Effect of acetaminophen on heme metabolism in rat liver

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Abstract

Background and aims: Acetaminophen (APAP) or paracetamol is a hepatotoxic drug through mechanisms involving oxidative stress. To know whether mammalian cells possess inducible pathways for antioxidant defense, we have to study the relationship between heme metabolism and oxidative stress. Methods: Fasted female Wistar rats received a single injection of APAP (3.3 mmol kg⁻¹ body weight) and then were killed at different times. Heme oxygenase-1 (HO), δ-aminolevulinic acid (ALA) synthase, ALA dehydratase, and porphobilinogenase activities, lipid peroxidation, GSH, catalase and glutathione peroxidase, were measured in liver homogenates. The antioxidant properties of bilirubin and S-adenosyl-L-methionine were also evaluated. Results: APAP increased lipid peroxidation (115% ± 6; S.E.M., n = 12 over control values) 1 h after treatment. GSH reached a minimum at 3 h (38% ± 5) increasing thereafter. At the same time antioxidant enzymes reached minimum values (catalase, 5.6 ± 0.4 pmol mg⁻¹ protein, glutathione peroxidase, 0.101 ± 0.006 U mg⁻¹ protein). HO induction was observed 6 h after treatment reaching a maximum value of 2.56 ± 0.12 U mg⁻¹ protein 15 h after injection. ALA synthase (ALA-S) induction occurred after enhancement of HO, reaching a maximum at 18 h (three-fold the control). ALA dehydratase activity was first inhibited (31 ± 3%) showing a profile similar to that of GSH, while porphobilinogenase activity was not modified along the whole period of the assay. Administration of bilirubin (5 μmol kg⁻¹ body weight) or S-adenosyl L-methionine (46 μmol kg⁻¹ body weight) 2 h before APAP treatment entirely prevented the increase in malondialdehyde (MDA) content, the decrease in GSH levels as well as HO and ALA-S induction. Conclusion: This study shows that oxidative stress produced by APAP leads to increase in ALA-S and HO activities, indicating that toxic doses of APAP affect both heme biosynthesis and degradation. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Heme metabolism; Oxidative stress; Acetaminophen; Bilirubin; S-adenosyl-L-methionine

Abbreviations: ALA, δ-aminolevulinic acid; APAP, acetaminophen; DTNB, 5,5′ dithio-bis-(2-nitrobenzoic acid); MDA, malondialdehyde; NAPQI, N-acetyl-p-benzoquinoneimine; PBG, porphobilinogen; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances.

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1. Introduction

Acetaminophen (APAP), also known as paracetamol, is one of the most extensively employed analgesics-antipyretics worldwide. Because of its minimal gastric toxicity, it has found increasing use in recent years as a substitute for acetylsalicylic acid. APAP is safe at therapeutic doses, but whether accidental or intentional, poisoning with this drug is frequent [1]. The induced APAP hepatotoxicity is already evident, as indicated by increased levels of alanine–leucine-transaminase, 24 h after a single injection to rats of 400–500 mg APAP per kg body weight (3.3–4.0 mmol kg⁻¹) [2] and its toxicity is associated with the depletion of hepatic GSH, followed by covalent binding of the reactive metabolite N-acetyl-p-benzoinoneimine (NAPQI), produced by cytochrome P450, to tissue proteins [3,4]. When GSH levels are low, the metabolite fails to be detoxified by conjugation, it accumulates and causes liver injury [5,6]. Lipid peroxidation resulting from oxidative stress contributes to the initiation and progress of liver damage [7,8] and recently reported data suggest that APAP hepatotoxicity is mediated by an initial metabolic oxidation, covalent binding and subsequent activation of macrophages to form reactive oxygen and nitrogen species [9]. Protection against oxidation is provided by glutathione and also by a system of soluble and enzymatic cellular defenses [10,11].

Heme oxygenase, the rate limiting enzyme in the heme degradation pathway, is induced in animal tissues (particularly liver) by many factors such as heme compounds, metal ions, and others [12–15]. The induction of heme oxygenase is entirely prevented by administration of several antioxidants such as α-tocopherol and allopurinol [15]. Recently, it has been demonstrated that bilirubin is a physiological protector against oxidative stress in the liver of CoCl₂, CdCl₂, CuSO₄ treated rats [16–18], in an ischemia-reperfusion model of rat liver [19], and in neonatal plasma [20]. Antioxidant effects suggest that oxidant species play a major role in the induction of heme oxygenase either directly or by GSH depletion [21,16,15]. An increase in heme oxygenase activity will enhance bilirubin formation. Unconjugated bilirubin is an efficient scavenger of reactive oxygen species (ROS) and its increase could be a response to initial oxidative stress [21,16,22,19].

