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Paracetamol (Acetaminophen) Cytotoxicity in Rat Type II Pneumocytes and Alveolar Macrophages *In Vitro*

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ABSTRACT. Paracetamol (acetaminophen, APAP) liver and kidney cytotoxicity is associated with bioactivation by P450 and/or prostaglandin H synthetase (PGHS) to a reactive metabolite, which depletes GSH, covalently binds to proteins, and leads to oxidative stress. Although APAP may also damage the lung, little is known about the mechanism by which this occurs. We studied the *in vitro* toxicity of APAP and its effect on the intracellular GSH level in rat type II pneumocytes (freshly isolated or 24-hr-old) and alveolar macrophages. Cytotoxicity was evaluated by changes in membrane integrity (lactate dehydrogenase, [LDH] assay) as well as by mitochondrial metabolic activity (reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT), following a 20-hr incubation with APAP (1–20 mM). APAP caused a concentration-related decrease in MTT reduction and LDH retention in the fraction of attached cells, which was associated with an increase in LDH activity in the medium and in the fraction of non-attached cells. The order of susceptibility was: freshly isolated type II pneumocytes > alveolar macrophages > 24-hr-old type II pneumocytes. A time- and concentration-dependent decrease in intracellular GSH occurred in freshly isolated type II pneumocytes and alveolar macrophages exposed to subtoxic ($\leq$ 1 mM) APAP concentrations. In 24-hr-old type II pneumocytes, there were no changes in intracellular GSH concentration after APAP exposure. Potassium ethyl xanthate (a P450 inhibitor) and indomethacin (a PGHS inhibitor) significantly decreased APAP-induced GSH depletion in freshly isolated type II pneumocytes and alveolar macrophages, suggesting that P450 and/or PGHS are involved in APAP bioactivation in these cells. BIOCHEM PHARMACOL **59**;11:1467–1475, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. acetaminophen; paracetamol; type II pneumocytes; alveolar macrophages; *in vitro* toxicity; GSH

Paracetamol (acetaminophen, APAP§) is a commonly used analgesic and antipyretic and one of the first described examples of bioactivation-dependent toxicity. Because the mechanism of APAP-induced toxicity involves the disruption of several biochemical pathways (depletion of GSH, covalent modification of target proteins, oxidative stress, chromosomal aberration, apoptotic DNA fragmentation, disruption of calcium homeostasis), it has commonly been used to study mechanisms of cell toxicity [1–6]. At therapeutic doses, APAP is relatively safe, but high doses of the drug cause fulminant liver and kidney cell necrosis in humans [7] and experimental animals [1]. This acute liver and kidney toxicity is generally considered to be associated with P450-mediated APAP bioactivation to a reactive metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI) [2, 3]. The main P450 isozymes involved in APAP bioactivation in the liver are CYP3A4, CYP2E1, CYP1A2, and CYP1A1 [8–10]. In addition to P450, several reports have shown the ability of PGHS to catalyse the metabolism of APAP to a reactive metabolite, which binds to proteins and GSH [11, 12]. PGHS-dependent metabolism of APAP has been suggested to be important in APAP kidney toxicity [13].

Although the liver and the kidney are the principal targets for APAP-induced toxicity, there is evidence to suggest that the lung is also at risk following APAP overdose [14–16]. In humans, lung injury is common in patients with APAP-induced fulminant hepatic failure, but the mechanism of this toxicity is still unclear [17]. Price *et al.* [18] described a case of fatal APAP overdose in a 16-year-old woman in whom the autopsy findings included liver necrosis, proximal renal tubular necrosis, and diffuse alveolar pulmonary damage. APAP-induced pneumonitis has also been observed [19]. There is evidence for the depletion of pulmonary GSH, arylation of lung proteins, and the *in situ* formation of APAP reactive metabolite(s) in experimental animals treated with APAP [20–22]. Hart *et al.* [16] found a similar distribution for anti-APAP and

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§ *Abbreviations:* APAP, acetaminophen, paracetamol; CYP, cytochrome P450; DMEM, Dulbecco's modified Eagle's medium; DNase I, deoxyribonuclease I; FBS, fetal bovine serum; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PEX, potassium ethyl xanthate; and PGHS, prostaglandin H synthetase.

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anti-CYP2E1 antibodies in the lung. Bronchiolar epithelial (Clara cell) necrosis has been observed in mice after toxic APAP dose [23]. Because of their P450 and PGHS activity [24–26], type II pneumocytes and alveolar macrophages are a potential target of APAP toxicity. Type II pneumocytes synthesise and secrete surfactant, control the volume and composition of the epithelial lining fluid, proliferate and differentiate into type I pneumocytes after injury in order to maintain the integrity of the alveolar wall, and are able to metabolise many xenobiotic compounds [27]. Alveolar macrophages are considered to be the first line of cellular defence against inhaled agents, but activated alveolar macrophages and their secretory products may also contribute to a variety of lung disorders induced by xenobiotics [28].

