

Protein expression of kidney and liver bilitranslocase in rats exposed to mercuric chloride—A potential tissular biomarker of toxicity



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HIGHLIGHTS

- We examined BTL expression in liver and kidney from HgCl₂ treated rats.
- Mercuric treatment induced a decrease in BTL protein expression.
- BTL is postulated as a new tissular biomarker of mercuric toxicity.

ARTICLE INFO

Article history:

Received 27 October 2013

Received in revised form

18 November 2013

Accepted 20 November 2013

Available online 25 December 2013

Keywords:

Bilitranslocase

Nephrotoxicity

Hepatotoxicity

Mercuric chloride

Tissue biomarker

ABSTRACT

Bilitranslocase (BTL) is a plasma membrane carrier that transports organic anions of physiological and pharmacological interest. It is expressed in basolateral plasma membrane of kidney and liver. BTL has been recently described as a marker of transition from normal tissue to its neoplastic transformation in human kidney. Inorganic mercury is a major environmental contaminant that produces many toxic effects. Previous reports have described an interaction between BTL and mercuric ions. This study was designed to evaluate the renal and hepatic expression of BTL in rats exposed to a nephrotoxic and hepatotoxic dose of HgCl₂. Male rats were treated with a single injection of HgCl₂ at a dose of 4 mg/kg body wt, i.p. (HgCl₂ group). Control rats received the vehicle alone (Control group). Studies were carried out 18 h after injection. Afterwards, the kidneys and livers were excised and processed for histopathological studies or immunoblot (homogenates and crude membranes) techniques. In rats treated with HgCl₂, immunoblotting showed a significant decrease in the abundance of BTL in homogenates and plasma membranes from kidney and liver. BTL decrease of expression might reflect the grade of damage in renal tubule cells and in hepatocytes. Thus, BTL might be postulated as a new biomarker of tissue toxicity induced by mercury.

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1. Introduction

Mercury (Hg) is a highly toxic metal that results in a variety of adverse health effects including neurological, renal, hepatic, respiratory, immune, dermatologic and reproductive effects. It is found in the environment in several physical and chemical forms: elemental mercury (Hg⁰), inorganic mercury (mainly mercuric chloride, HgCl₂) and organic mercury (mainly methylmercury, CH₃Hg⁺, and ethylmercury, CH₃CH₂Hg⁺). Hg⁰, CH₃Hg⁺ and CH₃CH₂Hg⁺ can be converted to inorganic mercury inside the body. The mercuric ion, Hg²⁺ is the main toxic species

in liver and kidney (Zalups, 2000; Torres, 2013). HgCl₂ induces acute kidney injury in humans and experimental animals where glomerular hemodynamics and, more specifically, the pars recta of the proximal tubule are affected. The injury is most prominent in the pars recta (S2 and S3 segments) at low doses, with involvement of pars convoluta of the proximal tubule and distal segments of the nephron at higher doses (Zalups, 2000; Stacchiotti et al., 2003; Torres, 2013).

HgCl₂ also accumulates in the liver, causing oxidative stress, mitochondria depolarization and presumably uncoupling leading to ATP depletion, bleb formation, and ultimately cell death (Nieminen et al., 1990; Ghosh and Sil, 2008; Bashandy et al., 2011).

Bilitranslocase (BTL) is a plasma membrane carrier located in the sinusoidal domain of hepatocytes (Sottocasa et al., 1996). It has also been found to be expressed in the absorptive epithelia of the gastrointestinal tract (Passamonti et al., 2003, 2009) and in the kidney. In the kidney, BTL was detected in the basolateral

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domain of proximal tubular cells by immunohistochemistry (Elias et al., 1990). It has been shown that BTL has an active role in the transport of many organic anions through the cell membrane. BTL binds a variety of substrates like bilirubin, nicotinic acid, bromosulfophthalein (BSP) and anthocyanins (Passamonti et al., 2009). The expression and function of BTL in renal and hepatic cells under physiological and pathological conditions has not yet been fully elucidated. In this connection, we have recently demonstrated an increased expression of BTL in kidneys from rats with extrahepatic cholestasis (Brandoni et al., 2010). Moreover, Montanic et al. (2013) have recently described BTL as a marker of transition from normal tissue to its neoplastic transformation in human kidney.

