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EXPERIMENTAL STUDIES

Hepatotoxic Effects of Acetaminophen. Protective Properties of Tryptophan Derivatives

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Abstract—Rat intoxication with acetaminophen (APAP) (500–1500 mg/kg body weight, intragastrically) caused a considerable dose-dependent decrease in reduced glutathione (GSH) level in both liver cell cytoplasm and mitochondria (at the dose 1500 mg/kg body weight by 60% and 33%, respectively). The decrease in cytoplasmic GSH level was more pronounced than in mitochondria. Despite of significant mitochondrial GSH depletion we did not observe any inactivation of the mitochondrial enzymes: succinate dehydrogenase, α-ketoglutarate dehydrogenase, glutathione peroxidase, and also any decrease in the respiratory activity of liver mitochondria isolated from APAP-intoxicated rats. We have investigated hepatoprotector properties of tryptophan derivatives, melatonin and N-acetyl-nitrosotryptophan (a nitric oxide donor). The pineal gland hormone, melatonin, a known antioxidant (10 mg/kg body weight), did not prevent intramitochondrial GSH, but decreased the APAP hepatotoxicity evaluated as the decrease in the activity of marker enzymes of hepatic damage, ALT and AST and total bilirubin content in blood plasma of intoxicated rats, whereas NNT did not exhibit any hepatoprotective effects.

Key words: acetaminophen, melatonin, tissue respiration, oxidative stress, hepatotoxicity, hepatoprotectors

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INTRODUCTION

Overdose of acetaminophen (paracetamol; APAP) widely used as an analgesic and analgesic/antipyretic drug causes essential damage of the centrilobular zone of liver [1]. Simultaneously, APAP is used as a model toxin in studies on mechanisms of hepatotoxicity and search for new hepatoprotectors. N-acetylcysteine still remains the most effective clinical tool preventing APAP hepatotoxicity [2].

Mechanisms of APAP hepatotoxicity are widely studied and methods to prevent and correct APAP-induced liver damage are actively developed. It is known that a significant proportion of APAP undergoes conjugation with glucuronic acid or sulphate followed by subsequent excretion, however, some proportion of APAP is metabolized by the hepatic cytochrome P450 system [3]. The latter results in formation of a highly reactive derivative, N-acetyl-*p*-benzoquinoneimine, which rapidly reacts with reduced glutathione (GSH) [3]. Thus, APAP metabolism causes rapid depletion of hepatic glutathione [4]. The reactive APAP metabolite also covalently modifies (arylates) cell proteins [5]. It is suggested that the developing oxidative stress, reactive oxygen and nitrogen species are also involved into damage and death of hepatocytes. For example, there is evidence that peroxynitrite acts as an active mediator of the APAP-induced necrosis of hepatocytes [6]. At the same time, mechanisms of cell death initiated by metabolic conversions of APAP leading to glutathione depletion remain unknown [2]. Knowledge of sequence of events from target modification to necrotic cell death would provide better understanding of hepatotoxicity and modes of its correction. Earlier it was demonstrated that impairments of oxidative phosphorylation in liver mitochondria and therefore energy homeostasis are directly associated with APAP-induced hepatotoxicity [7]. Exposure of isolated mouse hepatocytes to toxic concentrations of APAP (5 mM) resulted specific damage of mitochondrial complexes I and II with simultaneous decrease of mitochondrial and cytoplasmic ADP [7].

The aim of this study consisted in further investigation of mechanisms of APAP hepatotoxicity in rats, search of possible hepatoprotectors, comparison of pathobiochemical processes induced in cytoplasm and mitochondria by administration of large doses of APAP, elucidation of possible applicability of the tryptophan derivatives, melatonin (Mel) and N-acetyl-nitrosotryptophan (NNT) as hepatoprotectors.

