility that a variation in apical and basolateral excretion of APAP-glu between groups could result from differences in endogenous synthesis of the conjugate, we also determined the activity of UGTs involved in APAP metabolism. UGT activity towards APAP was measured as described (Kessler et al., 2002) except that

palmitoyl lysophosphatidylcholine (PLPC, 0.15 mg/mg protein) was used to fully activate the microsomal suspension and D-Saccharic acid 1,4-lactone (2 mM) was routinely included in the incubation media to inhibit enzymatic hydrolysis of APAP-glu (Catania et al., 1998). At the end of the incubation, all mixtures were deproteinized with perchloric acid as described above and APAP-glu detected in the supernatants by HPLC.

Statistical analysis. Data are presented as mean ± SD. Statistical analysis was performed using the Student *"t"* test. Values of $p < 0.05$ were considered to be statistically significant.

RESULTS

Biliary secretory function.

EE treatment caused an increase in the liver weight/body weight ratio with respect to controls (3.83 ± 0.21 vs 3.37 ± 0.06 respectively, $p<0.05$, n=3), due to both decreased body weight (~8%) and increased liver weight (~5%), as was reported by others (Koopen et al., 1998; Crocenzi et al., 2001; Sánchez Pozzi et al., 2003). Serum ALP activity also increased in response to EE treatment (333 ± 24 vs 260 ± 47 U/L, for EE and control groups, respectively, $p<0.05$, n=3). Table 1 shows that the basal bile flow and bile acid and glutathione excretion rates were reduced by EE as reported (Koopen et al., 1998; Crocenzi et al., 2001, Sánchez Pozzi et al., 2003), and that the hepatic content of glutathione was not different between groups.

Expression of Mrp2 and Mrp3.

As shown in western blot studies (Fig 1), Mrp2 protein expression was significantly reduced (-41%) following EE treatment. In contrast, EE produced a marked (+200%) increase in Mrp3 protein expression (Fig 1). The selective induction of Mrp3 by EE was confirmed by immunoflourescence (Fig 2). Although no quantitative analysis of Mrp3 expression was performed, these images clearly demonstrate that basolateral immunoreactivity was increased and extended from perivenous to periportal hepatocytes, as was observed in experimental obstructive cholestasis (Donner and Keppler, 2001; Soroka et al., 2001).

Mrp3 transport activity in isolated perfused liver (IPL).

The relative contribution of Mrp3 to APAP-glu transport was estimated in IPL. Basal bile flow was significantly lower in livers from EE group than from controls (0.99 ± 0.08 and 0.31 ± 0.01 µl/min/g of liver, respectively, $p<0.05$, n=3-4). Apical and basolateral

excretion and liver content of APAP-glu are depicted in Fig 3. The upper panel shows that biliary excretion of APAP-glu was diminished by EE at all periods studied. Thus, at the end of the perfusion period (60 min), cumulative biliary excretion of the conjugate was significantly decreased by 80% in response to EE treatment (see inset). In contrast, cumulative excretion of APAP-glu into the perfusate was increased by 45% in EE group when compared to controls (see middle panel). Increased basolateral secretion of APAP-glu does not seem to be merely a consequence of impaired Mrp2 activity, because the hepatic content of this metabolite was significantly lowered (-36%) rather than increased by EE pretreatment (see bottom panel). These data indicate a more efficient global elimination of the conjugate from the liver cell, agreeing well with up-regulation of Mrp3.