The aim of the present work was to evaluate if the expression of BTL is altered in kidneys and livers from rats exposed to mercuric chloride.

2. Materials and methods

2.1. Experimental animals

Male Wistar rats aged from 110 to 130 days old were used throughout the study. Rats were treated with a single injection (i.p.) of HgCl_2 at a dose of 4 mg/kg body wt (w/v in 1 mL saline/kg) (HgCl_2 group) (Goering et al., 2000; Stacchiotti et al., 2004; Torres et al., 2011). Control rats received the vehicle alone (1 mL saline/kg) (Control group). The studies were performed 18 h after the injection. All animals were allowed free access to a standard laboratory chow and tap water, and housed in a constant temperature and humidity environment with regular light cycles (12 h) during the experiment. All experiments were conducted according to NIH Guide for the Care and Use of Laboratory Animals.

Different sets of animals were used for: biochemical determinations, histopathological studies, preparation of homogenates and plasma membranes from kidneys and livers and renal clearance studies. On the day of the experiment the animals were anesthetized with sodium thiopental (70 mg/kg body wt, i.p.).

2.2. Biochemical determinations

On the day of the experiment, blood was collected from the heart in heparinized syringe and urine was collected from the urinary bladder with a syringe immediately after opening the abdominal cavity. Plasma samples were used to measure urea and aspartate aminotransferase (AST) levels as indicative parameters of global renal and hepatic function, respectively. Plasma urea and aspartate aminotransferase levels were determined employing commercial kits (Wiener Laboratory; Rosario, Argentina). Total mercury determination in urine samples was performed using an Atomic Absorption Spectrophotometer Perkin Elmer AAnalyst 300 measurement by cold vapor, Flow Injection Analysis System, FIAS 100 – Perkin Elmer (Littlejohn et al., 1975). The Hg^{2+} was reduced by the treatment with stannous chloride to Hg^+ , which was then measured by cold vapor atomic absorption at 254 nm with a Hg monitor. The gas used was argon. In order to avoid sulphide and chloride interferences the urine samples were pretreated with potassium permanganate and with hydroxylamine hydrochloride, respectively. Standards for Hg^{2+} were prepared daily from appropriate dilution of the stock solution produced by solving HgCl_2 p.a. in a nitric acid solution (1.354 g/L). The detection limit of the instrument was estimated to be 1 $\mu\text{g}/\text{L}$.

2.3. Histopathological studies

Histopathology of livers and kidneys was performed after fixing in 10% neutral-buffered formaldehyde solution for 4 h and embedding in paraffin; 4-mm thick sections were processed for routine staining with hematoxylin-eosin.

2.4. Preparation of kidney and liver plasma membranes

Total plasma membranes were isolated from kidneys as previously described in our laboratory (Di Giusto and Torres, 2009). Liver plasma membranes were obtained as previously described (van Amelsvoort et al., 1978; Brandoni et al., 2010). Aliquots of the membranes were stored immediately at -80°C until use. Each preparation represents the kidney or the liver of one rat, respectively. For each experimental group, four different preparations were prepared. Protein quantification of samples was performed using the method of Sedmak and Grossberg (1977).

2.5. Electrophoresis and immunoblotting

Homogenates and crude membranes from kidney and liver were boiled for 3 min in the presence of 1% 2-mercaptoethanol, 2% SDS (sodium dodecyl sulfate). Samples were applied to an 8.5% polyacrylamide gel, separated by SDS-PAGE, and then electroblotted to nitrocellulose membranes. To verify equal protein loading and transfer between lanes Ponceau Red was employed as previously described (Hazelhoff et al.,

2012; Bulacio and Torres, 2013). The nitrocellulose membranes were incubated with 5% nonfat dry milk in phosphate-buffer saline containing 0.1% Tween 20 (PBST) for 1 h. After being rinsed with PBST, the membranes were then incubated overnight at 4°C with a non-commercial rabbit anti-sequence antibody against BTL previously used in other published studies (Brandoni et al., 2010). The membranes were incubated for 1 h with a peroxidase coupled sheet anti-rabbit IgG (Amersham, Buckinghamshire, UK) after further washing with PBST. Blots were processed for detection using a commercial kit (Pierce ECL Western Blotting Substrate, Thermo Scientific, Rockford, USA). A densitometric quantification of the Western blot signal intensity of membranes was performed.