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MATERIALS AND METHODS

Forty male Wistar rats (200–250 g) were used in experiments. Animals adapted to the experimental condition of 12 : 12-h light (since 08.00 a.m.) dark (since 20.00 p.m.) cycles for one week and subdivided into 5 groups: control, APAP, APAP + NNT, APAP + Mel, NNT. At 9.00 animals of the groups APAP, APAP + NNT, and APAP + Mel were treated intragastrically (i.g.) with APAP (1500 mg/kg body weight) as 1% starch suspension; animals of control and NNT groups received 1% starch suspension (i.g.). After 4 and 6 h animals of the groups APAP + NNT and NNT received intraperitoneal (i.p.) injection of NNT (2 mg/kg), animals of the group APAP + Mel received i.p. injection of melatonin (10 mg/kg) and animals of control, and APAP groups received i.p. injections of 0.9% NaCl (in equivalent volumes as NNT and melatonin solutions). Melatonin (8.6 mg) was dissolved just before experiment in 150 μl of ethanol and then total volume was adjusted to 2 ml with 0.9% NaCl. NNT (1.86 mg) was dissolved in 90 μl of ethanol and then total volume was adjusted to 2 ml with 0.9% NaCl. Animals were decapitated 24 h after APAP administration and blood and tissues samples were used for analysis.

The following reagents were used in this study: N-acetyl-5-methoxytryptamine (melatonin), succinate (potassium salt), L-glutamate (sodium salt), ADP (disodium salt), sucrose, KH_2PO_4, KCl, $MgSO_4$, NaCl, $NaNO_2$, ethylenediaminetetraacetate (EDTA), Tris-HCl, 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent), ethyl acetate, trichloroacetic acid (TCA). N-acetyl-D,L- tryptophan (Sigma-Aldrich, USA, or Steinheim, Germany), APAP—Panadol (paracetamol, GlaxoSmithKlain)

Synthesis of N-acetyl-nitrosotryptophan. N-acetyl-D,L- tryptophan (526 mg) and sodium nitrate (162 mg) were mixed in a tube containing 20 ml of bidistilled water for 2 h in the dark at room temperature. The resultant yellow color reaction mixture was cooled to 1°C and then 10 ml of 1 M HCl (cooled to the same temperature) was added. The formed yellow sediment was then extracted with ethyl acetate (60 ml, 1°C). The organic layer was separated and resultant solution was evaporated in vacuum at room temperature. The yield of N-acetyl-N-nitroso-D,L-tryptophan was about 500 mg. Purity of the synthesized preparation was controlled by thin layer chromatography and spectrophotometry ($\varepsilon_{335} = 6100\ M^{-1}\ cm^{-1}$).

Mitochondria isolation and registration of mitochondrial respiration. After decapitation and collection of blood samples the non-perfused livers were rapidly removed on ice (0–4°C), dried with filter paper, weighed and homogenized in the isolation medium containing 0.25 M sucrose, 0.02 M Tris-HCl and 0.001 M EDTA, pH 7.2, at 4°C. Mitochondria were isolated by the method of differential centrifugation [8]. The nuclear fraction of hepatic cells was removed by homogenate centrifugation at 600 *g* for 10 min and the resultant supernatant was centrifuged at 8500 *g* for 10 min at 4°C for mitochondria sedimentation. The mitochondrial pellet was washed twice in the isolation medium and resuspended in this medium to a protein concentration determined by the Lowry method [9] of 35–40 mg/ml.

