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Research Article

Effect of Dietary Protein Deficiency on the Activity of Cytochrome P450 Enzyme Systems in the Liver of Rats of Reproductive Age Under Acetaminophen-Induced Injury

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Abstract

In this study, we aimed to evaluate the effect of dietary protein deficiency on the activity of cytochrome P450 enzyme systems - p-hydroxylation, N-demethylation, N-oxidation in the liver of rats of reproductive age under acetaminophen-induced injury. Within this topic, we investigated the content of cytochrome P450 and the rate of its inactivation in the inactive form of P420, as well as the intensity of generation of superoxide anion radical under experimental conditions.

During the experiment, the experimental animals consumed a semi-synthetic diet AIN-93 in accordance with the recommendations of the American Institute of Nutrition. In order to model the alimentary protein deprivation rats received a low-protein diet daily for 28 days, which contained 1/3 of the generally accepted daily requirement of protein. After four weeks of keeping animals on an experimental diet, acute toxic injury with acetaminophen was modelled. The toxin was administered at 1250 mg/kg of animal weight as a suspension in a 2% solution of starch gel once a day for 2 days.

We found that acetaminophen toxic injury in the study group of rats leads to an increase in CYP450 and significant activation of microsomal monooxygenases in the liver with a simultaneous redistribution of hydroxylation and oxidation reactions in favor of oxidative N-dealkylation and N-oxidation, accompanied by excessive formation of NAPQI as opposed to non-toxic 3-OH-APAP.

At the same time, acetaminophen toxic lesion of protein-deficient animals is accompanied by weakening of detoxification potential of the liver. We have shown a decrease in p-hydroxylase and N-demethylase activity against the background of direct N-oxidation of drug xenobiotic, as evidenced by the growth of N-oxygenase activity. The decrease in CYP450 content under these experimental conditions is associated with an increase in the rate of its inactivation and transition to the inactive form, cytochrome P420.

It should be noted that the administration of toxic doses of acetaminophen is a key factor in the intensification of superoxide generation, regardless of the amount of protein in the diet.

Keywords: Cytochrome P450; P-Hydroxylation; N-Demethylation; N-Oxidation; Superoxide Anion Radical; Acetaminophen; Alimentary Protein Deficiency; Liver; Rats of Reproductive Age

Introduction

Today, due to various socio-economic (unbalanced diet, consumption of proteins of low biological value, diets, vegetarianism), medical (diseases of the digestive and excretory systems, postoperative and hypercatabolic conditions), technological (loss of essential nutrients during food production) factors the issue of development and course of nutrient-associated pathologies is particularly acute. Exogenously induced metabolic disorders can underlie the emergence and deepening of pathobiochemical processes of different etiologies, as well as lead to the formation of endogenously induced nutrient deficiency states [1,2].

Protein-energy deficiency in randomized studies in recent years has been recognized as a virulence factor in the severe form of COVID-19 because it is accompanied by a decrease in antibody production, which leads to a longer persistence of the virus [3,4]. It is believed that the low pool of available proteins also results in a decreased amount of functional active immunoglobulins and gut-associated lymphoid tissue (GALT), which play a role in gut-mucosal defense against infection [5]. In addition, the literature [6] notes that the lack of protein in the diet can be considered as one of the causes of induced changes in taste and smell caused by coro-

navirus disease. The peculiarities of the course of the disease have pronounced regularities in different age groups of the population [7]. Therefore, unbalanced nutrition can increase the risk of complications, reducing the functional reserves of the organism and its adaptation potential not only in persons with concomitant pathology, but also with age-related changes [8].

However, in the literature there are only occasional data on the influence of nutritional factors on the risk of hepatotoxic reactions in the intake of medicinal xenobiotics. Hepatotoxicity of drugs is realized in two ways: as a direct cytotoxic effect or individual intolerance. In the first case, the reaction is predictable, stereotypic and dose-dependent and is characterized mainly by hepatocellular damage. Such a scenario, for example, is realized against the background of acetaminophen (paracetamol) intake [9,10].