Of the two known mammal liver heme oxygenase isoenzymes, heme oxygenase-1 (HO) and heme oxygenase-2, only HO is inducible, so that in the present article we will only refer to HO.

Most of the enzymes involved in the heme pathway are subjected to a fine regulation. A dysfunction in one of them leads to diseases generically known as ‘porphyrias’ [23]. Each porphyria is associated with the hypoactivity of one of these enzymes, coupled with the induction of δ-aminolevulinic acid (ALA) synthetase, the rate limiting enzyme in this metabolic pathway, which is regulated by the intracellular heme pool by feedback and repression mechanisms [23]. All of these events lead to the accumulation of intermediates, such as ALA, porphobilinogen (PBG) and/or porphyrinogens, depending on the enzyme affected.

Because several pathological processes involve cellular oxidation, it is of great interest to elucidate whether mammalian cells possess inducible pathways for antioxidant defense.

In view of these considerations, we have carried out a study on the effect of a toxic dose of APAP on the early steps of porphyrin biosynthesis, heme oxygenase induction and the associated oxidative stress, its possible modulation by bilirubin accumulation, the protective effect of S-adenosyl-L-methionine.

2. Materials and methods

2.1. Materials

APAP, NADPH, GSH, GSSG, 5,5’ dithio-bis-(2-nitrobenzoic acid) (DTNB), thiobarbituric acid, glutathione reductase, bilirubin, S-adenosyl-L-methionine, 5-aminolevulinic acid (ALA) were from Sigma Chemical (Saint Louis, MO), tert-butyl hydroperoxide was from Aldrich Chemical (Phillipsburg, NJ); PBG was obtained enzymatically according to Sancovich et al. [24]. All other chemicals were of analytical grade.
2.2. Animals

Fasted female albino Wistar rats (120–150 g) were injected intraperitoneally (i.p.) with a single dose of APAP (3.3 mmol kg\(^{-1}\) body weight). APAP was dissolved in saline solution in a boiling water bath and used after cooling at 37°C. Bilirubin was prepared as follows: solutions in 0.1 M NaOH were freshly prepared before administration, adjusted to pH 7.4 with phosphate buffer and diluted with saline solution. \(\text{S}-\text{adenosyl L-methionine}\) was dissolved in saline solution. Both, bilirubin (5 \(\mu\)mol kg\(^{-1}\) body weight) and \(\text{S}-\text{adenosyl L-methionine}\) (46 \(\mu\)mol kg\(^{-1}\) body weight), were injected i.p. 2 h before APAP treatment. Controls were carried out by injection of saline solution.

3. Methods

3.1. Enzyme preparations and assays

Rats were anesthetized with sodium pentobarbital (50 mg kg\(^{-1}\) body weight, i.p.) and killed 1, 3, 6, 9, 12, 18, 24 and 48 h after injection of APAP. A fraction of the liver (approximately one-third) was excised and immediately homogenized (1:3, w:v) in a solution containing 0.9% NaCl, 0.1 mM per Tris–HCl pH 7.4, 0.5 mM per EDTA, for ALA synthase (ALA-S) determination [25]. The remainder of the organ, previously perfused with ice-cold saline solution, was removed and homogenized in a Potter–Elvehejm homogenizer using different solutions. For heme oxygenase assay, homogenate preparation and enzyme activity determination were performed according to Llesuy and Tomaro [16]. Microsomal HO was obtained as described elsewhere [26]. Catalase and glutathione peroxidase activities were determined spectrophotometrically in liver homogenates prepared in a medium consisting of 140 mM KCl and 25 mM potassium phosphate buffer (pH 7.4), and centrifuged at 600 \(\times\) g for 10 min. The supernatant, a suspension of preserved organelles, was used as homogenate. Catalase activity was determined by measuring the decrease in absorbance at 240 nm [27], glutathione peroxidase activity following NADPH oxidation at 340 nm [28]. ALA dehydratase activity was determined by measuring the formation of PBG at 555 nm [29], while porphobilinogenase activity was determined by measuring spectrophotometrically the formation of uroporphyrins [30] and using the expression defined by Rimington [31].