The rate of mitochondrial respiration was registered polarographically [10] using a laboratory-made oxygen Clark-type electrode and a hermetic polarographic cell of 1.15 ml at 26.5°C. The polarographic oxygen electrode was calibrated by air bubbling through the cell (air pO_2) and then with gaseous nitrogen (pO_2 = 0 mm Hg). Mitochondrial suspension (1 mg of protein/ml) was placed in a cell containing the incubation medium (0.125 M sucrose, 0.02 M Tris-HCl, 0.05 M KCl, 0.02 M KH_2PO_4, 0.005 M $MgSO_4$, 0.001 M EDTA, pH 7.5); respiratory substrates (5 mM succinate, 4 mM L-glutamate) and ADP (180 μM) were added after the mitochondrial suspension. The rate of mitochondrial respiration was calculated for the following metabolic states: V_1 – the rate of endogenous (basal) respiration; V_2 – the rate of substrate-dependent respiration; V_3 – the rate of phosphorylation-coupled respiration (after ADP addition); V_4 – the rate of mitochondrial respiration after consumption of added ADP. The following parameters characterizing coupling of mitochondrial respiration and phosphorylation were also determined: the acceptor control ratio ($ACR = V_3/V_2$, the respiratory control ratio ($RCR = V_3/V_4$) and the phosphorylation coefficient ADP/O. The rate of mitochondrial respiration at various metabolic states were expressed as ng-atoms of oxygen/min per 1 mg of mitochondrial protein.

Study of biochemical parameters. Reduced glutathione (GSH) and protein SH group (PSH) content was determined in hepatic mitochondrial suspensions and postmitochondrial fractions by the method of Ellman [11] using the molar extinction coefficient $\varepsilon_{412} = 13600\ M^{-1}\ cm^{-1}$. Mitochondrial suspension (0.1 ml) was mixed with 0.1 ml of 25% TCA and subjected to three freeze-thaw cycles. After centrifugation of samples at 6000 *g* for 3 min an aliquot of the supernatant (0.15 ml) was mixed with 1.2 ml of 0.5 M phosphate buffer (pH 7.8) and 50 μl of the Ellman's reagent (5 mM). The sediment was used for determination of protein-glutathione mixed disulfides (PSSG) as described by Rossi et al. [12]. Determination of GSH concentration in the postmitochondrial supernatant was performed without freeze-thaw procedure.

For glutathione peroxidase assay 0.1 ml of mitochondrial suspension was resuspended in 0.1 ml of H_2O, subjected to three freeze-thaw cycles [13], and then diluted with 10 volumes of the isotonic phosphate buffer (150 mM NaCl, 10 mM Na_2HPO_4, pH 7.4). An aliquot (20 μl) of the resultant sample was used for determination of enzyme activity by the method of

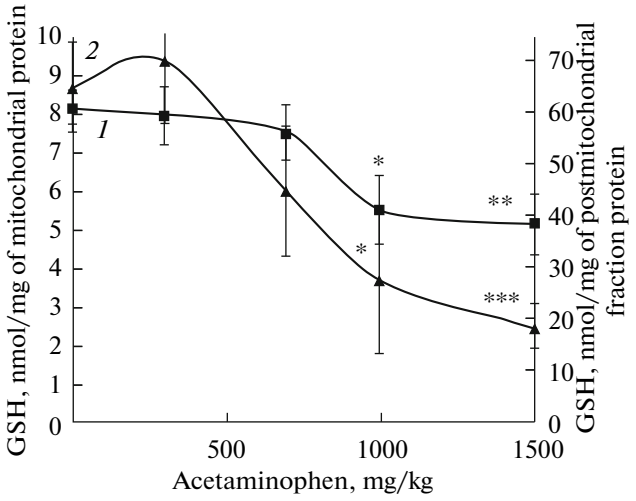

Fig. 1. The toxic liver damage of rats treated with acetaminophen: the dependence of reduced glutathione content in rat liver mitochondria (*1*) and postmitochondrial fraction (*2*) on the dose of the administred preparation. Asterisks show statistical significance versus control: * – $p < 0.05$; ** – $p < 0.01$; *** – $p < 0.001$, respectively.