In the current experimental protocol, APAP-glu, the common Mrp2 and Mrp3 substrate, is generated endogenously from the unconjugated parent drug. Because EE was found to modify its own glucuronidation through down-regulation of UGTs (Crocenzi et al., 2001; Sánchez Pozzi et al., 2003), it was important to rule out the possibility that EE alters APAP glucuronidation. UGT activity towards APAP was not affected by EE treatment (4.19 ± 0.25 vs 3.93 ± 0.33 nmol/min/mg protein, for control and EE groups respectively, n=3). Accordingly, as shown in Fig 1, EE treatment did not affect expression of UGT1A6, the main isoform involved in APAP glucuronidation, nor the intensity of the bands revealed with the 1A antibody that recognizes most relevant isoforms involved in conjugation of phenol derivatives (Ikushiro et al., 1995). More importantly, the total amount of APAP-glu (bile+perfusate+liver) detected at the end of liver perfusion did not differ between groups (2342 ± 790 and 2320 ± 135 nmol/g liver,

for control and EE groups, respectively), confirming similar rates of APAP glucuronidation by the intact liver.

DISCUSSION

In the present work, we evaluated the effect of EE pretreatment on the expression and activity of Mrp3 in an attempt to clarify the mechanisms underlying the estrogen-induced impairment in biliary secretory function. In agreement with previous studies (Koopen et al., 1998; Crocenzi et al., 2001; Sánchez Pozzi et al., 2003), we observed that EE administration decreased bile flow, as a result of a moderate decrease in bile salt excretion and a dramatic reduction in glutathione biliary excretion, the main determinant of bile salt independent bile flow (see Table 1). Similarly, in the IPL model we observed a decrease in bile flow and a substantial decrease in biliary excretion of APAP-glu following EE treatment. This latter finding could be attributed to decreased expression of Mrp2 and/or to induction of Mrp3. Lack of intrahepatic accumulation of APAP-glu in livers from EE animals would support a role for Mrp3 induction. This interpretation is consistent with partitioning of APAP-glu for efflux via Mrp3 vs Mrp2 following EE treatment as consequence of the higher affinity of APAP-glu for Mrp3 respect to Mrp2 (Xiong et al., 2000; Manautou et al., 2004).

Under normal conditions, bile salts and glucuronides are predominantly excreted into bile through Bsep and Mrp2, respectively (Kullak-Ublick et al., 2000), thus contributing to bile flow formation. Under experimental cholestatic conditions, such as bile duct ligation and EE-induced cholestasis, expression of these apical transporters is reduced in association with impaired biliary secretory function. For example, EE induced a reduction in ATP-dependent transport of taurocholate and S-(2,4-dinitrophenyl) glutathione by canalicular vesicles (Bossard et al., 1993), as well as decreased expression of Bsep (Lee et al., 2001; Micheline et al., 2002) and Mrp2 (Trauner et al., 1997; Crocenzi et al., 2001). Decreased biliary secretion of bile salts is expected to lead

to an increase in their intrahepatic levels. However, several findings offer evidence against significant intrahepatic accumulation of bile acids in EE-induced cholestasis. First, EE-treatment decreases the sodium-dependent taurocholate cotransporter (Ntcp) expression (Simon et al., 1996), taurocholate uptake in basolateral vesicles (Bossard et al., 1993), bile salt synthesis (Koopen et al., 1999) and the bile salt pool size (Crocenzi et al., 2001). Second, the serum ALP level, which is normally increased by intrahepatic bile salt accumulation, is only moderately increased by EE (+28%), in contrast with the significant increase (+160%) observed in obstructive cholestasis (Carreras et al., 2003). Because some bile salts are substrates of Mrp3 (Akita et al., 2002), up-regulation of Mrp3 by EE could also contribute to reduce their intracellular accumulation. Accordingly, Koopen et al. (1999) and Kamisako and Ogawa (2005) reported increased levels of total bile salts in serum from EE-treated rats.

