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Microsomal oxidative damage promoted by acetaminophen metabolism

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ABSTRACT

Adverse reactions of acetaminophen have been associated to oxidative stress, which may be elicited by reactive oxygen species (ROS) and/or production of the metabolite NAPQI. Both phenomena would arise through the activity of liver cytochrome P450 (CYP450) system, but their contribution to this oxidative stress is yet to be clarified. A NADPH oxidase activity has been proposed in rat liver microsomes. This activity may be due to the presence of NAD(P)H oxidase (NOX) isoforms in liver endoplasmic reticulum. Both NOX and the CYP450 system activities can catalyze ROS generation using NADPH as a cofactor. Therefore, acetaminophen biotransformation, which requires NADPH, may promote ROS generation through either activity or both. To discriminate between these possibilities, rat liver microsomes were incubated with acetaminophen and NADPH in the presence or absence of specific inhibitors. Incubation with NADPH and acetaminophen elicited lipid peroxidation and decreased thiol content and glutathione-*S*-transferase (GST) activity. The NOX inhibitors apocynin and plumbagin prevented all these phenomena but the decrease in thiol content. In contrast, this decrease was completely prevented by the specific CYP450 system inhibitor SKF-525A. These data suggest that ROS generation following incubation of microsomes with acetaminophen and NADPH appears to be mainly caused by a NOX activity. In light of these data, toxicity of acetaminophen is discussed.

1. Introduction

Acetaminophen is a drug widely used as analgesic and antipyretic that elicits very few adverse reactions at its usual therapeutic dosage. Acute overdose due to indiscriminate consumption or suicide attempts, cause liver failure (Chen et al., 1998; Gunnell et al., 1997; Neuman, 2002). Most of the administered acetaminophen (90%) is conjugated with sulfate and glucuronic acid, in reactions catalyzed by sulfotransferase and UDP-glucuronyltransferase, respectively. Metabolites generated through these reactions are ultimately excreted in the urine (Epstein et al., 1991). A small percentage (5%) is excreted without biotransformation in the urine. The remaining 5% is biotransformed through the cytochrome P450 (CYP450) oxidative system, mainly localized in the liver endoplasmic reticulum. The CYP450 system catalyzes, in the presence of NADPH, the oxidation of acetaminophen into the toxic metabolite *N*-acetyl-*p*-quinone-imine (NAPQI). Microsomal glutathione-*S*-transferase (GST) catalyzes the conjugation of this reactive metabolite with GSH, decreasing its toxicity (Manov et al., 2004).

Acetaminophen overdose can lead to saturation of sulfotransferase and UDP-glucuronyltransferase enzymes. In this condition, excess of acetaminophen is metabolized by the CYP450 system, increasing NAPQI concentration. This can lead to the saturation of GST and the accumulation of NAPQI in the cell. Enzymatic and non-enzymatic conjugation of excess NAPQI with GSH can promote depletion of the latter, decreasing the cellular antioxidant capacity, which leads to oxidative stress (Moore et al., 1985). Clinical manifestations of these phenomena are kidney and liver failure, clotting diseases, acidosis, brain edema, heart failure and ultimately death (Mutimer et al., 1994; O'Grady et al., 1991).

The CYP450 system is one of the main metabolic pathways of generation of reactive oxygen species (ROS). Detection of ROS in rat liver microsomes incubated with acetaminophen and NADPH has been reported (Fischer and Mason, 1984, Fischer et al., 1985 a,b). The source of these ROS has yet to be clarified. NADPH itself can lead to an increase in microsomal lipid peroxidation. It has been proposed that the source of ROS would be due to a NADPH oxidase activity in rat liver microsomes (Dubin et al., 1987, 1991). This activity may correspond to NAD(P)H oxidase (NOX), which are represented by a family of enzymes that catalyze ROS generation (Brown and Griendling, 2009). Noteworthy, NADPH is the required cofactor for acetaminophen biotransformation through the CYP450 system and also for NOX activity. Thus, it is

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possible that ROS detected following incubation of microsomes with acetaminophen and NADPH, could be generated through the activities of NOX, the CYP450 system, or both.

