Format for ANSWERING REVIEWERS

August 27th, 2013

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name:3342-review.doc).

Title: Regulation of hepatic EAAT-2 glutamate transporter expression in human liver cholestasis

Author: Mustapha Najimi[1], Xavier Stéphenne[1], Christine Sempoux[2] & Etienne Sokal[1]

Name of Journal: *World Journal of Gastroenterology*

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The manuscript has been improved according to the suggestions of reviewers:

Reviewer 1

The authors investigated glutamate excitatory amino-acid transporter 2 (EAAT2), expression in cholestasis. EAAT2 has been extensively investigated before in nervous tissue but this is the first report concerning human liver cell line. Congratulations for the authors for selecting such topic. They investigated the effect of PMA-induced PKC-mediated experimental cholestasis on the EAAT2 expression in HepG2 and demonstrated its down-regulation which was abolished by using PKC inhibitor. On the other hand, they demonstrated EAAT2 up-regulation in rat with ligated bile duct and in human cholestatic liver disease. The authors clearly demonstrated the alteration of glutamate handling by EAAT2 in cholestatic liver disease in humans both at the intrahepatic level as in PFIC and extrahepatic level as in BA. The study design is clear and the methodology used is appropriate and clearly described. They used different methodologies to confirm the results; substrate uptake assay, western blotting, IF, IHC and RT-PCR. Figures are clearly expressing the results in logical sequence that is in accordance with scientific thinking. The conclusion is strongly based on the obtained results from the experiments.

Comments to the author:
Major

1- EAAT2 expression was focal in PFIC2 (Fig 7B) and membranous in PFIC3 (Fig 7D). The authors need to explain the elevated levels of GGT in PFIC3 and its low levels in PFIC2. This point need to be elaborated.

Hepatocytes are the principal liver cells producing bile (a complex fluid containing salts and phospholipids). Bile is delivered it into the intestine via the bile ducts where acting like detergents in order to dissolve fat and help vitamins absorption. Alteration of gene expression in PFIC causes poor bile flow including salts (in PFIC1 deficiency) or phospholipids (PFIC3 deficiency). The consequent accumulation of such bile substances in the liver leads to hepatic damage and cholestasis. In PFIC3 (MDR3 deficiency) GGT is present in large quantities in the canalicular and

bile-duct membranes. When those are damaged, GGT is released into the bile. GGT then leaks across the wall of the biliary tract into the blood and the concentrations of GGT activity in the serum rise. GGT is lacking in the canalicular membranes in PFIC-1 reason why it is not released. In PFIC-2, bile acids are not normally pumped into bile although GGT is present in the canalicular membranes. Without the detergent action of bile acids, GGT cannot be released.

The information has been added in the discussion part

2- What factors might affect the localization of EAAT2 in human liver cells? Please discuss this point in details.

According to its important intermediary metabolic role in liver ammonia detoxification, gluconeogenesis and acid-base balance, the control of hepatic glutamate transport may significantly modulate its availability and the related intrahepatic metabolic processes. Indeed, glutamate transport across the liver sinusoidal membrane is currently recognized to be important for controlling liver nitrogen flux by modulating the perivenous glutamine synthesis (Haussinger, 1990). The activity of glutamate transporters in liver sinusoidal membranes is influenced by a variety of factors in vivo among which those altering plasma amino acid composition or Na^+ electrochemical gradient across the sinusoidal membrane. Modulation of both the degree and the distribution of such amino acid transporter expression within the liver may enhance the range of hepatic response to a variety of physiological and physio-pathological challenges to whole-body nitrogen metabolism as demonstrated after starvation, diabetes and glucocorticoids treatment (Low et al, 1992).

The information has been added in the discussion section

3- Scale bar is not indicated on the photographs

The scale bar is now inserted in the figures.

4- Figure 4A, canalicular localization of MRP2, the figure is not convincing

For more clearness, a merged photo of MRP2 and EAAT2 co-immunostaining has been used in figure 4A.

5- Figure 7, magnification seems to be more than 200x in Fig 7C (more like 400x). Please indicate expression by arrows especially in Fig 7A and 7B

We confirm that the magnification is 200x (the size of nuclei is the same for all pictures). Hepatocytes only look bigger in the BA group than other groups.