2.6. Renal clearance studies

These studies were performed as previously described (Brandoni and Torres, 2009). Rats were anaesthetized as described. Femoral vein and artery were cannulated and a bladder catheter (3 mm, i.d.) for urine collection was inserted through a suprapubic incision. A priming dose BSP (12 mg/kg body weight) in 1.5 mL of buffer phosphate pH 7.40 was administered through the venous catheter. A solution containing BSP (0.05 g/L) and buffer phosphate pH 7.40 was infused through the venous catheter using a constant infusion pump (KDS210; KD Scientific, New Hope, PA) at a rate of 0.1 mL/h/kg body weight. The clearance of BSP and the excreted load of BSP were calculated by conventional formulas for each animal. BSP concentrations in serum and urine were determined spectrophotometrically by alkalization with 0.1 M NaOH. The volume of urine was determined gravimetrically.

2.7. Materials

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were analytical grade pure.

2.8. Statistical analysis

Statistical analysis was performed using an unpaired t test. When variances were not homogeneous, a Welch's correction was used. $p < 0.05$ was considered significant. Values are expressed as means \pm SEM. For these analyses, GraphPad (San Diego, CA) software was used.

3. Results

Body weight, kidney weight, kidney/body weight ratio, liver weight and liver/body weight ratio are shown in Table 1. Kidney/body weight ratio increased significantly in HgCl_2 group in comparison to Control rats. The increase in the kidney/body weight ratio observed in HgCl_2 group is presumably due to edema, as observed in other experimental models of acute renal failure and nephrotoxic acute renal failure (Green et al., 2000; Di Giusto et al., 2008, 2009; Torres et al., 2011).

Urea plasma levels, aspartate aminotransferase (AST) activity in plasma and total mercury levels in urine are shown in Table 2.

Table 1

Body weight, kidney weight, kidney/body weight ratio, liver weight and liver/body weight ratio in Control ($n = 4$) and HgCl_2 ($n = 4$) rats.

	Control	HgCl_2
Body weight (g)	379 ± 10	399 ± 4
Kidney weight (g)	2.69 ± 0.20	3.03 ± 0.12
Body weight/kidney weight ($\times 10^{-3}$)	6.49 ± 0.11	$8.27 \pm 0.31^{\text{a}}$
Liver weight (g)	11.26 ± 0.09	10.69 ± 0.24
Liver weight/body weight ($\times 10^{-3}$)	27.95 ± 0.47	26.72 ± 0.78

Results are expressed as the mean \pm SEM.

^a $p < 0.05$ compared with Control group.

Table 2

Urea plasma levels, aspartate aminotransferase (AST) activity in plasma and total mercury (Hg) levels in urine from Control ($n = 4$) and HgCl_2 ($n = 4$) rats.

	Control	HgCl_2
Urea (g/L)	0.46 ± 0.03	$1.12 \pm 0.20^{\text{a}}$
AST (IU/L)	16 ± 1	$38 \pm 5^{\text{a}}$
Hg ($\mu\text{g}/\text{mL}$)	Non detected	3.91 ± 0.57

Results are expressed as the mean \pm SEM.

^a $p < 0.05$ compared with Control group.

Table 3

Renal clearance (Cl_{BSP}) and Excreted Load of BSP (EL_{BSP}) in Control ($n = 4$) and HgCl_2 ($n = 4$) rats.

	Control	HgCl_2
Cl_{BSP} (mL/min/100 g body wt)	0.0120 ± 0.002	$0.0018 \pm 0.00008^{\text{a}}$
EL_{BSP} ($\mu\text{g}/\text{min}/100 \text{ g}$ body wt)	0.080 ± 0.014	$0.012 \pm 0.0034^{\text{a}}$

Results are expressed as the mean \pm SEM.

