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Hepatic ethoxyresorufin O-deethylase activity of liver microsomal cytochrome P-450 (CYP1A1) in young and adult rats treated with paracetamol and β-naphthoflavone

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Abstract

Background: Age-related differences in the ethoxyresorufin O-deethylase (EROD) activity of CYP1A1 and its inducibility in rats may determine the toxic potential of acetaminophen. This study was carried out to compare the effects of acetaminophen (APAP) and β-naphthoflavone (βNF) on CYP1A1 activity in young and adult rats.
Methods: Young and adult rats (n = four / group) were treated with different doses of APAP. Likewise groups of young and adult rats were treated with a single dose of β-naphthoflavone (βNF, 67 mg / kg b.w). EROD was measured in microsomal fraction using resorufin as the substrate.
Results: The results showed that a single i. p. injection of APAP (25 mg / kg B.W.) failed to alter liver microsomal EROD in young and adults. Whereas, in adults treated with 250 and 450 mg APAP / kg B.W, liver CYP1A1 was elevated to about 45 and 60% respectively. The rate of CYP1A1 induction in young rats with single dose of APAP (450 mg/kg B.W) was approximately 32%. Induction in CYP1A1 was noticed 4 h after APAP injection and returned to normal levels in 24 h. The inducibility of CYP1A1 in rats treated with a toxic dose of APAP was comparable to the data obtained from animals treated βNF, 67 mg / kg b.w.
Conclusions: These results together with our previous reports indicate a similar pattern of changes in CYP1A1 in both the age-groups treated with toxic doses of APAP may suggest that the inducible CYP1A1 can equally contribute to protection against liver damage in young and adult rats.

Keywords: Age; CYP1A1; Paracetamol; EROD; Hepatotoxicity

Introduction

Paracetamol or acetaminophen (APAP) is a widely used antipyretic and analgesic drug, which in overdose can cause extensive hepatic and renal damage (Mitchel et al., 1973; Bessems and Vermeulen, 2001). APAP is metabolized primarily by conjugation with glucuronic acid and sulfate in the liver of many species. Less than 5% of the dose is metabolized by hepatic cytochrome P-450 to *N*-acetyl-*p*-benzoquinone imine (NAPQI), a chemically reactive metabolite. According to McGill and Jaeschke (2013), the mechanism of APAP hepatotoxicity is through NAPQI binding to proteins, particularly to mitochondrial

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proteins leading to oxidative stress and mitochondrial dysfunction (McGill and Jaeschke, 2013).

The initial oxidative stress activates JNK in turn; JNK translocates into the mitochondrial permeability transition (MPT) and rupture to the outer membrane causing release of the endonucleases apoptosis-inducing factor (AIF) and endonuclease G (EndoG) which can degrade nuclear DNA.

NAPQI is responsible for glutathione (GSH) depletion and subsequently causes protein binding which is a critical event in the toxicity (Jollow et al., 1973; James et al., 2009; Hinson et al., 1990; Holme et al., 1984). Alternatively the metabolite can be conjugated with GSH and is rendered non-toxic (Allameh and Alikhani, 2002; Gregus et al., 1988).

Earlier, we have reported the effect of age on drug metabolizing factors (Behroozikhah et al., 1989; Allameh et al., 1997). Moreover, we showed that the factors involved in antioxidant system in blood and liver are readily induced in young rats in response to APAP and menadion derivatives (Ansari-Hadipour et al., 2003; Ansari-Hadipour et al., 2004; Dadkhah et al., 2006).

Cytochrome P-450 (CYP) is a group of microsomal hemoproteins that catalyze the oxidative, peroxidative and reductive metabolism of a wide variety of endogenous and exogenous compounds. This superfamily enzyme is divided into families and subfamilies according to homologies in their nucleic acid sequences (Kanamura and Watanabe, 2000). The human CYP1A subfamily consists of two members; CYP1A1 and CYP1A2. The CYP1A1 is primarily expressed in the liver and induced by a series of heterocyclic and polycyclic aromatic hydrocarbons (PAH) (Grover, 1986; Shimada et al., 2002).