3.2. Lipid peroxidation

Lipid peroxidation in the liver was determined by measuring the rate of production of TBARS [expressed as malondialdehyde (MDA) equivalents]. One vol. of homogenate was mixed with 0.5 vol. trichloroacetic acid (15% w:v) and centrifuged at 2000 \(\times\) g for 10 min. Supernatant (1 ml) was mixed with 0.5 ml thiobarbituric acid (0.7% w:v) and boiled for 10 min. After cooling, sample absorbance was read spectrophotometrically at 535 nm. MDA concentration was calculated using a \(\varepsilon\) value of 1.56 \(\times\) 10\(^5\) M\(^{-1}\) cm\(^{-1}\) [32].

3.3. Endogenous hepatic GSH content

Total glutathione (GSH plus GSSG) was determined in liver homogenates after precipitation with 2% perchloric acid and using yeast–glutathione reductase, DTNB and NADPH, at 340 nm. GSSG was determined by the same method in the presence of 2-vinylpyridine and GSH calculated from the difference between total glutathione and GSSG [33].

3.4. Protein determination

Protein concentration was evaluated by the method of Lowry et al. [34] using bovine serum albumin as standard.

3.5. Statistics

Figures in the text and tables indicate mean value ± S.E.M. Differences between control and treated animals were analyzed using Student’s \(t\)-test, taking \(P < 0.05\) as significant.
4. Results

Administration of APAP to female rats at dosages of 4 mmol kg$^{-1}$ resulted in 25% mortality within 24 h of injection [2], hepatic APAP concentration can be calculated to be about 2 μmol g$^{-1}$ liver, 4–6 h after, and then decreasing. The dose here used is considered to be toxic, non-lethal and recovery is expected to occur within 18–24 h after treatment.

Time course studies on the effect of APAP on lipid peroxidation and hepatic GSH content are shown in Fig. 1. As indicated by MDA levels, lipid peroxidation increased reaching a maximum (115% ± 6) at 1 h after APAP injection, decreasing thereafter and returning to control levels 18 h after the injection.

Liver GSH was depleted in animals treated with APAP (Fig. 1). GSH levels were reduced to 20% with respect to control animals 1 h after APAP injection, maximum depletion (38% ± 5) was found 3 h after, and at 15 h it returned to near normal levels. Injection of saline solution did not produce any significant change in either of the parameters considered here along the whole period of the assay (data not shown).

Table 1 shows that the activities of the antioxidant enzymes, catalase and glutathione peroxidase were significantly diminished between 1 and 6 h after APAP injection, apparently reaching their minimum value at 1, 9 h after they began to increase, and at 24 h enzyme activities showed control levels.

Enhancement of lipid peroxidation, depletion of GSH content and reduction of catalase and glutathione peroxidase activities seem to be events closely related and taking place before HO induction. In Fig. 2 we can see that heme oxygenase induction was detected 6 h after treatment, reaching a maximum of 412% ± 20 at 15 h, showing that induction occurred once the active oxygen species have increased and the components of the antioxidant defense system (GSH, catalase and glutathione peroxidase) were reduced.

On the other hand, when we have a look at the time profile of ALA-S activity (Fig. 2) we observe an early decline (31% ± 4), 3 h after treatment, then it recovers to control values at 6 h, and then it is induced 55% at 9 h, reaching a maximum at 18 h (210%). Between 24 and 48 h after injection values were within control levels. In vitro experi-
Fig. 2. Time course of APAP effect on ALA-S activity (●) and on HO activity (○). Rats were treated as described in Section 2 and killed at the indicated times. Enzyme units for ALA-S and HO are defined, respectively, as the amount of enzyme producing 1 nmol of ALA per 60 min or 1 nmol of bilirubin per 30 min under the standard incubation conditions. Symbols indicate mean values from 12 rats and bars indicate S.E.M. * No significant differences (P > 0.05) as assessed by Student’s t-test. Values measured in control animals were the same as those at 0 time (ALA-S, 0.11 ± 0.01 nmol ALA per mg protein; HO, 0.5 ± 1 U mg⁻¹ protein).

Fig. 3. Time course of APAP effect on porphobilinogenase activity (●) and on ALA dehydratase activity (○). Rats were treated as described in Section 2 and killed at the indicated times. Enzyme units for porphobilinogenase and ALA dehydratase are defined as the amount of enzyme producing 1 nmol of uroporphyrin per 60 min or 1 nmol of PBG per 60 min, respectively, under the standard incubation conditions. Symbols indicate mean values from 12 rats and bars indicate S.E.M. * No significant differences (P > 0.05) as assessed by Student’s t-test, except for porphobilinogenase where all the values represented were not significant. Values measured in control animals were the same as those at 0 time (ALA dehydratase, 6.7 ± 0.2 nmol PBG per mg protein; porphobilinogenase, 0.031 ± 0.005 nmol porphyrins per mg protein).