^a $p < 0.05$ compared with Control group.

Urea and AST concentrations increased in HgCl_2 rats, confirming the presence of kidney and liver damage, respectively.

Fig. 1A shows kidney histological damage in HgCl_2 group, where tubular dilatation, vacuolated cells and cell desquamation were observed. **Fig. 1B** shows liver histological damage in HgCl_2 group, where radial pattern disruption of hepatocytes and numerous and dispersed zones of fibrosis were observed. These observations corroborated the morphological kidney and liver damage that underwent animals with HgCl_2 treatment, as previously reported (Di Giusto et al., 2009; Di Giusto and Torres, 2010; Bashandy et al., 2011; Wadaan, 2009).

Kidney homogenates (KH), kidney plasma membranes (KPM), liver homogenates (LH) and Liver plasma membranes (LPM) from Control and HgCl_2 animals were subjected to immunoblot analyses for BTL protein. **Fig. 2** shows a decrease in the abundance of BTL in KH and KPM from HgCl_2 as compared with Control rats. As shown in **Fig. 3**, BTL abundance in LH and in LPM from HgCl_2 animals was also significantly lower as compared with Control ones.

Table 3 shows that excreted load of BSP (EL_{BSP}) and renal clearance of BSP (Cl_{BSP}) decreased in HgCl_2 rats as compared with Control ones.

4. Discussion

The human body is continuously exposed to a great variety of xenobiotics, via food, drugs and environment. Excretory organs

such as kidney and liver defend the body against the potentially harmful effects of these compounds by biotransformation into less active metabolites and excretory transport processes. The kidneys and liver are the major routes for organic anion elimination (Srimaroeng et al., 2008; Burckhardt, 2012).

It has been observed that blockade of one elimination pathway (bilateral nephrectomy or bile duct ligation) is followed by a sufficient compensation of drug excretion via the alternative elimination route (Fleck and Bräunlich, 1995; Ahn and Nigam, 2009; Wu et al., 2011). It must also be mentioned that impairment of liver or kidney functions can subsequently cause syndromes characterized by failure of the alternative elimination organ (Fleck and Bräunlich, 1995; Kramer, 1997; Bataller et al., 1998).

Numerous compounds, such as drugs, environmental substances, plant and animal toxins, and metabolites of both foreign and endogenous origins, are classified as organic anions. These compounds can affect or regulate the level of expression of drug transporters (Srimaroeng et al., 2008; Burckhardt, 2012).

BTL is a plasma membrane organic anion carrier located at the basolateral domain of renal tubular plasma membrane, sinusoidal domain of the liver cell and in the gastric mucosa (Elias et al., 1990; Sottocasa et al., 1996). BTL has been indicated as the protein responsible for the liver electrogenic uptake of cholephilic organic anions (such as BSP, thymol blue, bilirubin) and flavonoids (Passamonti et al., 2009). BTL has also been demonstrated to be involved in the renal transport of BSP, bilirubin and anthocyanins (Vanzo et al., 2008). In this way, BTL contributes to the hepatic and renal elimination of exogenous organic anions (such as BSP), endogenous metabolites (such as bilirubin) and anthocyanins (Vanzo et al., 2008).

Inorganic mercury is still widely used in certain types of batteries and continues to be an essential component of fluorescent light bulbs (Clarkson, 1997). Humans and animals can be exposed to mercury through contaminated water and food (Magos and Clarkson, 2006). Dental amalgam is the major source of mercury