In addition, the inclusion of paracetamol in the regimen of first-line antipyretics in the treatment of COVID-19 becomes relevant [11]. Despite the wide use of acetaminophen as an effective analgesic/antipyretic, in high therapeutic doses the drug can cause hepatotoxicity [12,13]. The mechanism of the adverse effect of acetaminophen on the liver is usually complex and, in most cases, insufficiently studied. Development of hepatotoxicity is often the reason for deviations from treatment protocols. Paracetamol toxicity is due to the accumulation in hepatocytes of a highly reactive intermediate metabolite - N-acetyl-p-benzoquinone imine (NAPQI) under the action of cytochrome P450 enzymes (CF 1.14.14.1, CYP450) [12].

In the literature [14] there is evidence that the biotransformation of paracetamol in phase I and II changes with age due to differences in the activity of the main metabolic pathways. For example, during neonatal and infantile age, the rate of glucuronidation is lower than in adults, with a compensatory increase in the sulfation rate. There are significant differences in CYP450 maturation depending on the isoenzyme spectrum. Evidence is that young children have relative immaturity in CYP2E1 metabolism, which significantly reduces NAPQI formation.

Although the mechanism of acetaminophen toxicity is well known, clinical and biochemical consequences of age-related differences in biotransformation of the xenobiotic can't always be assessed. In this regard, we investigated the activity of cytochrome P450 system in the liver of reproductive age rats under the condition of acetaminophen-induced lesion against the background of dietary protein deficiency.

Materials and Methods

Animals

Experiments were conducted on white, nonlinear rats weighing 170-200 g (n = 110). All animals were of reproductive age (130-150 days), which corresponds to the developmental stage of the human body at 23-25 years [15].

All animal procedures were performed according to international recommendations of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasburg, 1986), «General Ethical Principles for Animal Experiments», approved by the First National Congress on Bioethics (Kyiv, Ukraine, 2001). Animals were kept in sterile conditions in plastic cages with sandy bedding and *ad libitum* access to water.

During the experiment, animals consumed a semi-synthetic diet AIN-93 in accordance with the recommendations of the American Institute of Nutrition on the principle of pair-feeding (Table 1) [16].

Casein was used as a complete protein containing the whole complex of essential amino acids. Given that the index of the biological value of casein is 0.8 due to deficiency of sulfur-containing amino acids, L-cysteine was additionally added to the diet as an amino acid supplement.

The acute toxic injury was induced by oral administration of acetaminophen in the form of a suspension in a 2% starch gel solution at a daily dose of 1250 mg/kg for the last 2 days of the experiment by gastric intubation [17].

The research model involved dividing the animals into groups

- Animals kept on a semi-synthetic diet balanced for all nutrients - control group (C).
- Animals kept on a semi-synthetic low-protein diet for 28 day prior to the experiment (1/3 of the normal daily protein requirements) (LPD);
- Animals that received acute acetaminophen-induced toxic injury (TI) after being fed a complete semi-synthetic diet
- Animals with acute toxic injury modeled against the background of alimentary protein deficiency (LPD/TI).

Cervical dislocation of the studied animals under light ether anesthesia was performed on days 29 and 31 of the experiment.

Isolation and evaluation of purity of subcellular fractions

Microsomal fraction of liver cells was precipitated according to the method, described in [18]. The degree of contamination of the microsomal fraction by membranes of other cellular compartments was assessed by comparative determination of the activity of 5'-nucleotidase (EC 3.1.3.5) as a specific marker of plasma membranes and succinate dehydrogenase (EC 1.3.5.1) as a marker of mitochondria inner membrane. The experiments used a microsomal fraction with a purity of at least 90%.

Enzymatic activity

p-Hydroxylase activity of cytochrome P450 was estimated by determining the rate of p-hydroxylation of aniline by the amount of p-aminophenol formed [18]. The reaction mixture contained: 40 mM Tris-HCl buffer, pH 7.3, 16 mM $MgCl_2$, 3 mM NADPH, about 2 mg of microsomal protein. The reaction was initiated by the addition of aniline. Samples were incubated at 37°C for 20 min with constant shaking. After deproteinization with 15% TCA, 10% Na_2CO_3 and 2% phenol in 0.2 N NaOH solution were added to the supernatant. The extinction of the experimental samples was determined on a CARY 60 spectrophotometer (USA) at λ = 630 nm, considering a molar extinction coefficient of 13.3 $mM^{-1}\times cm^{-1}$.