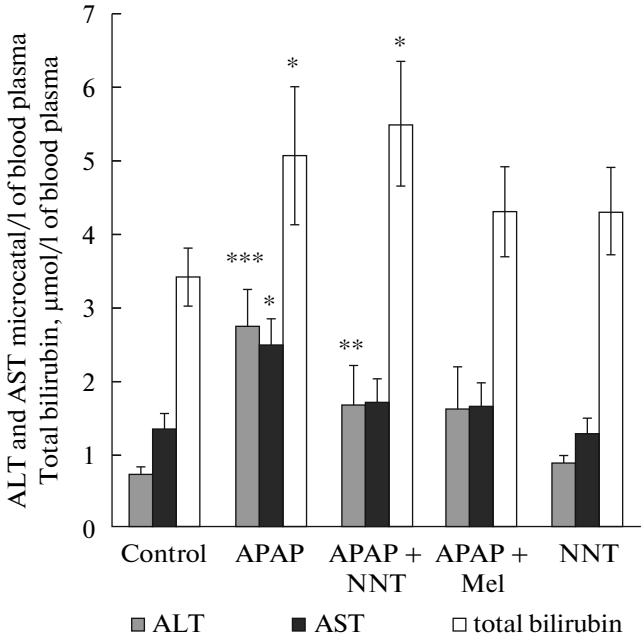

Fig. 2. The protector effect of tryptophan derivatives (two intraperitoneal administrations of 2 mg/kg NNT and 10 mg/kg melatonin) on acute intoxication of rats induced by APAP (1500 mg/kg in 1% starch suspension): alanine aminotransferase activity, aspartate aminotransferase activity and total bilirubin in blood plasma. Asterisks show statistical significance versus control: * – $p < 0.05$; ** – $p < 0.01$; *** – $p < 0.001$, respectively.

Martinez et al. [14]. Catalase activity was assayed by the method of Aebi [15]. Briefly, 1.0 ml of 54 mM H_2O_2 and 2.0 ml of 50 mM phosphate buffer (pH = 7.8) were added to a cuvette and optical density was registered at 240 nm for 1 min at 24°C. Enzyme activity was expressed as micromoles of degraded H_2O_2/min per 1 mg of protein using coefficient of molar extinction of H_2O_2 of 36 M^{-1} cm^{-1}.

Mitochondrial succinate dehydrogenase activity was determined by the rate 2,6-dichlorophenolindophenol reduction, α-ketoglutarate dehydrogenase activity by the rate of NAD^+ reduction [16].

Activity of marker enzymes of hepatic damage, ALT and AST and total bilirubin content in blood plasma were determined using Pliva-Lachema reagent kits (Czech Republic).

Statistical analysis. Resultant values of the registered parameters in the groups studied followed the normal distribution law of variation distribution and they were analyzed by the methods of variation statistics using the Student's t test. Results are shown as the mean of 8–10 determinations ± SD.

RESULTS AND DISCUSSION

APAP intoxication of rats resulted in a dose-dependent depletion of liver cytoplasmic GSH (Fig. 1); the dose of APAP of 1500 mg/kg decreased GSH content by 60% ($p < 0.05$). Simultanelusly we found a marked decrease in liver mitochondrial GSH content, which was not, however, lower than in cytoplasm. At the dose 1500 mg/kg the decrease in mitochondrial GSH was 33% ($p < 0.05$). It should be noted that there are significant differences in cytoplasmic and mitochondrial GSH concentrations; according to our assays, the mitochondrial GSH level represent 12–15% of the cytoplasmic level. It is known that reduced glutathione is synthesized in hepatocyte cytoplasm and then some its proportion is transported into mitochondrial matrix, where GSH plays a decisive role in protection against electrophilic and oxidative stress [17, 18]. Controlling free radical generation in the respiratory chain, GSH determines development of pathological processes and cell death induced by intoxication [17]. Depletion of mitochondrial glutathione may be associated with impairments in its transport from cytoplasm to mitochondria induced by intoxication [19].