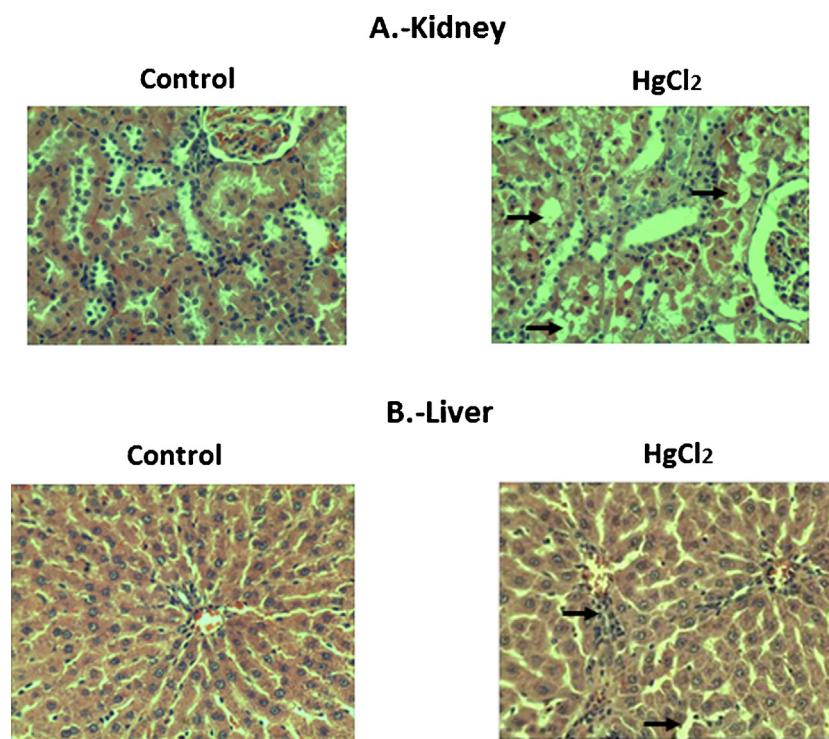


Fig. 1. (A) Kidney histology from Control and HgCl_2 rats (hematoxylin–eosin). Photos are representative of samples obtained from four rats from each experimental group. In HgCl_2 group tubular dilation, vacuolated cells and cell desquamation was observed (arrows). Magnification $\times 200$. (B) Liver histology from Control and HgCl_2 rats (hematoxylin–eosin). Photos are representative of samples obtained from four rats from each experimental group. In HgCl_2 group radial pattern disruption of hepatocytes and numerous and dispersed zones of fibrosis were observed (arrow). Magnification $\times 200$.

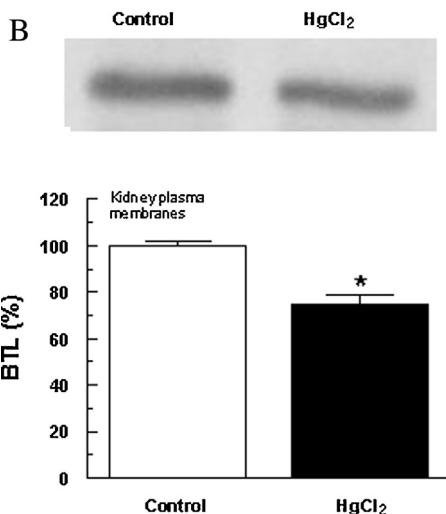
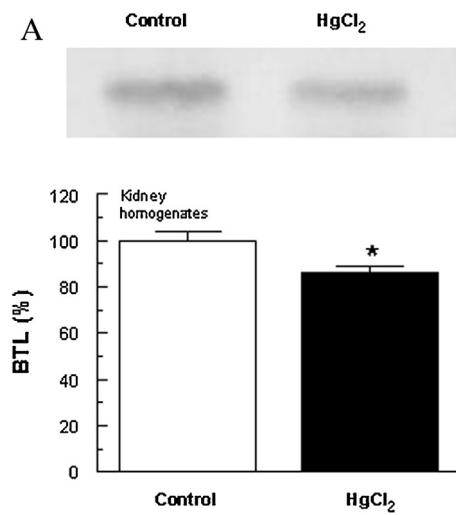


Fig. 2. Western blot analysis for BTL protein in kidney homogenates and in kidney membranes from Control and HgCl₂ rats. BTL was identified using polyclonal antibodies as described in Section 2. Control levels were set at 100%. Each column represents mean \pm SEM from experiments carried out in four different preparations for each experimental group. (*) $p < 0.05$.

vapor exposure in the general population and an association between the number of amalgam fillings and the concentrations of inorganic mercury in blood and urine has been reported (Clarkson, 1997; Magos and Clarkson, 2006).