N-oxygenase activity of cytochrome P450 was estimated by the amount of formed N-oxide dimethylaniline capable of reduction at pH 2.5 to dimethylaniline. The latter was transformed into *p*-nitrosodimethylaniline with an absorption maximum at 420 nm when $NaNO_2$ was added. Composition of the reaction mixture: 40 mM Tris-HCl buffer, pH 7.6, 16 mM $MgCl_2$, 3 mM NADPH, about 1.5 mg of microsomal protein. The reaction was initiated by the addition of dimethylaniline. After incubation the reaction was stopped by addition of 0.9 N $HClO_4$. The pH of supernatant was adjusted to 9.4 using a portable pH tester HI 98103 Checker (Germany). To extract unoxidized dimethylaniline, the experimental samples were extracted with diethyl ether. Subsequently, the pH was adjusted to 2.4 with a 5% TCA solution and 0.1 M $NaNO_2$ was added. The samples were incubated at 60C, thereafter their extinction was measured at λ=420 nm with the molar extinction coefficient of p-nitrosomethylaniline (N, N-Dimethyl-4-nitrosoaniline) being 8.2×10^3 $cm^{-1} \times M^{-1}$.

N-demethylase activity of cytochrome P450 was evaluated by determining the demethylation rate of dimethylaniline by the amount of formaldehyde formed. The content of the components in the reaction mixture and the course of determination by the time the reaction was stopped coincided with that described in determining the N-oxygenase activity of cytochrome P450. The reaction was stopped by adding a mixture of equal volumes of 25% $ZnSO_4$ and saturated $Ba(OH)_2$ solution. The content of formaldehyde in the supernatant was determined by color reaction with Nashe reagent. The color intensity was recorded at 412 nm, taking into account the formaldehyde molar extinction coefficient of 1.5 $mM^{-1}\times cm^{-1}$.

Content and rate of cytochrome P450 inactivation

To determine the content of cytochrome P450, we used the method of Omura and Sato described in the literature [19].

The rate of cytochrome P450 conversion to its inactive form (cytochrome P420) was determined by recording differential absorption spectra of reduced hemoprotein carboxycomplexes after barbotage of experimental samples with CO for 1 min followed by addition of $Na_2S_2O_6$. The inactivation rate was calculated as the difference of optical absorbance at 420 nm and 450 nm ($\Delta A_{420-450}$) [19].

Intensity of superoxide anion radical generation

The formation of superoxide radical anions (O_{2^-}) was recorded photometrically using nitroblue tetrazolium (NBT) test at 540 nm [20]. To the microsomal fraction in isotonic phosphate buffer was added 18 mM NADPH, incubated at 37°C, followed by 0.2% NST and solvent (chloroform - dimethyl sulfoxide in a 1:2 ratio).

The protein content in the experimental samples was determined by the Bradford method.

Statistical data analysis

Statistical analysis of the data was performed using Microsoft Office Excel 2016 (activation key JXTBB-4NX7D-B2PBT-32HKF-WFG9) and *STATISTICA 10.0*. Post hoc analysis between groups was performed using one-way analysis of variance (ANOVA) with Tukey's test. The values obtained in the groups of experimental animals were compared with the control using Student's t-test. Comparisons of two independent samples with non-normal distributions of values were made using the nonparametric MannWhitney U-test. For this purpose we calculated the arithmetic mean of independent determinations and standard deviations. The data were considered significant when $p \leq 0.05$.

Results

The results of our studies indicate the absence of statistically probable differences in changes in the enzymatic activities of cytochrome P450 as compared with the values of the control in the liver of protein-deficient rats (LPD) (Figure1). At the same time, we found that administration of toxic doses of acetaminophen to animals fed complete (TI) and low-protein (LPD/TI) diets was accompanied by multidirectional changes in *p*-hydroxylase, *N*-demethylase and *N*-oxygenase activities of cytochrome P450 as compared with controls (Figure 1).

