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The impact of serotonergic system dysfunction on the regulation of P4501A isoforms during liver insufficiency and consequences for thyroid hormone homeostasis

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ABSTRACT

This study aimed to evaluate the impact of serotonergic system dysfunction on the regulation of cytochrome P4501A (CYP1A) during liver insufficiency. A rat model of liver insufficiency with a dysfunctional serotonergic system was developed. To induce liver insufficiency, animals were treated with nitrosodiethylamine (DEN) at 50 mg/kg of body weight twice a week for 7 weeks. To induce serotonergic system dysfunction, the animals were fed a tryptophan-free diet for 3 days.

Serotonergic system dysfunction during liver insufficiency generated the aryl hydrocarbon receptor (AhR) activation and the "superinduction" of the AhR target genes: *CYP1A1*, *CYP1B1* and *UGT1A*, with a concomitant increase in CYP1A1 protein and activity. CYP1A2 gene expression was simultaneously down-regulated, with a concomitant decrease in CYP1A2 protein and activity. A significant reduction in TRβ receptor levels, together with a simultaneous increase of TRα receptor gene and protein level (mainly TRα2 isoform) after serotonergic system dysfunction, suggests that the serotoninergic system is involved in the regulation of CYP1A isoforms without influence from thyroid hormones during liver insufficiency.

The interplay between the serotonergic system and the regulation of CYP1A isoforms, which are downstream targets of AhR activation, is dependent on hepatic function and can be observed without influence from thyroid hormones.

1. Introduction

Cytochrome P4501A (CYP1A) is a highly conserved subfamily without important functional polymorphisms that participates in both the activation and detoxification of carcinogens and drugs in the liver (Rodriguez-Antona and Ingelman-Sundberg, 2006). The *CYP1A* gene subfamily comprises three genes: *CYP1A1*, *CYP1B1* and *CYP1A2*. *CYP1A1* and *CYP1B1* are highly inducible in the liver, whereas *CYP1A2* is expressed endogenously and constitutively in the liver (Nelson et al., 1996). Moreover, CYP1A2 mRNA, protein and enzymatic activity levels are decreased during liver insufficiency, e.g., in patients with nonalcoholic fatty liver disease (NAFLD) (Fisher et al., 2009), cholestasis (Klein et al., 2010), or cirrhosis (George et al., 1995).

In fact, the regulation of CYP1A isoforms, which is among the most important mechanisms of protection against mutagenesis, carcinogenesis and toxicity, is controlled by different factors. The enzymes of phase I (CYP1A isoforms, e.g., *CYP1A1*, *CYP1B1* and *CYP1A2*) and II (e.g., UDP-glucuronosyl transferase *UGT1A*) are induced by the aryl hydrocarbon receptor (AhR), which is a ligand-activated transcription factor under the control of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling (Whitlock, 1999; Tan et al., 2002; Chen et al., 2005). Liver-specific transcription factors that are part of the hepatic nuclear factor (HNF) regulatory network are also engaged in the regulation of CYP1A isoforms expression (Cheung et al., 2003; Eeckhoute et al., 2004). In addition, the expression of CYP1A isoforms depends on hepatic function and can be altered by different exogenous (e.g., toxins, drugs) or endogenous (neurotransmitters, hormones) factors. Moreover, different factors such as serotonin (5HT) and vascular endothelial growth factor (VEGF) can activate the same set of signaling kinases, including MAPK/ERK, upon

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stimulation of 5HT receptors (e.g., 5HT2A and 5HT2B receptors), either directly or indirectly via the activation of protein kinase C (PKC), which seem to have an important role in liver function because of the observed putative up-regulation of both the 5HT2A and 5HT2B receptors in response to activation in hepatic stellate cells (HSCs) (Hoyer et al., 1994; Ruddell et al., 2008; Zamani and Qu, 2012). In addition, 94% of serotonin in the body is synthesized outside the central nervous system (CNS) by enterochromaffin cells and is rapidly taken up and stored by platelets (Ruddell et al., 2008). Moreover, the studies in rats have demonstrated that the serotonergic system is an unknown earlier factor that can influence the activity and protein levels of CYP1A isoforms in the liver (Kot and Daniel, 2009; 2011; Kot et al., 2012). The depletion of serotonin using *p*-chloroamphetamine (PCA, a serotonergic neurotoxin), *p*-chlorophenylalanine (PCPA, an inhibitor of serotonin synthesis) or a tryptophan-free diet contributed to increased CYP1A2 protein and activity. Furthermore, *p*-chlorophenylalanine has been found to increase the serum concentrations of total thyroxin in rats and thyroidectomized animals treated with triiodothyronine, which suggests some role for the serotonergic system in the regulation of thyroid hormone homeostasis (Masalova and Sapronov, 2009).

As mentioned above, several factors can influence the regulation of CYP1A isoforms; nevertheless, a possible mechanism linking the serotonergic system and the induction of CYP1A isoforms during liver insufficiency has not yet been elucidated. Because liver insufficiency is associated with an increased risk of NAFLD, as well as predisposition to hepatocellular carcinoma, the aim of this study is to evaluate the impact of serotonergic system dysfunction on the regulation of cytochrome CYP1A isoforms during liver insufficiency, as well as the consequences for thyroid hormone homeostasis.