Minor

1- The authors used DMEM for HepG2 cell culture; was it high or low glucose medium, please indicate the composition of the DMEM, cat. Number and supplier

The basal medium used for HepG2 cell culture is DMEM 4.5 g/L glucose (Invitrogen, ref 41965-039). The information is added in the M&M section

2- What was the type of culture plates? Please provide type and provider details.

The cultures plates used for HepG2 cell culture are provided by Greiner Bio-one (CELLSTAR® flasks).

The information is added in the M&M section

3- In immunoblot analysis, what kind of sample buffer used (reducing or non-reducing?) is the 5% SDS-gel was in-house made or ready-made? Please indicate the provider details in case of ready-made one.

The samples have been suspended in loading buffer containing DTT (125 mM Tris–HCl, 50 mM dithiothreitol, 4% sodium dodecyl sulphate, 20% glycerol, 0.01% bromophenol blue, pH 6.8). The 5% SDS-gels used are home made.

The information has been added in the corresponding paragraph of the M&M section

4- During immunohistochemistry, what was the type of secondary antibody, please indicate provider details.

For immunostaining experiments, EnVision^{+} System- HRP Labelled Polymer Anti-Rabbit (DAKO) has been used the reveal the rabbit polyclonal immunoreactivity.
The information has been added in the corresponding paragraph of the M&M section

5- What was the statistical software used for data analysis?

GraphPad prism was used for statistical data analyses. The information was added in the corresponding paragraph of the M&M section

6- English language, punctuations and typos need to be revised.

The manuscript has now been carefully reviewed and corrections made accordingly

7- The authors sometimes wrote "immunohistochemistry" and others "immune-histochemistry", sometimes "immunostaining" and others "immune-staining", some times "SEM" and others "S.E.M.". Please unify.

Modifications have been inserted

Reviewer 2

This study investigates the regulation of glutamate transporter EAAT2 in liver cells under cholestasis condition. To this end, HepG2 cell culture, liver of bile duct ligated rats, and human cholestatic patients were used. Using a combination of molecular and pharmacological approaches, the authors evidenced that activation of protein kinase C signaling in HepG2 cells decreased both substrate affinity and maximal transport velocity of EAAT2 transporter. Immunofluorescence study evidenced an intracellular translocation of the EAAT2 transporter. Expression of the EAAT2 transporter was instead increased in hepatocytes of rat livers that underwent bile duct ligation, and in human liver specimens manifesting cholestasis. In human samples, the surface expression of the EAAT2 transporter appears to be particularly increased in patient with high level of gamma-glutamyl transpeptidase, as observed in biliary atresia and progressive familial intrahepatic cholestasis type 3. The conclusion of the authors is that a potential cross talk exists between glutamate transporter and bile system. The study is well planned and conducted. The appropriate controls are in place, and the conclusion of the authors is supported by the data reported here. The discrepancy between the results obtained in HepG2 cells and those reported in the other experimental models is not clear. As the authors speculate, this discrepancy may depend on the experimental conditions or models used in the study (i.e. immortalized cells vs. in vivo or ex vivo specimens). No major flaws are noticed in the study.

Minor points:

1. Is there any evidence that under cholestatic conditions or biliary atresia protein kinase C signaling is downregulated in human subjects?

After careful analysis of literature data, no documented information was available so far with respect to PKC down regulation in human liver cholestasis and biliary atresia. Connection between PKC & GGT & Glutathione pathways may lead to potential reciprocal regulation

See also answer below

2. Alternatively, could the redox state of HepG2 cells vs. that the human specimens explain the increased expression of gamma-GT and consequently the different expression of EAAT2 in the various experimental conditions?