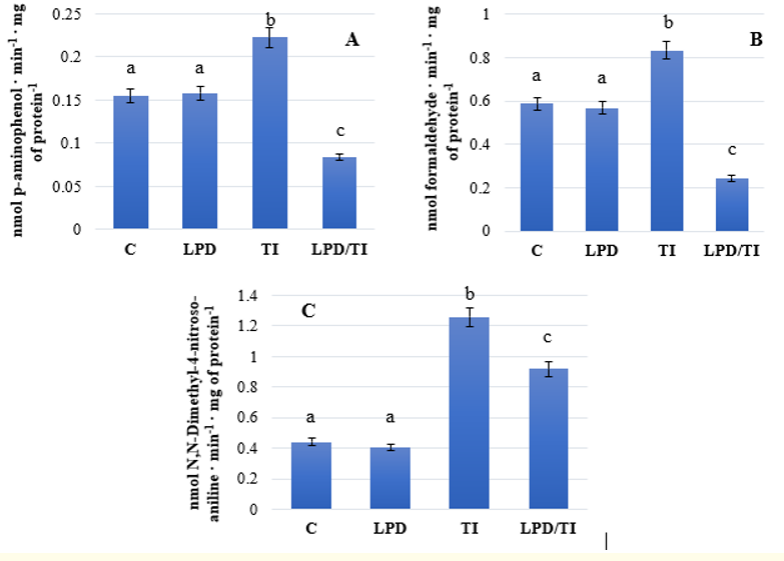

Figure 1: *p*-hydroxylase (A), N-demethylase (B) and N-oxygenase activities (C) of cytochrome P450 activity in the microsomal liver fraction of rats under acetaminophen toxic injury against the background of alimentary protein deprivation.

a, b, c-values denoted by these letter indices are statistically significantly different $P < 0.05$.

In particular, animals with acetaminophen-induced lesions previously maintained on a complete semi-synthetic diet (TI) recorded increased *p*-hydroxylase (30.2%) (Figure 1A), *N*-demethylase (29.4%) (Figure1B), and *N*-oxygenase (63.3%) (Figure 1C) cytochrome P450 activities in the microsomal fraction of rat liver as compared with those of controls.

As to LPD/TI group of animals, microsomal oxidation activity by *p*-hydroxylation decreased compared to the values of the control (45.7%) and the values obtained in the TI group of rats (62.2%) (Figure1A). Thus, the decrease in *p*-hydroxylase activity in protein-deficient rats with toxic lesions as compared to the experimental group of animals that were administered toxic doses of the medicinal xenobiotic and against the background of consuming a complete diet can be considered as a reliable fact of direct dependence of the established changes on the amount of dietary protein.

As to N-demethylase and N-oxygenase activities in the liver microsomal fraction of LPD/TI animals, we recorded their multidirectional changes compared to the control, characterized by a 58.6% decrease in the N-dealkylation reaction rate (Figure1B) with a simultaneous 51.5% activation of N-oxidation (Figure1C).

Thus, the increased p-hydroxylase (Figure1A), N-demethylase (Figure1B), and N-oxygenase (Figure1C) activities of cytochrome P450 in the microsomal fraction of the rat liver with APAP-induced lesion (TI) that we identified are probably associated with an increase in cytochrome P450 (35.9%) compared to controls (Figure 2).

When assessing the content of CYP450 in the experimental group of TI+LPD rats, we recorded a statistically probable decrease in this index compared to the control (43%) and animals

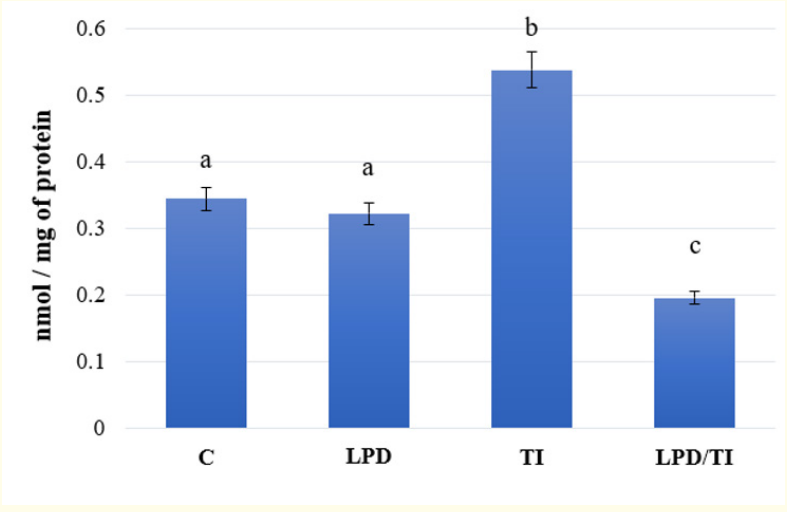

Figure 2: Cytochrome P450 content in the microsomal fraction of the rat liver during acetaminophen toxic injury on the background of alimentary protein deprivation.

with APAP-induced lesions that consumed a diet balanced in all nutrients (63.5%) (Figure2). A consequence of the decrease in cytochrome P450 content is an increase in the rate of its inactivation and transition into the inactive form, cytochrome P420, only in TI+LPD animals (Figure 3).