Although APAP may also damage the lung, little is known about the mechanism by which this occurs. The aim of the present study was to investigate the *in vitro* toxicity of APAP and its effect on intracellular GSH levels in rat type II pneumocytes and alveolar macrophages. More generally, the present studies with the model compound, APAP, should contribute to a better understanding of bioactivation-dependent cytotoxicity in the lung.

MATERIALS AND METHODS

Chemicals and Materials

Acetaminophen, indomethacin, trypsin type I (EC 3.4.21.4, Cat. No. T-8003), GSH, pyruvic acid, NADH, metaphosphoric acid (HPO_3), bovine serum albumin, Percoll, EDTA, MTT, DMSO, and *o*-phthaldialdehyde were purchased from Sigma. DMEM, FBS, penicillin–streptomycin solution (10,000 U/mL and 10,000 μg/mL, respectively), fungizone (250 μg/mL), and L-glutamine (200 mM) were obtained from GIBCO. DNase I (EC 3.1.21.1) was purchased from Boehringer Mannheim GmbH. Protein assay dye solution was obtained from Bio-Rad. All other chemicals were purchased from U.C.B. Tissue culture plates (96- and 24-well plates) and 60- and 100-mm diameter culture dishes were purchased from Corning.

Animals

Male Wistar rats with a mean weight of 198 g (range 174–244 g) were used. The animals were obtained from an in-house strain and were maintained in a conventional animal house with 12-hr dark/light cycles in metal cages with a wired bottom. Animals were allowed free access to standard laboratory diet and tap water.

Cell Isolation

TYPE II PNEUMOCYTES. A population of enriched type II pneumocytes was isolated from rat lung according to the methods of Richards *et al.* [29] and Hoet *et al.* [30], which include lung perfusion, trypsin digestion, Percoll gradient centrifugation, and differential adherence. The rats were anaesthetised with 90 mg/kg pentobarbital intraperitoneally and euthanized by exsanguination. The lungs were perfused with saline solution (0.9% [w/v] NaCl) via the pulmonary artery and ventilated 5 times with 8–10 mL of air by means of a syringe attached to the cannula, placed in the trachea. The left atrium was cut to release the perfusate. The lungs were excised and lavaged via the trachea with warm (37°) saline (5 times 3.5 mL/100 g body weight). The lungs were trypsinised (250 mg trypsin in 100 mL PBS with calcium and magnesium, i.e. PBS^+: 130 mM NaCl, 5.2 mM KCl, 10 mM glucose, 10.6 mM HEPES, 2.6 mM Na_2HPO_4, 1.9 mM $CaCl_2$, 1.29 mM $MgSO_4$, pH 7.4, per rat lung) for 30 min at 37°. The trachea and the main bronchi were removed, the lungs were chopped, and 5 mL FBS and 3 mg DNase I were added. After shaking and filtering, the cell suspension was layered onto a discontinuous Percoll gradient (density 1.089 and 1.040 g/mL) and centrifuged at 250 *g* for 20 min (10°). The creamy layer above the heavy gradient was collected, rinsed in PBS^+, suspended in DMEM supplemented with 10% FBS (v/v), 100 U/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL fungizone, 2 mM glutamine, and 75 μg/mL DNase I, and incubated for 1 hr in a 60-mm diameter culture dish in a CO_2 incubator (5% CO_2, 37°) to let the macrophages attach. The unattached cells were spun down at 250 *g* for 10 min (10°) and resuspended in the final DMEM. In most experiments, mainly at the start of the project, identification of type II pneumocytes was carried out with the alkaline phosphatase stain; Clara cells were identified by nitroblue tetrazolium staining. In these experiments, an average of 11×10^6 cells were isolated per animal (range 7.5–19 $\times$ 10^6) of which 75 ± 4% (N = 8) were type II cells, the remainder being alveolar macrophages (approx. 10%), Clara cells (approx. 6%), or unidentifiable cells. The cell viability, assessed by trypan blue exclusion, was 97%. All the experiments done on cells referred to as "freshly isolated type II pneumocytes" were carried out immediately after their isolation. The "24-hr-old type II pneumocytes" were used 24 hr after their isolation and plating. They were cultured in DMEM supplemented with 10% FBS (v/v), 100 U/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL fungizone, and 2 mM glutamine.