Normally, hepatic Mrp3 expression is low in rat and humans, but is induced under cholestatic conditions such as bile-duct ligation (Donner and Keppler, 2001; Soroka et al., 2001), in Mrp2-deficient rats (Hirohashi et al., 1998; Johnson et al., 2005), after treatment with specific xenobiotics or drugs (Xiong et al., 2002; Slitt et al., 2003; Ghanem et al., 2005) and in Dubin-Johnson patients (König et al., 1999). The present data demonstrated that EE also induces Mrp3 protein expression and activity. We postulate that EE treatment increases basolateral efflux of conjugated organic anions and bile salts, thus competing with their normal canalicular transport. In consequence, this could affect both bile acid dependent and independent fractions of bile flow. It is not known, however, whether Mrp3 induction results from a direct action of EE on *Mrp3* gene transcription or is a consequence of the cholestatic syndrome. It is interesting to note that Mrp3 expression is not increased in pregnancy, despite a decrease in Mrp2

expression (Cao, et al, 2002). Estrogen levels accumulate only modestly in pregnancy (from 20 to ~80 pg/ml; Bridges, 1984), suggesting that the high estrogen levels in EE-treated animals act directly to increase Mrp3 expression.

In contrast to what was reported in rats, Mrp3 is constitutively well expressed in hepatocytes from normal mice, and no induction of Mrp3 mRNA was observed in liver from Mrp2 null mice (Chu et al., 2006). Interestingly, these authors also found that mRNA and protein expression of basolateral Mrp4 was significantly increased in liver of Mrp2 null mice. It was demonstrated that human MRP4 is also able to transport bile acids (Zelcer et al., 2003). In consequence, induction of this transporter may represent a compensatory mechanism to deal with reduced apical secretion of these solutes in conditions of altered bile flow formation. In support to this assumption, Denk et al. (2004) have reported up-regulation of Mrp4 in liver from bile duct ligated rats. While induction of Mrp3 and Mrp4 likely compensates for absent or reduced Mrp2 activity in the rat, Mrp4 possibly plays a major compensatory role in mice under these same circumstances. Whether Mrp4 is induced in response to EE administration, either in rats or mice, remains unknown.

As reviewed by Kroemer and Klotz (1992), women taking oral contraceptives steroids present a faster elimination of APAP associated with an increased metabolic clearance. This was tentatively explained by stimulation of phase II metabolism of APAP by contraceptive steroids leading to increased urinary elimination of APAP-glu. Recently, we demonstrated increased APAP-glu renal elimination with reduced biliary excretion after repeated administration of APAP to rats (Ghanem et al., 2005). This was attributed to increased hepatic basolateral vs canalicular secretion of APAP-glu as a consequence of significant induction of Mrp3 relative to Mrp2. A decreased entero-hepatic

recirculation of APAP was also observed together with decreased drug hepatotoxicity. Although the dose of estrogens in contraceptive pills is much lower than that administered to rats to induce cholestasis, women consume contraceptive estrogens chronically and an induction of MRP3 can not be discounted. An eventual preferential basolateral vs apical hepatic excretion of APAP-glu may contribute to explain previous findings in women, as reviewed by Kroemer and Klotz (1992).

In conclusion, we observed up-regulation of Mrp3 by EE administration in rats, leading to enhanced sinusoidal efflux of APAP-glu. This may represent a compensatory mechanism to prevent intracellular accumulation of common Mrp substrates as a consequence of reduced Mrp2 activity.

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FOOTNOTES

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FIGURE LEGENDS

Fig 1. Immunodetection of Mrps and UGTs.

Transporters were detected in mixed plasma membranes and UGTs were detected in microsomal membranes. Equal amounts of total protein (15 µg for Mrp2 and UGTs detection and 30 µg for Mrp3 detection) were loaded in all lanes. These amounts 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. Data on densitometric analysis represent means ± SD of 3 rats per group.

* Significantly different from controls, $p<0.05$.

Fig 2. Detection of Mrp3 by immunofluorescence.

The images show *in situ* detection of Mrp3 at the basolateral domain in liver cells from control (A) and EE-treated rats (B). Pictures are representative images from at least 3 independent experiments per group. White bar represents 40 µm.

Fig 3. APAP-glu excretion in the isolated perfused liver.