Therefore, at the next stage of the study, we analyzed the level of superoxide anion radical production in the microsomal fraction of the rat liver. It was found that the introduction of toxic doses of acetaminophen acted as a key factor in the intensification of superoxide generation regardless of the amount of protein in the diet (Figure 4). Thus, in the TI group of animals, the anion radical content increased by 22.3%, and in the TI/LPD group of rats by 26.6% compared with the control values.

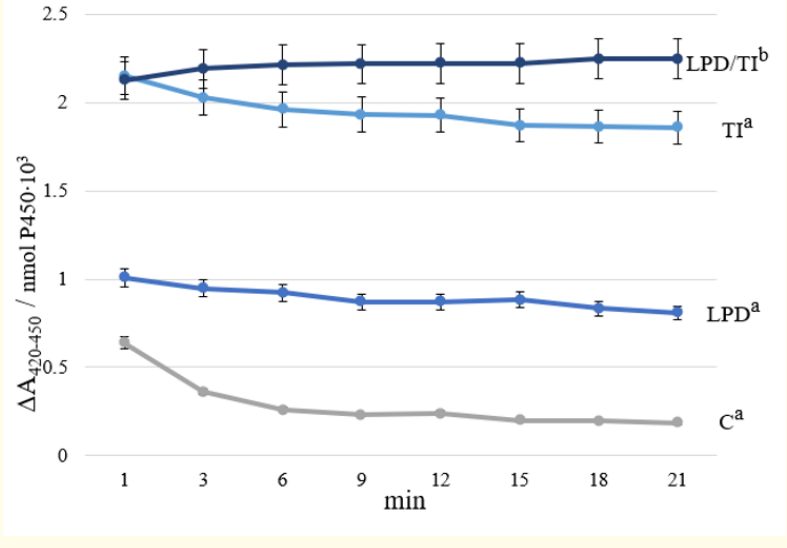

Figure 3: Cytochrome P450 inactivation rate in the microsomal fraction of the rat liver during acetaminophen toxic injury on the background of alimentary protein deprivation.

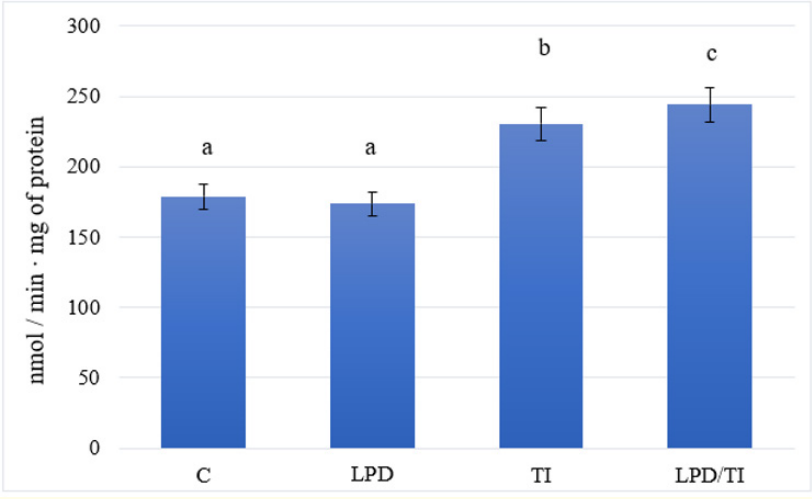

Figure 4: Intensity of superoxide anion radical generation in the liver microsomal fraction of rats during acetaminophen toxic damage on the background of alimentary protein deprivation.