ALVEOLAR MACROPHAGES. The bronchoalveolar lavage fluid from the same animals was centrifuged (250 *g*, 10 min, 10°) and the pellet was suspended in DMEM supplemented with 10% FBS (v/v), 100 U/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL fungizone, and 2 mM glutamine. An average of 8×10^6 alveolar macrophages per rat lung (range 4.6–14 $\times$ 10^6) was obtained. The cells were plated and incubated for 2.5 hr at 37° to allow alveolar macrophages to adhere. After removal of the medium and the non-adhering cells, alveolar macrophages were supplied with the final DMEM.

Experimental Protocols

Protocol 1 was used to test APAP cytotoxicity in rat type II pneumocytes and alveolar macrophages. Rat type II cells and alveolar macrophages were incubated (5% CO_2, 37°) in 96-well plates in DMEM (100,000 cells/200 μL/well) containing 10% FBS (v/v), 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL fungizone. Cell toxicity was determined after a 20-hr incubation with APAP (1–20 mM, dissolved in the medium), using the MTT and LDH assays.

Protocol 2 was used to examine the effect of APAP on intracellular GSH content. The cells were incubated in 24-well plates (600,000 cells/well) in 1200 μL DMEM containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL fungizone. The cells were incubated from 0 to 4 hr with 1 mM APAP or for 2 hr in the presence of different APAP concentrations (range 0.01–1 mM).

Protocol 3 was designed to study the effect of P450 and PGHS inhibitors on APAP-induced GSH depletion. PEX was used as an inhibitor of P450 [31, 32], and indomethacin as a PGHS inhibitor. The cells were preincubated for 10 min with PEX (50 or 100 μM), indomethacin (1 or 10 μM), or 100 μM PEX and 10 μM indomethacin. After this preincubation, the cells were exposed for a further 2 hr to 1 mM APAP in the presence of PEX and/or indomethacin.

Analytical Methods

LDH ASSAY. At the end of the incubation, the medium was collected and centrifuged (100 *g*, 10 min, 4°) to separate the non-attached cells from the medium. The attached and non-attached cells were lysed with 200 μL 0.2% (v/v) Triton X-100 in PBS^+. LDH activity was measured in the medium (LDH release) and in the fractions of attached and non-attached cells (LDH retention). The rate of decrease in absorbance at 340 nm, which reflects NADH consumption and LDH activity, was measured [33] using a Beckman DU65 spectrophotometer. LDH activity in the different fractions was calculated as a percentage of total activity. Correction was made for the LDH activity in FBS and the inhibitory effect of APAP on LDH.

MTT ASSAY. The reduction of yellow water-soluble MTT to blue-insoluble MTT formazan derivative by the mitochondrial dehydrogenases was measured according to the method of Mosmann [34]. The medium was removed, and the cells were washed with PBS^+ and incubated with 200 μL 0.05% (w/v) MTT solution for 2 hr. The cells were washed again and the intracellular formazan derivative was dissolved in 200 μL DMSO (30 min, room temperature). The absorbance at 550 nm (reference at 655 nm) was measured spectrophotometrically using a Microplate reader (Benchmark, Bio-Rad). The results were expressed as a percentage of the absorbance of control cells.

GSH DETERMINATION. Intracellular GSH content was measured according to the method of Hissin and Hilf [35], which uses *o*-phthaldialdehyde as a fluorescent reagent. Cells were deproteinised in 200 μL metaphosphoric acid (5% [w/v] HPO_3 in 0.1 M phosphate, 0.005 M EDTA buffer, pH 8.0). Samples were centrifuged and the supernatant was removed and immediately frozen at −80° for a maximum of 20 hr. For GSH assay, 160 μL of the supernatant was mixed with 2215 μL 0.1 M phosphate, 0.005 M EDTA buffer (pH 8.0), and 125 μL *o*-phthaldialdehyde (74.5 mM in methanol) and incubated for 20 min at room temperature. Fluorescence was measured at an excitation of 350 nm and an emission of 426 nm using a Shimadzu RF-5001PC fluorescence spectrophotometer. A standard curve using GSH was prepared for each run. GSH content was expressed as nmol per milligram cell protein. The preliminary results showed that the fluorescence of the GSH-*o*-phthaldialdehyde complex in the presence of HPO_3 was stable for more than 60 min when 125 μL of 74.5 mM *o*-phthaldialdehyde was used. The use of 5% (w/v) trichloroacetic acid instead of HPO_3 decreased the intensity of the fluorescence and the sensitivity of the measurement. The HPO_3 did not influence the determination of cell protein.