The major classes of CYP are known to be involved in APAP bioactivation are 2E1, 1A2, 3A4, and 2A6 (James et al., 2003; Patten et al., 1993). The hepatotoxicity of APAP is dependent on activities responsible for the activation of this drug and the efficiency of detoxification pathways. These enzymes are often induced in response to in vivo administration of foreign compounds in animals.

The inducibility of phase I drug metabolizing enzymes, particularly CYP could be considered as a defence mechanism against liver damages caused by APAP. Although several studies have described the toxic consequences of APAP treatments in adults, the effect of age, particularly young age on resistance/susceptibility to APAP-induced liver damages is not well understood. This study was carried out to find out the differences in APAP-related activation of liver CYP1A1 by APAP and BNF in immature and adult rats and the consequences of CYP1A1 induction and

APAP activation leading to liver injuries.

Materials and Methods

Chemicals

Paracetamol or acetaminophen (APAP), β-nicotinamide adenine nucleotide phosphate, reduced form (β-NADPH), 7-ethoxy-resorufin, Resorufin, β-naphthoflavone (βNF) were purchased from Sigma Chemical Co., USA. Dimethyl sulfoxide (DMSO), 3, 3-methylen-bis (4-hydroxycoumarin), sucrose and dicumarol, were from E. Merck, Germany. All other reagents and solvents used were of analytical grade.

Animals and treatments

Male albino rats of Wistar strain were used throughout this study. Animals were obtained from Pasteur Institute of Iran, Tehran, Iran and maintained in our animal house facilities. Adult animals were 3–5 months of age, weighing 250 ± 10 g and growing rats were 16 ± 4 days old, weighing 25 ± 3 g. The animals maintained on a commercial pellet food and tap water *ad libitum.*

Animals in each age-group were divided into five groups (four rats / group). In the treated groups each animal was given a single i.p injection of APAP dissolved in warm normal saline phosphate buffer (100 mM, pH 7.0). The final dose of APAP injection to young and adult rats was 25, 250, and 450 mg/kg BW A group of rats treated with equal volume of saline phosphate buffer alone was considered as control group. The volume of injection for each young and adult rat was adjusted to 250 and 1000 µl respectively.

Likewise a group of rats were treated with a single dose of βNF (67 mg / kg BW) and considered as the positive control group. At different time intervals (30 min, 4, 6, 12 and 24 h) blood samples were collected by heart puncture under light ether anaesthesia and transferred to small tubes containing citrate buffer, plasma was separated and stored in freezer at −70 ◦C for further use. Liver tissues were also removed and tissues were collected and processed for preparation of microsomal fraction.

Tissue preparation and processing

Liver tissues were excised, rinsed with buffer, cut into pieces with scissors and homogenized in phosphate buffer (100 mM, pH 7.4). Then the homogenate was centrifuged at 9000g for 20 min at 4 ºC. The supernatant was removed and centrifuged at 100000g for 60 min in an ultracentrifuge at 4ºC. The cytosolic fraction was separated and the pellet was rinsed, resuspended in KCl (1.15%) and spun again. The microsomal pellet was resuspended in phosphate buffer (pH 7.4) containing 0.25% sucrose and stored in small aliquots at -70 ˚C. Likewise, microsomal fraction was prepared from the two age-groups pre-treated with βNF

(a classic inducer of CYP) and processed for measuring CYP1A1 activity.

Ethoxyresorufin O-deethylase (EROD) assay

CYP1A1 activity was determined spectro-fluorometrically using EROD assay according to the method described by Smeets et al. (1999) (20). An aliquot (100 µl) of the microsomal fraction was added to 900 µl of EROD reaction mixtures (50 mM Tris buffer pH 7.8, 0.1 M NaCl, 20 µM dicumarol, 4 µM 7-ethoxyresorufin and 3 mM NADPH). The mixture was incubated for 10 minutes at 25ºC and then the increase in fluorescence (excitation: 530 nm, emission: 590 nm) was recorded. Resorufin formation was calculated by comparing the fluorescense intensity with those obtained from a series of standard resorufin solutions.

Other assays

Protein content of the microsomal sample was determined by Bradford protein assay (Bradford 1976), using bovine serum albumin as standard [21]. The liver damage enzyme markers, serum alanine transaminase (ALT) and aspartate amino transferase (AST) were determined by commercial kits (Zist Chemie, Tehran, Iran) using auto- analyzer (Technicon RA-1000).