To evaluate this postulate, rat liver microsomes were incubated with acetaminophen and NADPH. This condition provoked: microsomal lipid peroxidation, a decrease of protein thiols and in microsomal GST activity, a redox sensitive enzyme (Aniya and Anders, 1989; Letelier et al., 2010). ROS generation in the reaction mixture, was confirmed using enzymatic (superoxide dismutase and catalase) and non-enzymatic (mannitol) antioxidants. Contribution of the putative NOX activity to this ROS generation was assessed using the NOX inhibitors apocynin (Touyz, 2008) and plumbagin (Ding et al., 2005), and SKF-525A, specific inhibitor of the CYP450 system (Bondy and Naderi, 1994). Our data suggest that the NOX activity would be the main responsible of the occurrence of ROS in rat liver microsomes incubated with acetaminophen and NADPH. Oxidative stress as a mechanism underlying toxicity of acetaminophen is discussed.

2. Material and methods

2.1. Reagents

Acetaminophen, bovine serum albumin (BSA) fraction IV, apocynin [1-(4-hydroxy-3-methoxyphenyl) ethanone], catalase from bovine liver, catechin [(+)-cyanidol-3-(2R,3S)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-1(2H)-benzopyran-3,5,7-triol], 5,5′-dithiobis-(2-nitrobenzoic) acid (DTNB, Ellman's reagent), GSH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase from baker's yeast, mannitol, β-NADP, β-NADPH, *p*-nitroanisole, plumbagin (5-hydroxy-2-methyl-1,4-naphtoquinone), SKF-525A (proadifen), and superoxide dismutase (SOD) from bovine erythrocytes were obtained from Sigma–Aldrich Química Limitada (Santiago, Chile). Trichloroacetic acid (TCA), thiobarbituric acid (TBA), and Folin–Ciocalteu's reagent were purchased in Merck Chile (Santiago, Chile). 1-chloro-2,4-dinitrobenzene was obtained from ACROS Organics (New Jersey, NJ, USA). All other reagents were of the best analytical grade available.

2.2. Animals

Adult male Sprague–Dawley rats (200–230 g) were maintained at the vivarium of the Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile. Animals were kept in a normal pellet diet and water *ad libitum*, 12 h light:dark cycles and at 22 °C. All procedures were performed according to protocols approved by the Ethical Committee of the Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile and to the Guide for the Care and Use of Laboratory Animals (NRC, USA).

2.3. Isolation of rat liver microsomes

Microsomes were isolated by differential centrifugation, as previously described (Letelier et al., 2004). Protein concentration was assayed according to the Lowry method (Lowry et al., 1951), using BSA as standard.

2.4. Conditions for acetaminophen biotransformation through the CYP450 system

Microsomes (0.2 mg protein/mL) were incubated with 1 mM acetaminophen in 50 mM phosphate buffer, pH 7.4, and a NADPH generating system (6 mM glucose-6-phosphate, 0.6 mM NADP and glucose-6-phosphate dehydrogenase 0.15 U/mL). Blanks contained all reagents but microsomes. Samples and blanks were incubated for 10 min at 37 °C under constant agitation, prior to evaluate the oxidative phenomena on microsomal molecules.

2.5. Microsomal lipid peroxidation

The extent of microsomal lipid peroxidation following incubation of microsomes with acetaminophen and the NADPH generating system was estimated assaying thiobarbituric acid reactive substances (TBARS), as previously described (Letelier et al., 2005).

2.6. Microsomal thiol content

Thiol content was titrated in microsomes previously incubated with acetaminophen and the NADPH generating system, using DTNB as previously reported (Ellman, 1959; Sedlak and Lindsay, 1968).

2.7. Microsomal glutathione S-transferase (GST) activity

Conjugation of 1-chloro-2,4-dinitrobenzene with GSH, reaction catalyzed by GST, was assayed as previously described (Letelier et al., 2005), in microsomes previously incubated with acetaminophen and the NADPH generating system.

2.8. p-Nitroanisole O-demethylation activity

This reaction, catalyzed by the CYP450 system, was assayed as previously described (Letelier et al., 2009), in the absence or presence of 0.1 mM catechin.