In addition, mercury species (vapor, inorganic mercury and methylmercury) have been associated with human poisoning in gold mining areas (Magos and Clarkson, 2006).

It has been demonstrated that HgCl₂ acts as a potent toxic that affects numerous organs, including the kidney (Zalups, 2000; Stacchiotti et al., 2003; Di Giusto et al., 2009) and the liver (Nieminem et al., 1990; Ung et al., 2010; Wadaan, 2009; Bashandy et al., 2011).

In the present work we evaluated the renal and hepatic expression of BTL in rats treated with a nephrotoxic and hepatotoxic dose of HgCl₂. A single dose of HgCl₂ was administered 18 h prior to performing the experiments. Plasma urea and AST were measured as indicators of kidney and liver injury. In HgCl₂ group both parameters were increased. Histological damage was detected in kidneys and liver from HgCl₂ animals, thus confirming the nephrotoxicity and hepatotoxicity of the employed dose, as previously described

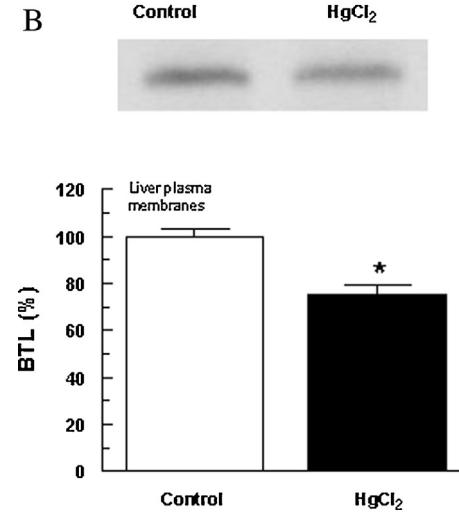
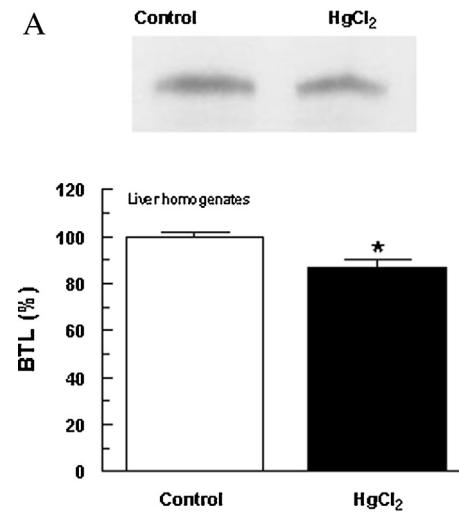


Fig. 3. Western blot analysis for BTL protein in liver homogenates and in liver from Control and HgCl₂ rats. BTL was identified using polyclonal antibodies as described in Section 2. Control levels were set at 100%. Each column represents mean \pm SEM from experiments carried out in four different preparations for each experimental group. (*) $p < 0.05$.

(Di Giusto et al., 2009; Di Giusto and Torres, 2010; Bashandy et al., 2011; Wadaan, 2009).

In this experimental model, the BTL protein expression decreased significantly in liver homogenates and in liver plasma membranes from rats treated with HgCl₂. On the other hand, the exposure to HgCl₂ also decreased the BTL protein expression in kidney homogenates and in kidney plasma membranes. This could be explained by a decrease in the protein synthesis or an increase in the degradation both in liver and kidneys as it was demonstrated for other transport proteins in this experimental model (Di Giusto et al., 2009; Di Giusto and Torres, 2010). Di Giusto et al. (2009) have described lower protein levels of Oat1 and Oat3 in basolateral membranes and lower Oat1 and Oat3 mRNA expression from kidneys of rat treated with HgCl₂ (5 mg/kg body wt, s.c.). Moreover, a decrease in the Oat5 mRNA and in Oat5 protein levels was also described in kidneys from rats in this experimental model (Di Giusto and Torres, 2010). Nevertheless, it is also possible that the overall-lower expression of BTL in both the liver and the kidneys might be due to the loss of intact parenchymal cells as documented by the histological analysis.