Statistical analysis

All the samples and standards were run in duplicate and the results are presented as mean ± SEM. Differences between control and treated animals were analyzed using Student's t-test, considering $P < 0.05$ as significant.

Results

Fig. 1 compares the time-course effect of a single low dose APAP (25 mg/kg B.W). These data show that the microsomal CYP1A1 activity (EROD assay) of both the age-groups is unaffected in rats treated with low dose APAP. However, as shown in figure 2, the microsomal EROD activity of both the age-groups was significantly ($P < 0.05$) induced following administration of a higher dose of APAP (250 mg/kg B.W). Induction in CYP1A1 activity was more obvious in young and adult rats treated with a toxic dose (450 mg/kg B.W) of APAP (Fig. 3). Regardless the age, the enzyme activity was increased significantly in rat liver microsomal preparation obtained from rats treated with a single high dose of APAP (450 mg/kg B.W).

When these data are compared with the results obtained from young and adult rats pre-treated with βNF, it was noticed that the changes in CYP1A1 activity at certain time points (4-6 h) after βNF injection is comparable to the data obtained from those treated with a high dose APAP (Fig. 4).

As shown in Table-1, the levels of the serum ALT and AST in untreated animals (control groups) was approximately 5-fold greater in

adults as compared to that in young rats. However, the levels of these enzymes were significantly elevated in rats treated with a single high dose APAP (450 mg / kg). The APAP-related changes in serum AST was limited to only young rats.

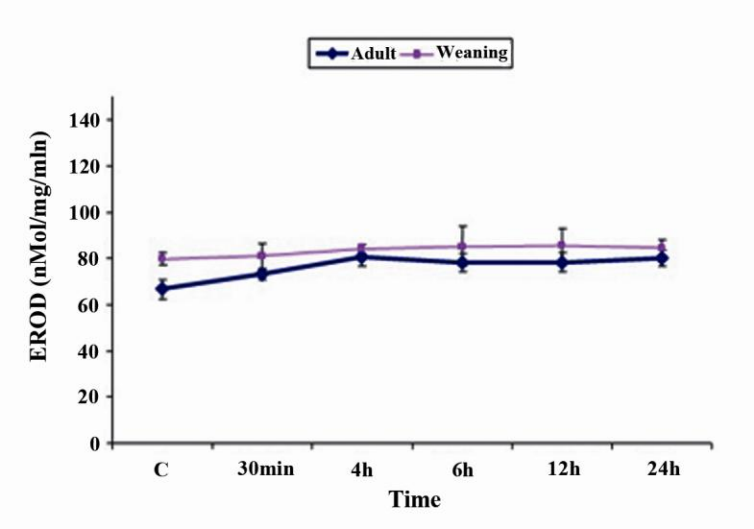

Fig. 1 Time-course changes in EROD in adult and young rats treated with a single low dose APAP.
Data are mean ± S.E.M. of four samples obtained from four animals in each group. Each rat received a single i.p dose of APAP dissolved in warm saline phosphate buffer (PBS). The final dose was 25 mg/kg BW. Controls received equal volume of buffer alone. The final volume of injection for young and adult was 250 and 1000 μl respectively.