2.9. Statistical analyses

Data are presented as the mean of at least four independent experiments ± SEM. Analyses of the significance of the differences in means were performed using *t*-Student test. Data were considered significantly different when $p < 0.05$. All statistical analyses were performed using GraphPad Prism, version 5.0.

3. Results

3.1. Microsomal lipid peroxidation

Preincubation of microsomes with acetaminophen in the presence of the NADPH generating system led to significant microsomal lipid peroxidation (Table 1). Catalase, almost completely prevented this phenomenon while superoxide dismutase (SOD) slightly decreased it (~8%, $p < 0.05$). Mannitol did not significantly decrease the observed microsomal lipid peroxidation (Table 1). On the other hand, the polyphenol catechin and the NOX inhibitors apocynin and plumbagin decreased this phenomenon in a similar

Table 1
Effect of antioxidants on microsomal lipid peroxidation elicited by acetaminophen and NADPH.

Condition	TBARS: nmol/min/0.2 mg microsomal protein	*p* value
Control	0.123 ± 0.005	–
100 U/mL SOD	0.114 ± 0.002	0.011
1 U/mL catalase	0.007 ± 0.002	<0.001
1 mM mannitol	0.116 ± 0.004	0.072

Microsomes were incubated with acetaminophen and the NADPH generating system as detailed in Material and Methods. Control: microsomes incubated in the absence of antioxidants. Lipid peroxidation was estimated from assaying TBARS as detailed in Section 2. Data represent the mean of at least 4 independent determinations ± SEM. Probability (*p*) values were calculated from the *t*-tests of each mean compared to the Control. SOD: superoxide dismutase.

extent, 80% (Fig. 1). The CYP450 system inhibitor SFK-525A, however, marginally prevented it (~15%, Fig. 1).

3.2. Microsomal protein thiol oxidation

As shown in Fig. 2, preincubation of microsomes with acetaminophen and NADPH significantly decreased (~30%) the total microsomal thiol content. This phenomenon was completely prevented by catechin and SKF-525A while apocynin and plumbagin failed to prevent it.

3.3. Microsomal GST Activity

Incubation of microsomes with acetaminophen and NADPH inhibited microsomal GST activity in about 60% (Fig. 3). This phenomenon was completely abolished by plumbagin and partially prevented (~15%) by catechin and SKF-525A (Fig. 3).

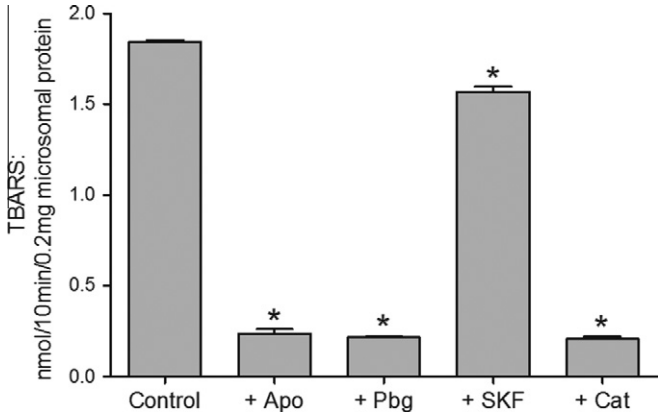

Fig. 1. Microsomal lipid peroxidation. Microsomes were incubated with either 3 mM apocynin (Apo), 1 μM plumbagin (Pbg), 50 μM SKF-525A (SKF), or 0.1mM catechin (Cat), for 5 min at 25 °C, prior to incubation with 1 mM acetaminophen and the NADPH generating system for 10 min at 37 °C. The extent of lipid peroxidation was assayed as detailed in Section 2. Data represent the mean of at least 4 independent experiments ± SEM. $^{*}p < 0.05$ compared to microsomes preincubated without inhibitors (Control bar).