The transport of APAP-glu, a common substrate for Mrp2 and Mrp3, was evaluated in the isolated perfused liver. The panels represent the biliary excretion of APAP-glu with time (top) or perfusate by 60 min (middle), and the APAP-glu hepatic content at the end of the experiment (60 min) (bottom). Inset in the upper panel represents the cumulative biliary excretion of APAP-glu by 60 min. Data are means ± SD of 3-4 rats per group.

* Significantly different from controls, $p<0.05$.

Table 1. Biliary secretory function.

	Control	EE
Bile flow (µl/min/g liver)	2.00 ± 0.17	0.98 ± 0.41*
Bile salt secretory rate (nmol/min/g liver)	58.2 ± 9.7	36.7 ± 15.1*
Biliary excretion of total glutathione (nmol/min/g liver)	2.53 ± 0.46	0.38 ± 0.26*
Hepatic content of total glutathione (nmol/g liver)	5984 ± 562	5391 ± 773

Results are expressed as mean ± SD (n = 3). * Significantly different from control group ($p<0.05$).

Fig 1

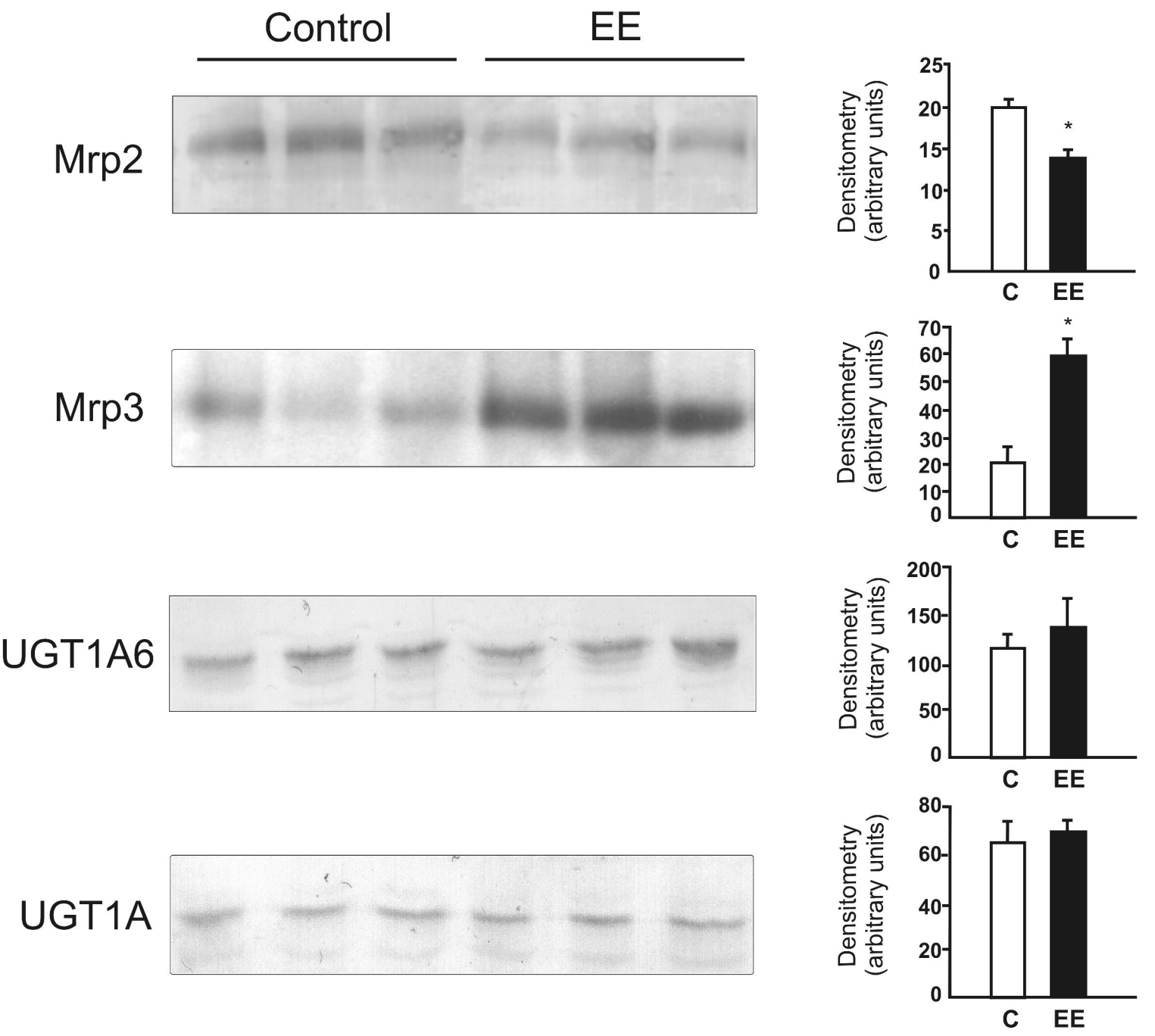
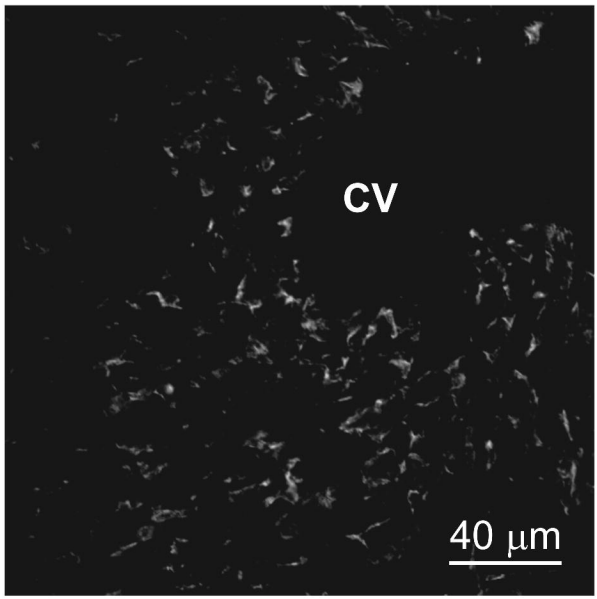