Figure 2 shows that the pronounced hepatotoxic effect was observed only at two APAP doses, 1000–1500 mg/kg. Activity of the marker enzymes of hepatic damage, and the level of total bilirubin in blood plasma of APAP (1500 mg/kg) intoxicated rats increased by 3.7-fold ($p < 0.001$), 1.9-fold ($p < 0.05$), and 1.5-fold ($p < 0.05$) in the case of ALT, AST, and total bilirubin, respectively. Earlier Reid et al. [20] postulated the key role of the developed oxidative stress, dissipation of mitochondrial potential, and a sharp increase in mitochondrial permeability transition (MPT) in APAP hepatotoxicity.

At the same time we did not find any evidence for significant damage in rat mitochondrial electron

Table 1. The toxic liver damage of rats treated with acetaminophen: the dependence of mitochondrial oxygen consumption parameters and activity of mitochondrial enzymes on the dose of administered acetaminophen

Parameters, characterizing mitochondrial state	Control	300 mg/kg	700 mg/kg	1000 mg/kg
Succinate dehydrogenase, nmol of succinate/min/mg of protein	51.5 ± 3.1	53.4 ± 5.3	56.0 ± 1.9*	56.0 ± 6.3
α-ketoglutarate dehydrogenase, nmol $K_3[Fe(CN)_6]$/min/mg of protein	34.0 ± 3.6	37.6 ± 2.2	36.1 ± 2.9	36.7 ± 6.8
Glutathione peroxidase, μmol GSH/min/mg of protein	0.72 ± 0.06	0.66 ± 0.09	0.82 ± 0.13	0.83 ± 0.11
ADP-dependent oxygen consumption (substrate: succinate), ng-atoms of oxygen/min/mg of protein	131 ± 24	158 ± 26	139 ± 24	151 ± 21
ADP-dependent oxygen consumption (substrate: glutamate) ng-atoms of oxygen/min/mg of protein	53.4 ± 16.3	57.4 ± 19.3	62.8 ± 22.1	58.3 ± 19.2
Respiratory control ratio (succinate)	4.6 ± 1.0	5.4 ± 2.0	6.1 ± 2.8	5.5 ± 2.1
Phosphorylation coefficient (succinate)	1.26 ± 0.23	1.15 ± 0.17	1.33 ± 0.20	1.26 ± 0.24

Note: Asterisk shows statistical significance versus control: * – $p < 0.05$.

Table 2. The content of protein sulfhydryl groups (PSH), protein-glutathione mixed disulfides (PSSG) in liver mitochondria and catalase activity in the liver postmitochondrial fraction of rats acutely intoxicated with acetaminophen and the protector effect of tryptophan derivatives

Parameters characterizing redox balance of liver cells	Control	APAP	APAP + NNT	APAP + Mel	NNT
PSH nmol/mg of protein	125 ± 8	123 ± 14	122 ± 11	118 ± 9	123 ± 10
PSSG nmol/mg of protein	0.76 ± 0.3	0.43 ± 0.23*	0.62 ± 0.32	0.63 ± 0.16	0.62 ± 0.25
Catalase, μmol H_2O_2/min/mg of protein	349 ± 84	204 ± 73*	261 ± 58*	229 ± 51*	349 ± 125

Note: Asterisk shows statistical significance versus control; * $p < 0.05$.

transport chain induced by APAP intoxication (Table 1). Although earlier it was found that 50 μM N-acetyl-*p*-benzoquinoneimine, a product of APAP metabolism, specifically inhibited mitochondrial succinate dehydrogenase (respiratory chain complex II) [7], we did not find any decrease in the activity of this enzyme in liver mitochondria isolated from APAP-intoxicated rats (up to the APAP dose of 1500 mg/kg) (Table 1). Also we did not observe any intoxication-induced inhibition of one of the key enzymes of the Krebs cycle, α-ketoglutarate dehydrogenase (Table 1). Moreover, at the doses 700–1500 mg/kg APAP caused a some increase (8–10%) in succinate dehydrogenase and α-ketoglutarate dehydrogenase activities.

The rate of state 3 respiration with succinate as a substrate increased by 20%; this resulted in the increase in respiratory control ratio (V_3/V_4) (Table 1). The phosphorylation coefficient remained unchanged. The latter suggests absence of significant changes in respiratory activity of liver mitochondria from APAP-intoxicated rats.