Table 2

In vitro effect of APAP on ALA-S activity*

<table>
<thead>
<tr>
<th>APAP concentration (mM)</th>
<th>Time (min)</th>
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<tbody>
<tr>
<td></td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>ALA-S (U mg⁻¹ protein)</td>
</tr>
<tr>
<td>0</td>
<td>0.052 ± 0.004</td>
</tr>
<tr>
<td>0.30</td>
<td>0.041 ± 0.002</td>
</tr>
<tr>
<td>0.50</td>
<td>0.024 ± 0.005</td>
</tr>
<tr>
<td>0.70</td>
<td>0.040 ± 0.002</td>
</tr>
</tbody>
</table>

* Enzymatic activity was assayed as described in the text. One U of the enzyme forms 1 nmol of ALA per 60 min under assay conditions. Data are mean ± S.E.M. of two different experiments using three replicates each time. *, Significant differences (P<0.05) as assessed by Student’s t-test.
values 12 h after APAP injection. It is worth to mention that time profile of ALA dehydratase inhibition resembles that of GSH depletion. Porphobilinogenase activity was not modified by APAP treatment along the whole period of the assay.

In Table 3 we show that administration of bilirubin, the end product of heme catabolism in mammals, or S-adenosyl l-methionine, precursor of sulfides and thiols including cysteine and GSH, 2 h before APAP injection, entirely prevented HO and ALA-S induction as well as the decrease in GSH and enhancement in MDA levels. It was also demonstrated that treatment with bilirubin or S-adenosyl l-methionine alone affected neither HO and ALA-S activities nor oxidative stress parameters.

5. Discussion

Recently there has been much interest in the possible contribution of oxidative stress to the initiation and/or progression of APAP-induced liver injury [9,35–37], yet, there was not much information regarding possible alterations in heme regulation and the synthesis of bilirubin, a known scavenger of ROS.

Our results indicate that a toxic dose of APAP can produce a sequence of events leading to the production of ROS, depletion of protein thiols, and alterations in both heme synthesis and degradation.

Soon after its administration, APAP provoked a significant decrease in GSH content and high increase in lipid peroxide levels in total liver homogenates, suggesting a close association between oxidative stress enhancement and GSH depression (Fig. 1). These results are in agreement with Minamide et al. [35], who have recently reported that augmented lipid peroxidation contributes to the APAP-induced hepatotoxicity at its very early stage of toxicity. The increased lipid peroxidation, together with the capacity of APAP to reduce GSH levels can contribute to the production rate of active oxygen species. A previous report [38] has shown that ROS cause oxidative damage to antioxidant enzymes (catalase and glutathione peroxidase) and hence their activities are diminished.

On the other hand, it has been reported that liver diminished GSH concentration precedes the induction of HO by metals and other agents [21,16,39,15].

Our results clearly demonstrate that ROS produced by APAP decrease GSH levels and then induce HO activity (Figs. 1 and 2), and it has also been observed that this induction would appear to occur once the ROS have increased and the antioxidant defense system (GSH, catalase and glu-

<table>
<thead>
<tr>
<th>Groups</th>
<th>MDA (nmol min⁻¹ mg⁻¹)</th>
<th>GSH content (µmol g⁻¹ liver)</th>
<th>HO (U mg⁻¹)b</th>
<th>ALA-S (U mg⁻¹)c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.10 ± 0.01</td>
<td>7.5 ± 0.2</td>
<td>0.50 ± 0.01</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>0.16 ± 0.01*</td>
<td>5.8 ± 0.2*</td>
<td>1.10 ± 0.06*</td>
<td>0.17 ± 0.01*</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>0.10 ± 0.01</td>
<td>7.3 ± 0.3</td>
<td>0.48 ± 0.01</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>Bilirubin 2 h before treatment</td>
<td>0.09 ± 0.01</td>
<td>7.2 ± 0.4</td>
<td>0.53 ± 0.02</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>S-adenosyl L-methionine</td>
<td>0.11 ± 0.01</td>
<td>8.3 ± 0.4*</td>
<td>0.51 ± 0.01</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>S-adenosyl L-methionine 2 h before</td>
<td>0.10 ± 0.01</td>
<td>7.5 ± 0.2</td>
<td>0.56 ± 0.01</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>treatment</td>
<td></td>
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</table>