The presence of free radicals, oxidative stress, and lipid peroxidation has been confirmed during cholestasis (Sokol et al. 1991, 1998; Parola et al. 1996; Pastor et al. 1997). This was attributed to the pro-oxidant potential of hydrophobic bile acids highly accumulated inside the hepatocytes (Baroni et al.1998; Sokol et al.1998) and a consequent dense inflammatory response (Seto et al. 1998).
An efficient antioxidant defence activity is closely related to maintenance of intracellular levels of glutathione. Because of its poor transport across membranes, glutathione level maintenance is dependent on equilibrium between consumption and de novo synthesis. The latter depends on an adequate supply of precursor amino acids like glutamate a part more likely via transporter activity (Paolicchi, 1996). Indeed, the free glutamate found in excreted bile is formed from the intra-biliary hydrolysis of GSH due to the hydrolytic reaction of GGT (McIntyre and Curthoys 1980). Glutamate formed in the canalicular bile may be transported back into the liver via the identified canalicular glutamate transport systems (Ballatori, 2006). Even the significance is still

unknown, a substantial intra-hepatic cycling of specific biliary constituents was proposed.
This complex interacting pathway (GSH-GGT-Glutamate) may be altered in HepG2 cells according to divergent role of GGT like in tumour progression (Paolicchi et al, 1996).
Furthermore, a relationship between PKC and GSH has been documented (Nitti et al, 2008). Indeed, negative regulation of PKC has been reported consequently to GSH depletion as in oxidative stress. Such depletion has been reported to remove a negative modulation of PKC and to provide a permissive environment for its activity (Ward et al. 1998). A decrease in liver GSH, induced by L-buthionine sulfoximine or diethylmaleate treatment, has also been accompanied by the inactivation of classic PKC isoforms and an increased activity of novel PKC isoforms in particular PKC-δ(Domenicotti et al., 2000).
These information have been added in the discussion part

Reviewer 3

Najimi et al. reported on the effect of PKC activation on EAAT2 localization and glutamate transport in HepG2 cells. They also studied EAAT2 localization under conditions of obstructive cholestasis in rat and human liver. Transport (substrate uptake assay), immunofluorescence, RT-PCR, and immunohistochemistry studies were performed. EAAT2 expression was found to be up-regulated in liver from rats subject to BDL and in human liver samples with chronic obstructive cholestasis. PMA treatment of HepG2 cells, leading to PKC activation, produced opposite effects. The most relevant and novel contribution of the manuscript is the finding on up-regulation of expression of EAAT2 in liver samples. Treatment with PMA of HepG2 cells failed to demonstrate the mechanism of such up-regulation.

Some important issues arise:

Major:

1. It is not clear whether EAAT2 is of basolateral or apical localization. In HepG2 cells the transporter is localized to the basolateral membrane (Fig 4), but the authors state that the background indicates apical localization.

After careful reviewing of the literature data, we come to the conclusion that studies focusing on liver ability to take up glutamate and where these transport systems were localized, still report controversial information. For the studies highlighting a measured glutamate transport activity in the liver, data were collected on both primary monolayer cultures of rat hepatocytes (Gebhardt & Mecke, 1983) and a mixed preparation of plasma membrane vesicles isolated from rat liver (Sips et al. 1982). In these studied models, the detected transport activities were presumed to be localized at the sinusoidal domain of the cell membrane. In these models, all domains of studied liver cell membranes were exposed to the substrate and no functional cell polarity was anymore maintained. Using isolated & purified hepatocyte canalicular and sinusoidal membranes, other authors documented L-glutamate sodium gradient-dependent transport predominantly at the canalicular domain of liver cell plasma membranes (Ballatori et al, 1986).
In our study, substrate was exposed to all membranes of HepG2 cells and the apparent Km (17 µM) of the Na^+-dependent high affinity system measured was quite comparable to what has been detected in primary isolated hepatocytes (21 µM) (Gebhardt & Mecke, 1983).
HepG2 cells also exhibit also significant functional hepatic metabolic activity and present cholestasis symptoms when MRP2 expression is down-regulated. All together these information support the use HepG2 cells in the current study.

All these information have been added in the discussion part

Also, what is the localization in immunohistochemistry photos (see Fig 6 and 7)? This is not clear from the images themselves, and no comment was added to the pictures on this specific point.

For those figures the staining reveals the presence of EAAT2 in all membranes of the hepatocytes after BDL in rat and in human. The information has been more detailed in the results and discussion sections.