Control
EE
Mrp2
Mrp3
UGT1A6
UGT1A
Densitometry (arbitrary units)
25
20
15
10
5
0
C
EE
*
70
60
50
40
30
20
10
0
200
150
100
50
80
60
40
20

Fig 2

Control



EE

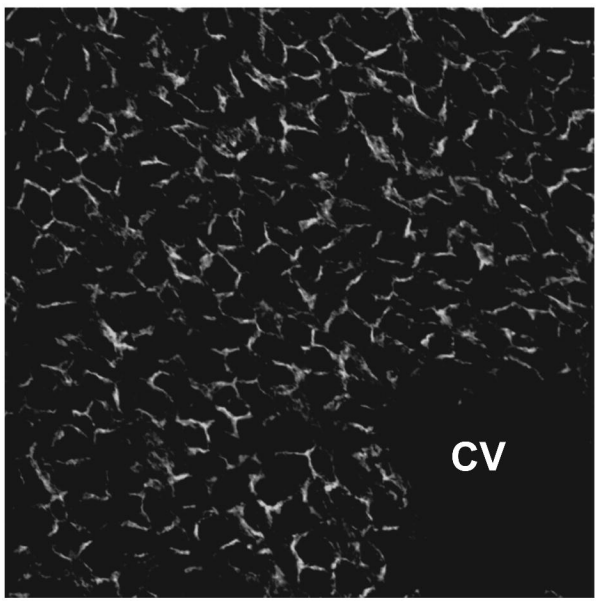


Fig 3