Discussion

The liver and, to a lesser extent, kidneys and intestines are known to be involved in the biotransformation processes of acetaminophen. At therapeutic doses, acetaminophen is preferentially metabolized in phase II biotransformation by glucuronidation (50-70%) and sulfation (25-35%) to form water-soluble metabolites, which are excreted by kidneys; less than 5% of APAP is excreted unchanged [21]; 5-10% of the drug is converted by cytochrome P450 to metabolite NAPQI. [22,23]. NAPQI is detoxified by its binding to the sulfhydryl group of glutathione (GSH) to form APAP-GSH, excreted in the urine as cysteine and mercapturate conjugates (APAP-cys) [21]. When toxic doses of acetaminophen are ingested, the conjugation (glucuronidation/sulfation) pathways become saturated, so most of the xenobiotic is metabolized by the cytochrome P450 system [21,23].

In addition, the literature [24,25] notes that isoenzymes of the monooxygenase system are capable of hydroxylating APAP to form the non-toxic metabolite catechol 3-hydroxyacetaminophen (3-OH-ARP) or oxidize to NAPQI, which has hepatotoxic properties. Thus, the maximum increase in N-oxygenase activity of CYP450 (Figure1C) in the microsomal fraction of the liver of reproductive-age rats with acetaminophen-induced lesion (TI) that we identified indicates enhanced formation of reactive NAPQI as opposed to 3-ON-AR aromatic hydroxylation under these experimental conditions (Figure1A).

The exact contribution of particular CYP isoforms to APAP bioactivation varies and depends on the concentration of the drug. In human liver microsomes, CYP2E1 and CYP1A2 were first reported to convert high doses of APAP to NAPQI [21], that reacts rapidly with reduced glutathione (GSH), leading to depletion of the liver stores of glutathione about 30%, as it was shown in previous studies [26]. Unconjugated NAPQI binds to proteins and sub-cell structures, resulting in induction of apoptosis and development of irreversible hepatocellular necrosis [23,21].

It should be taken into account that demethylation of dimethylaniline is a kind of N-dealkylation of xenobiotics by oxidation [27], which makes a certain contribution to NAPQI formation by increasing N-demethylase activity in the liver of rats injected with toxic doses of acetaminophen (TI) by 1.5 compared to controls (Figure1B).

It is known that one of the possible mechanisms of demethylation is oxidation of amine nitrogen and formation of N-oxide. Since CYP isoenzymes have different specificity and regioselectivity towards acetaminophen [24, 25], it has been experimentally proved that CYP2E1 mainly produces NAPQI, whereas CYP2A6 generates 3-OH-APAP/ NAPQI in a ratio of 3:1. It has been described [28] that activation of CYP2E1, 1A2, and 3A4 enzymes in the liver can lead to increased susceptibility to APAP-induced damage. Given the

changes in CYP450 activity in the liver of mice during the postnatal period [29], it has been suggested that the effect of APAP on P450 expression is age dependent. It has been demonstrated that APAP-induced lesions lead to a decrease in P450 expression (except for CYP2B10) in mice, thus affecting the metabolism and toxicity of drugs that are substrates for these enzymes when coadministered with APAP.

On the basis of the data we obtained, we can conclude that in reproductive-age rats fed a toxic dose of APAP (TI) against the background of a balanced diet, there is a functional redistribution of the rates of hydroxylation and oxidation reactions with a predominance of oxidative N-dealkylation and N-oxidation, which leads to excessive NAPQI formation.

It should be noted that despite the large number of isoforms and specific substrates, microsomal cytochromes P-450 catalyze the hydroxylation reaction in basically the same pattern. At the first stage, the oxidized form of the enzyme associates with the substrate to form an enzyme-substrate complex, as confirmed by spectral changes in the molecule [30,31]. Given that we used aniline as the substrate in this reaction, a type II complex is formed by the interaction of the amino group of the substrate (mainly amines) with the cytochrome P450 heme ferrum atom, forming ferrichemochromes. The heme ferrum in such complexes is in the six-coordinated state, with the oxygen binding site occupied by the substrate nitrogen. [19,32]. Since amino acids, particularly glycine, are the source of C and N used in porphyrin biosynthesis [33], it is possible that protein deficiency under these conditions will act as a key factor in the structural and functional changes of the enzyme-substrate complex.

The results of the changes in N-demethylase and N-oxygenase activity we obtained can be explained as follows. Type I substrates, which is dimethylaniline, interact with the low-spin form of cytochrome P450 [19,32]. Reliably, the decrease in dealkylation reactions (via N-demethylation) established by us is associated with changes in the formation of the type I complex due to disruption of the interaction of the hydrophobic site of the apoenzyme with the nonpolar substrate due to the lack of essential amino acids.