To induce liver insufficiency, animals were treated with nitrosodiethylamine (DEN). DEN is one of the most potent chemical hepatocarcinogens that is also formed *in vivo* after the ingestion of conventional foods, such as bacon or spinach (Fine et al., 1977). To induce serotonergic system dysfunction, the removal of dietary tryptophan was employed. The rate of serotonin synthesis is controlled by the availability of tryptophan, a naturally occurring amino acid found in foods, such as turkey and cheese, that binds to albumin in plasma (Culley et al., 1963; McMenamy, 1965). The administration of a tryptophan free-diet during over a three-days period cannot influence the plasma level of serotonin, in parallel with a lack of changes in the protein and activity level of CYP1A2 isoform (Kot et al., 2012). Thus, 3 days of tryptophan absence from the diet provides a great opportunity to specifically assess the role of the peripheral serotonergic system in the regulation of CYP1A isoforms, particularly during liver insufficiency, which is often associated with high concentrations of plasma serotonin (Culafic et al., 2007; Kot, 2015).

2. Materials and methods

2.1. Animals

Male Wistar rats (280 g) were purchased from Charles River, Hanover, Germany. The rats were housed in groups of four in standard cages under controlled light conditions (a 12-h light-dark cycle) in an acclimatized room. Animals were provided free access to water and rat chow and allowed to acclimate to the holding room for 5 days before the experiment. All procedures used in this study were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Local Bioethical Committee at the Institute of Pharmacology, Polish Academy of Sciences, Krakow, Poland.

2.2. General experimental design

Animals were divided into two groups: group A (N = 14) was phosphate-buffered saline (PBS)-treated, and group B (N = 24) was DEN-treated. DEN-treated animals were injected intraperitoneally with DEN (diethylnitrosamine) at 50 mg/kg of body weight, twice a week for 7 weeks. Next, the animals underwent a washout period for ten days to eliminate the acute effects of DEN. Then, group A was divided into two subgroups: subgroup A-diet received a tryptophan-free diet (C1069-free tryptophan diet, Altromin, Germany), whereas subgroup A-control received a control diet (C1069 control diet, Altromin, Germany) during the three following days. In parallel, group B was divided into two subgroups: subgroup B-diet received a tryptophan-free diet, whereas subgroup B-control received a control diet. All animals were weighed twice a week. The animals were sacrificed after a 3-day period of a dysfunction serotonergic system induced by a tryptophan-free diet. The livers were collected and stored at −80°C until use. Approximately 10 ml of the blood/sample was collected. All samples were obtained between 9.00 and 11.00 to minimize fluctuation in hormones. The blood was allowed to clot for 2 h at 4°C (39.2°F) and then centrifuged at 1073×*g* for 25 min at 4°C (39.2°F). Serum was isolated and stored at −20°C prior to use.

2.3. Chemicals

Diethylnitrosamine (DEN), PBS, serotonin (purity ≥ 98%), caffeine (BioXtra) and its metabolites, 7-ethoxyresorufin (purity HPLC ≥ 95%), resorufin (purity HPLC ≥ 95%), sucrose (BioUltra), NADPH (purity HPLC ≥ 93%), EDTA (BioUltra), $MgCl_2 \cdot 6H_2O$ (ACS) were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA). All other chemicals and solvents used were of high performance liquid chromatography (HPLC) grade and were purchased from Merck (Darmstadt, Germany). TEMED and other reagents used for Western blotting and electrophoresis were purchased from Bio-Rad Laboratories (Hercules, California, USA).

2.4. Histology and liver function tests

Tissue samples were infiltrated with 30% sucrose solution for cryoprotection before sectioning and hematoxylin and eosin (H&E) staining.

The serum concentration of biochemical markers of liver insufficiency, alpha-fetoprotein (AFP), alpha(2)-macroglobulin (M2G), aspartate transaminase (AST) and alanine transaminase (ALT), was determined by an ELISA kit (Cusiabo, Abcam). For the assessment of albumin, an index of hepatic synthesis, real-time quantitative PCR was performed.

2.5. Determination of serotonin in the serum

Serotonin serum levels were estimated using high-performance liquid chromatography (HPLC) with fluorescence detection. The supernatants from pooled untreated serum samples were diluted 1:100, 2:100, 3:100 and 4:100 with H_2O for calibration curve before determination of serotonin (5-HT) in serum. A stock solution of 5-HT (2.5 mM) was made by dissolving 0.5317 mg of 5-HT into 1 ml of H_2O. Stock solutions (0.0025–0.25 μM) for calibration curve were prepared by serial dilutions of the 2.5 mM stock solution with H_2O.

All serum samples (3:100; serum:H_2O) were filtered through a 0.2 μm membrane (1000×*g* × 5 min; Alltech). Immediately after filtration, the supernatants were analyzed for the content of 5-HT and its metabolite 5-HIAA. The serum was injected on an analytical column (C18 250 × 4.6 mm, 5 μm) from Alltech. The fluorescence detection was performed at an excitation wavelength of