* Bilirubin (5 µmol kg⁻¹) or S-adenosyl l-methionine (46 µmol kg⁻¹) were administered by i.p. injection 2 h before APAP treatment. Rats were killed 9 h after treatment. Enzymatic activity was assayed as described in the text.

b One U of the enzyme forms 1 nmol of bilirubin per 30 min under assay conditions.

c One U of the enzyme forms 1 nmol of ALA per 60 min under assay conditions. Data are mean ± S.E.M. of two different experiments using six rats each time. *, Significant differences (P<0.05) as assessed by Student’s t-test.
tathione peroxidase) were decreased (Fig. 1, Table 1). GSH is one of the main antioxidant compounds in liver [11], while catalase and glutathione peroxidase are the two antioxidant enzymes involved in the breakdown of H₂O₂ and therefore, play an essential role in reducing the oxidative damage in mammal tissues and organs. Moreover, it has been reported that substantial GSH depletion (up to 80%) and formation of glutathione conjugates are required for the induction of the identified as heme oxygenase 32 kDa protein [21,40,41,39,42]. Several authors have proposed that HO induction by various forms of oxidative stress constitute an antioxidant response operating by decreasing the levels of potential pro-oxidants and increasing the concentrations of antioxidant active bile pigments [16,43,22,19]. It is already accepted that unconjugated bilirubin is an efficient scavenger of ROS and its enhancement may be a response against initial oxidative damage.

It is widely accepted that the hepatocellular free heme pool controls the rate limiting enzyme ALA-S through mechanisms that involve a negative regulation on the transcription, translation and on its activity [23]. It should be noted that ALA-S induction occurs after the enhancement of HO activity. The former phenomenon may result from a diminution of the intracellular heme pool, due to either increased heme catabolism or decreased heme synthesis or both. Reduced synthesis of heme would be expected to lead to deficiencies in mitochondrial cytochromes with increased ROS generation [44]. Our results show that as a consequence of ROS generation at early times, APAP inhibits ALA-S and ALA dehydratase activities, which eventually produces diminished heme synthesis. Then, the activities of these enzymes return to normal values and occur the induction of HO [16]. These findings may reflect that APAP is both inhibiting heme biosynthesis and then increasing the catabolism of the pre-existing and newly formed heme by enhancing the activity of microsomal heme oxidation. Data in this report demonstrate that when heme degradation is stimulated, ALA-S makes up for the synthesis of bilirubin, by restoring the content of the diminished heme pool. These results also suggest that the inducing effect of APAP on ALA-S could not simple be interpreted as a consequence of the depletion of GSH. Rather, APAP could produce other specific effect on the machinery of ALA-S synthesis; therefore care should be taken in the evaluation of these experimental results. Inhibition of ALA dehydratase activity occurs concomitantly to GSH depletion. It is well known that ALA dehydratase is a thiol enzyme and its activity can be restored or stimulated in vitro by GSH and other thiol derivatives. Although porphobilinogenase is also a thiol enzyme, its sulphydryl groups are situated in non-exposed regions [45], therefore; no significant alterations in its activity would be expected by changes in GSH content as we have found here.

It is known that S-adenosyl L-methionine, via trans-sulfuration reactions is a good precursor of GSH, and because GSH constitutes the bulk of available liver sulphydryl groups for binding electrophilic species, it will function actively in the detoxification of xenobiotics, such as APAP [46]. Interestingly, as early as in 1973 Mitchell et al. [47], had demonstrated that APAP produced a dose-dependent depletion of hepatic GSH, and Stramentinoli et al. [48] had reported that administration of S-adenosyl L-methionine would prevent hepatic necrosis by toxic doses of APAP. Also decreased GSH levels and ALA dehydratase activity in blood of both patients and animals intoxicated with lead and alcohol, were returned to normal levels after S-adenosyl L-methionine administration [49,50]. The above considerations prompted us to investigate the effect of the administration of S-adenosyl L-methionine to APAP intoxicated rats. Also bilirubin, a well-known chain breaking antioxidant and scavenger of peroxyl radicals, was investigated. Our results show that independent treatment with both S-adenosyl L-methionine and bilirubin totally prevented ALA-S and HO induction, GSH depletion and enhancement of lipid peroxidation (Table 3).

In conclusion, our data show that ROS generation by APAP affects both heme biosynthesis and degradation. We also demonstrate that the cell possesses at least two inducible pathways against oxidative stress; both of them finally leading to increased bilirubin formation, which then exerts its antioxidant protective action.
Our findings would add a new insight into the effects of APAP on the hepatic heme metabolism.

Acknowledgements

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