It should be noted that we did not find any significant decrease in the content of protein SH- groups in the liver postmitochondrial fraction from APAP-intoxicated rats (Table 2). At the same time, during intoxication the content of liver glutathione-protein mixed disulfides decreased by 45% ($p < 0.05$). This may be attributed to decreased liver GSH content due to formation of glutathione conjugates with the APAP metabolite, N-acetyl-*p*-benzoquinoneimine rather than formation of oxidized glutathione GSSG. It is possible, that under these conditions there was no (significant) conjugation of the reactive APAP metabolite with cysteine residues of hepatic proteins. Intoxication insignificantly influenced (or even slightly increased) glutathione peroxidise activity but caused a marked inhibition (by 40%, $p < 0.05$) of liver mitochondrial catalase activity (Table 2). According to present findings, administration of toxic amounts of APAP causes necrotic (oncotic) death of hepatic cells, which is developed due to impaired functional activity of mitochondria and ATP depletion, impairments in calcium homeostasis, intensive DNA fragmentation, modification of cell proteins, and proteolysis [2].

One aim of this study was to search for hepatoprotectors preventing the APAP-induced liver damage. Earlier we found a hepatoprotector effect of the pineal gland hormone melatonin in carbon tetrachloride intoxicated rats. Administration of melatonin to the intoxicated rats prevented structural damage of the liver tissue, first of all hydropic and fat dystrophy, necrotic changes and leukocyte infiltration [21]. In this study we have investigated the tryptophan derivatives, melatonin and NTT.

N-acetyl-nitrosotryptophan is a nitric oxide donor. Earlier it was demonstrated that NCX, an NO-deriva-

tive of ursodeoxycholic acid that actively released NO in rat liver but not ursodeoxycholic acid prevented death of animals, necrotic and apoptotic death of hepatocytes, liver accumulation of the inflammatory mediators, IFN-γ, TNF-α during intoxication of mice caused by APAP administration (330 μmol/kg) [22]. Using mouse hepatocyte cell culture authors demonstrated that APAP-induced cell death correlated with hyperpolarization of mitochondrial membranes followed by their subsequent depolarization and cytochrome *c* translocation from mitochondria to cytosol, and procaspase-3 and -9 cleavage [22]. In our experiments pretreatment of rats with NNT and melatonin insignificantly influenced liver GSH oxidation in the intoxicated rats. The most significant effect of the administered tryptophan derivatives was the decrease in APAP-hepatotoxicity caused by melatonin: administration of melatonin caused a 1.7-fold decrease in ALT activity and 1.2-fold decrease of total bilirubin in blood plasma compared with intoxicated rats without such treatment (Fig. 2). In addition, administration of NTT and melatonin to APAP-intoxicated animals restored the level of protein-glutathione mixed disulfides (Table 2).

CONCLUSIONS

In accordance with numerous studies APAP intoxication resulted in a marked depletion of cytoplasmic and intramitochondrial GSH by 60 and 33%, respectively, at the dose of APAP of 1500 mg/kg. It should be noted that more pronounced depletion of cytoplasmic GSH compared with mitochondrial GSH possibly suggests increased formation of glutathione conjugates with N-acetyl-benzoquinoneimine, a product of APAP degradation formed by the microsomal oxidation system.

The decrease of hepatolysis (which accompanies APAP-induced intoxication) and increased levels of blood plasma ALT, AST, and total bilirubin, caused by melatonin represent the most important effect of tryptophan derivatives tested in this study.

In our experiments we did not find significant impairments of liver mitochondrial respiratory chain activity in rats intoxicated with APAP (1500 mg/kg). In spite of significant depletion of mitochondrial GSH we did not observe any inactivation of mitochondrial enzymes, succinate dehydrogenase, α-ketoglutarate dehydrogenase, and glutathione peroxidise.

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