Is EAAT2 abnormally localized to the basolateral membrane in HepG2 cells? If so, then the use of HepG2 cells is not appropriate.

See answers above

2. In addition, it is not clear how EAAT2 contributes to regulate glutamate intracellular pool and biliary excretion, and how this function depends on apical vs. basolateral localization. All these issues need to be clarified throughout the manuscript.

Liver is a major site of glutamate synthesis and display all the enzymatic pathways for that task. Accordingly, hepatic tissue concentrations are ~30 times higher than the plasmatic ones leading to believe that sinusoidal glutamate uptake activity is low.
High glutamate concentrations are also found in the bile not mostly originated from direct secretion as a free glutamate. These are generated within the biliary tree by the action of GGT on glutathione secreted into bile (Ballatori, et al, 1986). The glutamate transport system detected in the canalicular membrane may then serve to reclaim some of this amino acid.

See also answer 2 to reviewer 2

2. The order of presentation of the contents in the manuscript should be inverted, showing in first place the up-regulation of EAAT2 in liver samples, since this is the most relevant contribution of the manuscript. Secondly, they should present the model used to demonstrate the mechanism involved.

We do not agree with such proposal because i) the initial aim was to originally demonstrate in vitro in an accepted experimental cell model of cholestasis (Kubitz et al, 2004) that EAAT2 is modulated at the hepatic level, ii) the rationale of performing the in vivo studies was based on the obtained in vitro data in order to support the modulation of glutamate handling in vivo, iii) the pathological specimen used in the in vivo part have different clinical backgrounds which make difficult arguing the selection of the sole in vitro model we used in the current study.

3. It is obvious that treatment of HepG2 cells with PMA is not the right model to explore the mechanism responsible for up-regulation of EAAT2 found in liver samples. The authors should discuss alternative mechanisms or better yet, try to find the right model. Incubation of HepG2 cells (expressing NTCP) with cholestatic bile salts such as chenodeoxycholic or cholic acid is surely a more appropriate model. Regarding incorporation of alternative hypothesis to discussion section to explain such up-regulation, could be oxidative stress generated under cholestatic conditions a plausible explanation? Is EAAT2 indeed regulated by oxidative stress? It is known that bile salts accumulated in cholestatic liver and oxidative stress are causally related.

See answer to reviewer 2

Minor:

1. The English needs to be extensively revised all along the manuscript.

The manuscript has now been carefully reviewed and corrections made accordingly

2. What is the mechanism of action of PAO?

PAO has commonly been used to inhibit internalization of cell surface molecules. It is able at low concentrations (1-20 µM) to block clathrin dependent endocytosis (Gibson et al, 1989; Sturrock et al, 1990; Smit et al., 1995), macropinocytosis (Frost et al, 1989), phagocytosis (Massol et al, 1998) and membrane recycling (Bruneau & Akaaboune, 2006). Because of absence of ultrastructural studies, the exact mechanism of such an inhibition still remains unknown. PAO is a trivalent arsenical able to cross link with vicinal sulfhydryl groups (Frost et al, 1985) to form stable ring structures. Accordingly, it inhibits several other intracellular targets like Rho GTPases (Gerhard et al, 2003) as well as tyrosine phosphatases and depletes ATP stores which may induce actin cytoskeleton disorganization (Retta et al, 1996; Gerhard et al, 2003).

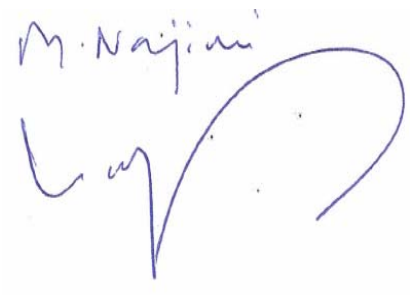
References have been added to support such information (page 11)

3. The term externalization refers to insertion?

Yes. The modification has been made for more clearness (page 11)

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology*.

Sincerely yours,



Mustapha Najimi, PhD
Laboratory of Pediatric Hepatology and Cell Therapy
Avenue Hippocrate 10/1301
1200, Brussels, Belgium
Phone : +3227645283
E-mail: Mustapha.najimi@uclouvain.be