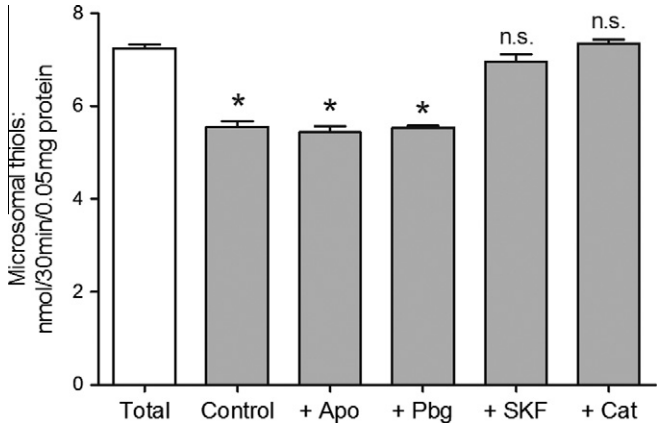

Fig. 2. Microsomal thiols. Microsomes were incubated with either 3 mM apocynin (Apo), 1 μM plumbagin (Pbg), 50 μM SKF-525A (SKF), or 0.1mM catechin (Cat), for 5 min at 25 °C, prior to incubation with 1 mM acetaminophen and the NADPH generating system for 10 min at 37 °C. Microsomal thiols were titrated as detailed in Section 2. Solid bars represent the microsomal thiols measured in the presence of acetaminophen. Data represent the mean of at least four independent experiments ± SEM. $^{*}p < 0.05$ compared to the total microsomal thiols, titrated in the absence of acetaminophen or NADPH (open bar). n.s.: not significantly different from the total microsomal thiols.

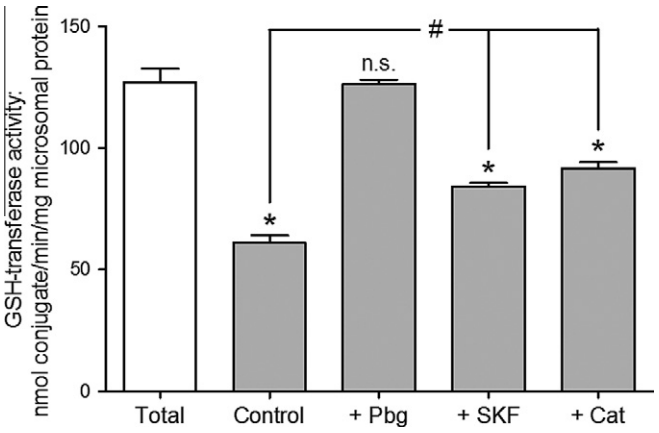

Fig. 3. Microsomal GST activity. Microsomes were incubated with either 1 μM plumbagin (Pbg), 50 μM SKF-525A (SKF), or 0.1 mM catechin (Cat), for 5 min at 25 °C, prior to incubation with 1 mM acetaminophen and the NADPH generating system for 10 min at 37 °C. Microsomal GST activity was assayed as detailed in Section 2. Data are expressed as nmol of GS-dinitrobenzene conjugate/min/mg and represent the mean of at least four independent experiments ± SEM. $^{*}p < 0.05$ compared to the control, without incubation with acetaminophen or inhibitors (left most control bar). $^{\#}p < 0.05$.

3.4. Effect of catechin on the actvity of the CYP450 system

SKF-525A (CYP450 system inhibitor) and catechin (antioxidant agent) prevented the occurrence of damage on microsomal thiol and GST in a similar extent. Catechin could also be substrate of CYP450 and thus, behave as an inhibitor of the biotransformation of acetaminophen. To evaluate this postulate, we assayed the effect of catechin on the *O*-demethylation of *p*-nitroanisole, a reaction catalyzed by this system. In the absence of catechin, this activity was 0.17 nmol of *p*-nitrophenol/min/mg microsomal protein; catechin decreased this activity in about 30%.

4. Discussion

Experimental evidences have shown the occurrence of ROS in rat liver microsomes incubated with acetaminophen and NADPH, conditions required for its biotransformation through the CYP450 system (Fischer and Mason, 1984, Fischer et al., 1985 a,b). This catalytic process has been suggested as the source of these ROS and the mechanism leading to the observed microsomal lipid peroxidation (Fischer and Mason, 1984, Fischer et al., 1985a,b). Nonetheless, a NADPH oxidase activity has been proposed to occur in rat liver microsomes. This putative activity would explain the microsomal lipid peroxidation elicited by NADPH alone (Dubin et al., 1991, 1987). NAD(P)H oxidases (NOX) catalyze ROS generation in the presence of molecular oxygen and NADPH (Brown and Griendling, 2009). NADPH is the cofactor of the CYP450 system and NOX activities. Therefore, one or both could be involved in ROS generation observed. The goal of this study was to evaluate the relative contribution of these activities to ROS generation leading to oxidative damage.