On the other hand, in the literature [21] it is described that prolonged starvation leads to redirection of acetaminophen metabolism from glucuronidation to the oxidation pathway. This can be explained by the fact that the main reactions of metabolism in the liver are shifted towards gluconeogenesis, which reduces the pool of glucogenic substrates for glucuronidation.

Thus, it can be assumed that alimentary protein deprivation enhances hepatotoxicity of acetaminophen after intake at excessive doses by direct N-oxidation.

It is clear that the phenotypic features of the metabolism of drug xenobiotics are determined by the genetic polymorphism of cytochrome P450 catalytic activity [34]. Monooxygenase enzymes, unlike other types of enzymes, are polyfunctional, which is determined by the presence of constitutive and inducible isoforms [35,36]. The inducer of CYP isoform biosynthesis is preferably a xenobiotic [35].

Administration of a subtoxic dose of APAP has been shown to increase CYP2E1 and CYP3A expression in adult rats [37]. Therefore, it can be assumed that the rate of hydroxylation, N-dealkylation, and N-oxidation in this case is due to the expression of the corresponding cytochrome P-450 isoforms in response to toxin intake. On the other hand, it should be noted that degradation of CYP3A occurs by ubiquitin-dependent proteolysis. Since APAP significantly reduces the level of polyubiquitinated CYP3A1/23 by inhibiting protein degradation, this leads to the accumulation of CYP3A [38].

The transition of cytochrome P450 to P420 is due to modification of the SH-groups of amino acids in the active center and is confirmed by our previous studies [39]. The inactive form of P420 is unstable and rapidly loses heme under oxygen, so the formation of P420 is one of the stages of cytochrome P450 degradation [40].

The inactivation of cytochrome P450 that we have identified, accompanied by a transition to a functionally inactive form, is probably associated with the formation of a reactive intermediate NAPQI by direct N-oxidation (Figure1C), which can covalently modify amino acid residues. with heme iron and lead to occlusion of the active site [41,30].

A decrease in cytochrome P450 can lead to abnormalities in the monooxygenase cycle. In this case, not all of the oxygen will be introduced into the substrate molecule; some of it will be released from the substrate-cytochrome P450-oxygen triple complex as a superoxide anion radical. [31,42].

It is believed that ROI formation in the microsomal system of cytochrome P-450 is possible at the 'NADPH - cytochrome P-450 reductase - cytochrome *b5*' site, as well as during the decay of reactive peroxo- (Fe^{3+}-O_2^{-}) and hydroperoxo-complexes (Fe^{3+}-HO_2), (Fe^{2+}-HO_2) [31,42]. Accordingly, the fact that NO-radicals, which compete with O_2^{-} for binding to heme, are effective inhibitors of cytochrome P-450 activity and O_2^{-} and H_2O_2 generation in rat liver microsomes, and is confirmed by our preliminary studies on NO-dependent system activation, should also be considered [43].

Conclusions

Given the role of CYP450 in the biotransformation of acetaminophen in animals, a study of the rate of hydroxylation and oxidation reactions involving microsomal monooxygenases under conditions of alimentary protein deprivation in response to intake of toxic doses of acetaminophen seems relevant.

We found that acetaminophen toxic injury in the study group of rats leads to an increase in CYP450 and significant activation of microsomal monooxygenases in the liver with a simultaneous redistribution of hydroxylation and oxidation reactions in favor of oxidative N-dealkylation and N-oxidation, accompanied by excessive formation of NAPQI as opposed to non-toxic 3-OH-APAP.

At the same time, acetaminophen toxic lesion of protein-deficient animals is accompanied by weakening of detoxification potential of the liver. We have shown a decrease in *p*-hydroxylase and N-demethylase activity against the background of direct N-oxidation of drug xenobiotic, as evidenced by the growth of N-oxygenase activity. The decrease in CYP450 content under these experimental conditions is associated with an increase in the rate of its inactivation and transition to the inactive form, cytochrome P420.

It should be noted that the administration of toxic doses of acetaminophen is a key factor in the intensification of superoxide generation, regardless of the amount of protein in the diet.

The data we obtained are important for understanding the contribution of each of the studied varieties of CYP450 activities to the overall process of biotransformation of toxic doses of acetaminophen during alimentary protein deprivation, as well as possible ways to correct impaired detoxification functions of the liver.

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