The relationship between renal and hepatic excretion of xenobiotics depends on the integrity of the elimination mechanisms.

Impairment of liver and kidney function can be followed by compensation via the alternative elimination route, changing the relationship between renal and hepatic excretion of drugs (Shitara et al., 2005; Kusuhasha and Sugiyama, 2009; Nigam et al., 2007). In this connection, it has been described an up-regulation of several transporters (such as BTL, BBBP, Oatp1 and Mrp2) in kidneys from rats with obstructive cholestasis which explained the dramatic increase in BSP renal elimination described in this pathology (Brandoni et al., 2006a, 2006b, 2010, 2012; Brandoni and Torres, 2012; Tanaka et al., 2002; Villanueva et al., 2006). BSP, an organic anion principally excreted in bile, is secreted negligibly by the kidney in non-pathological conditions (Fleck and Bräunlich, 1995). So, it was of interest to determine if the renal elimination of an organic anion preferentially excreted by the liver such as BSP would be altered after the hepatotoxicity caused by the treatment with HgCl₂. In this study we found a decrease in the excreted load of BSP and in the renal clearance of BSP. This could be explained by the decrease of BTL protein expression in kidney plasma membranes or by the irreversible inactivation of BTL-mediated transport by mercury ion, causing additional reductions in the renal tubular uptake of BSP. The depletion of free glutathione caused by HgCl₂ would favor the reaction of Hg ions with BTL protein thiols. The importance of thiol groups for the transport function of liver BTL is well documented (Passamonti et al., 1998). Moreover, the decrease in renal Oat3 expression previously described in HgCl₂ treated rats (Di Giusto et al., 2009) could also contribute to the lower renal BSP elimination, since BSP is also a substrate for Oat3 (Vanwert et al., 2010).

In case of mercury intoxication, the following effects favoring the cell survival (Sabolic, 2006; Aleo et al., 2005; Di Giusto et al., 2009) could be activated by remaining intact proximal tubule cells and in hepatocytes: increase of the intracellular levels of protective thiols, up-regulation of genes encoding for specific efflux pumps, capable of removing the Hg-GSH conjugates from the cells (Mrp1 and Mrp2), and down-regulation of genes encoding for transporters that mediate the mercury uptake into cells (Oat1 and Oat3). As said above, BTL decrease of expression might reflect the grade of necrosis (loss of cells). Thus, BTL might be suggested as a marker of tissue integrity. In this connection, Goljanin et al. (2008) have shown that BTL was severely down-regulated in clear cell renal carcinoma and Montanic et al. (2013) have recently described that a monoclonal mice anti-bilirubinase antibody, might be used as a research tool to assess BTL as a marker of transition from normal tissue to its neoplastic transformation in human kidney. The results obtained in the present work suggest that the change in protein levels of BTL might be postulated as a tissue biomarker of severe toxicity caused by mercury.

In summary, we demonstrated that mercuric toxicity in male rats induces a decrease in BTL protein expression in kidney and liver; and a marked decrease in the renal elimination of BSP. These results demonstrate that the compensation mechanisms in the excretion of xenobiotics between liver and kidneys are not put in evidence when the integrity of both elimination mechanisms is altered as in the case of mercury toxicity. The study of changes in the expression of BTL and other organic anion transporters in liver and kidney is of interest not only for understanding the molecular mechanisms that are triggered in these organs from exposure to mercury but also to identify potential therapeutic targets and potential new biomarkers of tissue integrity. In this case, BTL might be postulated as a new tissue biomarker of toxicity induced by mercury.

Conflict of interest

The authors state that there are no conflicts of interest.

Acknowledgements

This study was supported by the following grants: Fondo para la Investigación Científica y Tecnológica (FONCyT), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Universidad Nacional de Rosario (UNR).

The authors also thank Wiener Lab Argentina for analytical kits.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.toxlet.2013.11.022>.

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