Incubation of rat liver microsomes with acetaminophen and NADPH led to microsomal lipid peroxidation (Table 1, Fig. 1) confirming previous reports (Fischer and Mason, 1984, Fischer et al., 1985a,b). It also decreased microsomal protein thiols and inhibited microsomal GST activity (Figs. 2 and 3). These phenomena were inhibited by superoxide dismutase (SOD), catalase and catechin confirming ROS involvement in this damage. Thus, hydrogen peroxide and, to a lesser extent, superoxide anion are likely to be the species responsible for the observed microsomal lipid peroxidation. On the other hand, the NOX inhibitors apocynin

and plumbagin, significantly prevented microsomal lipid peroxidation (>80%) whereas the irreversible inhibitor of the CYP450 system SKF-525A marginally prevented it (~15%). These data suggest that ROS generation through NOX activity, rather than acetaminophen biotransformation through the CYP450 system, appears to be the major contributor of this oxidative damage.

The decrease (~30%) in microsomal thiol content elicited by incubation of microsomes with acetaminophen and NADPH was not prevented by NOX inhibitors (Fig. 2). It is necessary to note that NADPH alone also failed to decrease microsomal thiol content but did lead to microsomal lipid peroxidation (not shown). These data suggest that, in our assay conditions, ROS generated through NOX activity are not capable to oxidize microsomal thiols. This phenomenon however, was abolished by catechin (polyphenolic antioxidant) and SKF-525A (inhibitor of CYP450 system). These paradoxical results may be related to NAPQI generation. As an electrophilic metabolite, NAPQI may irreversibly bind to GSH and protein thiols in the cell (Saito et al., 2010). Thus, NAPQI may bind to microsomal thiols forming adducts that cannot be detected by titration with the Ellman's reagent. If NAPQI generation through CYP450 system is inhibited by SKF-525A, microsomal thiols would remain available for titration with the Ellman's reagent. Noteworthy, catechin behaved not only as an anti-lipid peroxidation agent but also inhibited the *p*-nitroanisole *O*-demethylating activity of CYP450 system. As such, catechin could also inhibit the generation of NAPQI, preventing the adduct formation and thus, the decrease of microsomal thiol content. More studies, however, are needed to address these postulates.

Since microsomal GST activity abolishes NAPQI reactivity, its inhibition may result in increased acetaminophen toxicity. Under our assay conditions, inhibition of GST activity may be the consequence of two events: (1) microsomal lipid peroxidation, which decreases microsomal GST activity (Letelier et al., 2010) and (2) competitive inhibition elicited by NAPQI given that it is also a substrate for microsomal GST. The observed decrease in GST activity was completely prevented by plumbagin but only partially by SKF-525A and catechin. Therefore, this phenomenon appears to be mainly elicited by the microsomal lipid peroxidation occurring under these conditions.

In summary, ROS detected when incubating rat liver microsomes with acetaminophen and NADPH would be mainly generated by NOX activity rather than the CYP450 system, as previously suggested (Fischer and Mason, 1984; Fischer et al., 1985a,b). On the other hand, excess NAPQI would lead to the consumption of GSH by adduct formation, which must be further metabolized for its excretion as a mercapturic acid. The ensued decrease in the cellular GSH pool may cause a higher risk of cellular damage by the unspecifc binding of NAPQI to thiol groups of proteins (Saito et al., 2010). It has been reported the use of *N*-acetylcysteine as an antidote to prevent liver necrosis by acetaminophen overdose (Atkuri et al., 2007). In addition, increasing the cellular content of metallothionein, a protein rich in thiol groups, can also protect mice from liver injury elicited by acetaminophen overdose (Saito et al., 2010). These evidences are in agreement with the idea that NAPQI accumulation in the organism is the main cause of acetaminophen toxicity.

Conflict of interest

None declared.

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