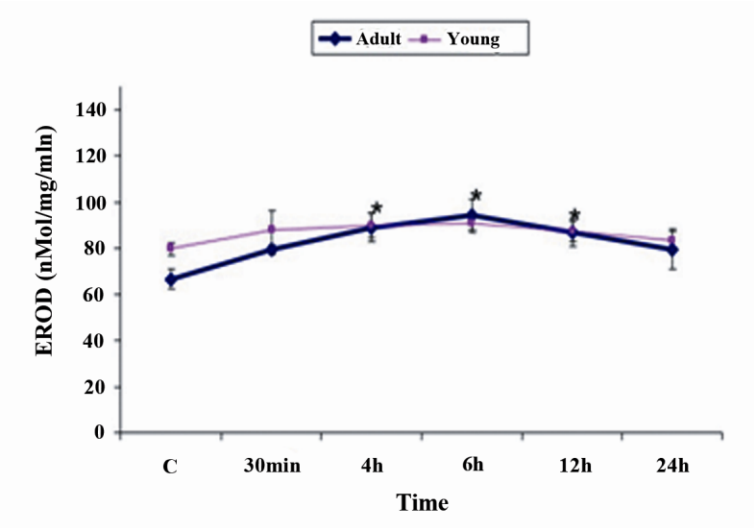

Fig. 2 Time-course changes in EROD in suckling and adult rats under single dose APAP treatment. Data are mean ± S.E.M. of six samples obtained from four animals in each group. Each rat received a single i.p dose of APAP dissolved in warm PBS. The final dose was 250 mg/kg BW. Control groups received equal volume of buffer. The final volume of injection for young and adult was 250 and 1000µl respectively. Asterisk (*) denote significantly different from the respective control group ($P < 0.05$).

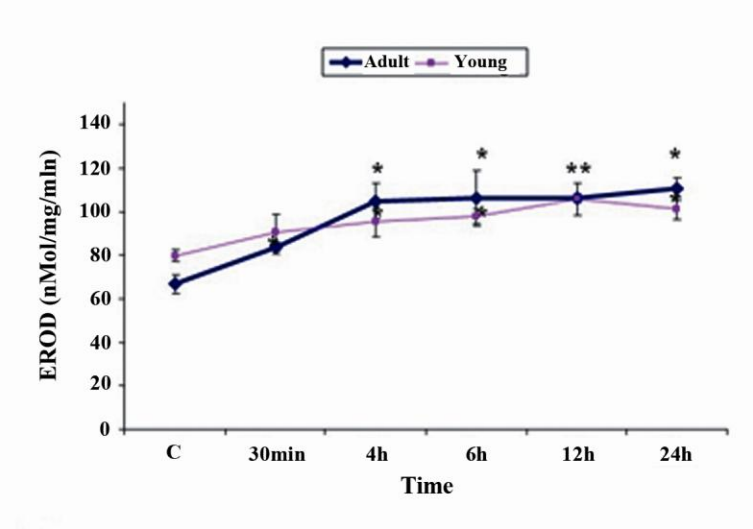

Fig. 3 Time-course changes in EROD in adult and young rats treated with a single high dose APAP. The results are presented as mean ± S.E.M. of four samples obtained from four animals in each group. Each rat received a single i.p dose of APAP dissolved in warm saline phosphate buffer. The final dose was 450 mg/kg BW. Controls received equal volume of buffer only. The final volume of injection for young and adult was 250 and 1000 µl respectively. Asterisk (*) denote significantly different from the respective control group ($P < 0.05$).

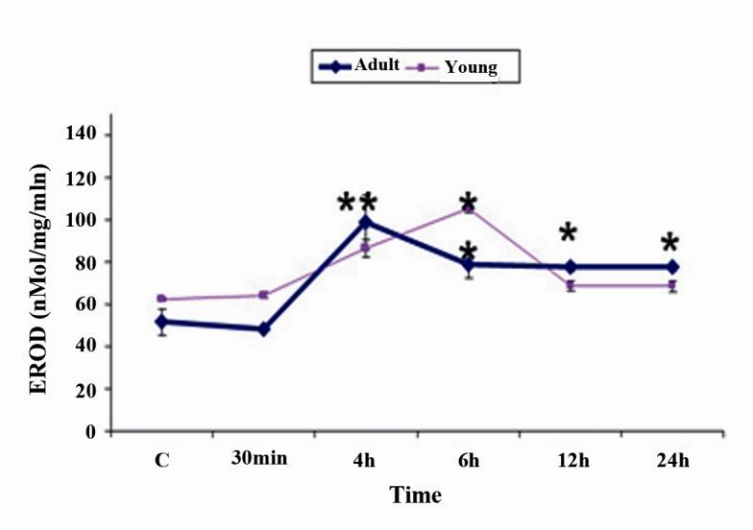

Fig. 4 Time-course changes in microsomal EROD activity in adult and young rats treated with βNF. Data are mean ± S.E.M. of four samples obtained from four animals in each group. Each rat received a single i.p dose of βNF (67 mg/kg BW) dissolved in DMSO and controls received an equal volume of DMSO alone. The final volume of injection for young and adult was 250 and 1000μl respectively. Asterisk (*) denote significantly different from the respective control group ($P < 0.05$).

Table 1 Effect of high dose APAP on serum ALT and AST levels in adult and young rats.

Marker		Young	Adult
ALT	Control	25	120
	APAP	35*	180*
AST	Control	132	375
	APAP	399*	456*

Results are mean ± SEM from four samples obtained from four individual rats. Variation between the samples was <15%. Treated groups were injected with a single dose of APAP (450 mg/kg BW). Asterisk (*) denote significantly different from the respective control group.

Discussion

Previously we showed that liver cytosolic glutathione S-transferase (GST) is differentially induced in growing and adult rats challenged with hepatotoxic agents. This finding was confirmed with different toxic agents such as aflatoxin B1 (Behroozikhah et al., 1989; Fatemi et al., 2006) and APAP (Allameh et al., 1997; Allameh et al., 2000). It was also shown that other defense mechanisms such as antioxidant factors are differentially activated in young and adult rats (Ansari-Hadipour et al., 2003; Ansari-Hadipour et al., 2004; Fatemi et al., 2006).

In the present study, we showed that the EROD activity representing CYP1A1 function is comparable in liver tissues of young and adult rats. The activation of CYP1A1 in young and adult rats was unaffected in response to a low dose APAP injection (25 mg/kg b.w). However, the enzyme was apparently induced in response to a toxic dose of the drug (500 mg/kg b.w). Based on EROD activity changes in liver CYP1A1 in adult and growing rats pretreated with a single dose of APAP (450 mg/kg B.W) was 60% and 32%, respectively as compared to corresponding controls. This data may suggest that the rate of APAP metabolism and formation of its reactive metabolite i.e. NAPQI is relatively higher in adult liver when compared to young rats. Formation of NAPQI alone does not imply the liver damages caused by APAP, since the NAPQI is readily conjugated to cellular glutathione (GSH) via GSH conjugation.

The pattern of the changes in CYP1A1 activity was comparable in young and adult rats. The enzyme activity reached to maximum (plateau value) during 4-6 h of APAP treatment, though the adult animals showed a relatively higher CYP1A1 inducibility in response to a high dose APAP (adult 60% and young 32% induction compared to respective controls).

APAP-related induction in CYP enzymes is probably not limited to CYP1A1, since multiple CYP enzymes are involved in metabolic activation of APAP. According to Kim et al., (2007) other cytochrome isoforms; CYP2E1 and CYP3A which are involved in APAP activation are induced in animals received a single sub-toxic dose of APAP (500 mg/ kg) (Kim et al., 2007). Over-expression of these enzymes in response to APAP treatments is believed to be comparable at both protein levels and catalytical activity (Kim et al., 2007; Kim et al., 2009) indicating a correlation between the APAP-dependent induction in CYP1A1 at protein and catalytic levels. The rate of CYP induction could be responsible for potentiating APAP toxicity in both the age groups, but the age-related differences in phase II drug metabolizing enzymes particularly GSH conjugation pathway can be considered as an important factor in modulation of APAP-

induced liver damages.

As shown in Table 1, although there was no significant differences in liver CYP1A1 induction in young and adult animals, the levels liver damage markers namely; serum ALT and AST were differentially altered in these age-groups. In this connection, increased level of AST (U / dl) in adult rats treated with APAP (450 mg/kg b.w) indicate the differences in liver damages caused by APAP, although the elevation in the level of AST was more in young rats as compared to that measured in adults.

With this limited information on liver damage markers it is difficult to assess the liver damage due to APAP overdose. However, measurement of other factors together with pathological examination of livers can provide further information.

Although there was no significant differences in CYP1A1 and the inducibility in adult and young rats, but induction in this class of enzyme such as CYP1A2 and CYP2E1 can be critical in biotransformation of chemicals known to be specific substrates for CYP1A1 (Shimada et al., 2002). In other words, presumably, the young and adult rats equally handle the hepatotoxic chemicals which undergo first phase of drug metabolism by CYP1A1. Further studies are needed to understand the developmental aspects of drug metabolizing enzymes directly involved in APAP activation and detoxification which may determine the susceptibility / resistance of the animals to the APAP toxicity.

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