PROTEIN DETERMINATION. The precipitated proteins were dissolved in 400 μL 1 N NaOH. The cell protein content was determined using the method of Bradford [36], after neutralisation with 0.333 N HCl. Bovine serum albumin was used as standard.

Statistical Analysis

The results are presented as means ± SD of three to six independent experiments. The data were statistically analysed by repeated measures ANOVA followed by Dunnett's comparison test or by Student's *t*-test for paired data (GraphPad Prism package). The minimum level of significance was considered to be $P \leq 0.05$. Where data are expressed as percentages of control, statistical analysis was performed before the data were transformed to percentages.

RESULTS

In Vitro *Toxicity of APAP*

After 20-hr incubation, APAP caused a concentration-related decrease in MTT reduction in rat type II pneumocytes (freshly isolated and 24-hr-old) and alveolar macrophages (Fig. 1). The reduction of MTT was significantly decreased compared to control at ≥ 5 mM APAP in freshly isolated type II pneumocytes and alveolar macrophages, and at ≥ 10 mM in 24-hr-old type II cells. APAP caused a significant decrease in LDH retention in the fraction of attached cells in freshly isolated type II pneumocytes, alveolar macrophages, and 24-hr-old type II pneumocytes at ≥ 5, ≥ 10, and ≥ 15 mM, respectively (Fig. 2A). This was associated with an increase in LDH activity in the

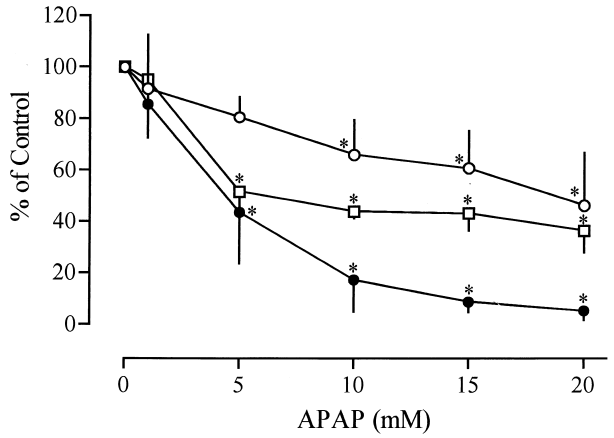

FIG. 1. MTT reduction in freshly isolated type II pneumocytes (-●-), 24-hr-hold type II pneumocytes (-○-), and alveolar macrophages (-□-) after 20-hr incubation with APAP (1–20 mM). The results are expressed as a percentage of control. Data are the means ± SD; N = 6 for freshly isolated and 24-hr-old type II pneumocytes; N = 3 for alveolar macrophages; repeated measures ANOVA followed by Dunnett's comparison test; *P ≤ 0.05 significant vs corresponding control.

fraction of non-attached cells (≥ 10 mM, freshly isolated type II pneumocytes and alveolar macrophages; ≥ 15 mM, 24-hr-old type II pneumocytes) (Fig. 2B). LDH activity in the medium (LDH release) was significantly increased at APAP concentrations ≥ 10 mM in freshly isolated type II pneumocytes, ≥ 15 mM in alveolar macrophages, and at 20 mM in 24-hr-old type II cells (Fig. 2C). Although the MTT method was more sensitive than the LDH assay, there was a good correlation between MTT reduction and LDH retention in the fraction of attached cells (R values > 0.7).

Effect of APAP on Intracellular GSH Level

A significant decrease in intracellular GSH occurred in freshly isolated type II pneumocytes and alveolar macrophages during a 4-hr exposure to 1 mM APAP (Fig. 3). In freshly isolated type II pneumocytes, the intracellular GSH level decreased by 40% during the first 2 hr, and a further small decrease was observed in the next 2 hr of incubation (Fig. 3A). In alveolar macrophages, APAP-induced GSH depletion reached a maximum after 2 hr (by 37%), with little recovery in the following 2 hr (Fig. 3B). In 24-hr-old type II pneumocytes, there were no significant changes in the intracellular GSH level during 4-hr exposure to APAP in concentrations from 1 to 15 mM (data not shown).

The effect of APAP concentration on the intracellular GSH content in type II pneumocytes (freshly isolated and 24-hr-old) and alveolar macrophages is shown in Fig. 4. Data are expressed as percent of the corresponding time-point control (not exposed to APAP). The GSH contents in controls were 16 ± 1, 22 ± 3, and 19 ± 4 nmol/mg protein for freshly isolated type II pneumocytes, 24-hr-old type II cells, and alveolar macrophages, respectively. A concentration-dependent decrease in intracellular GSH

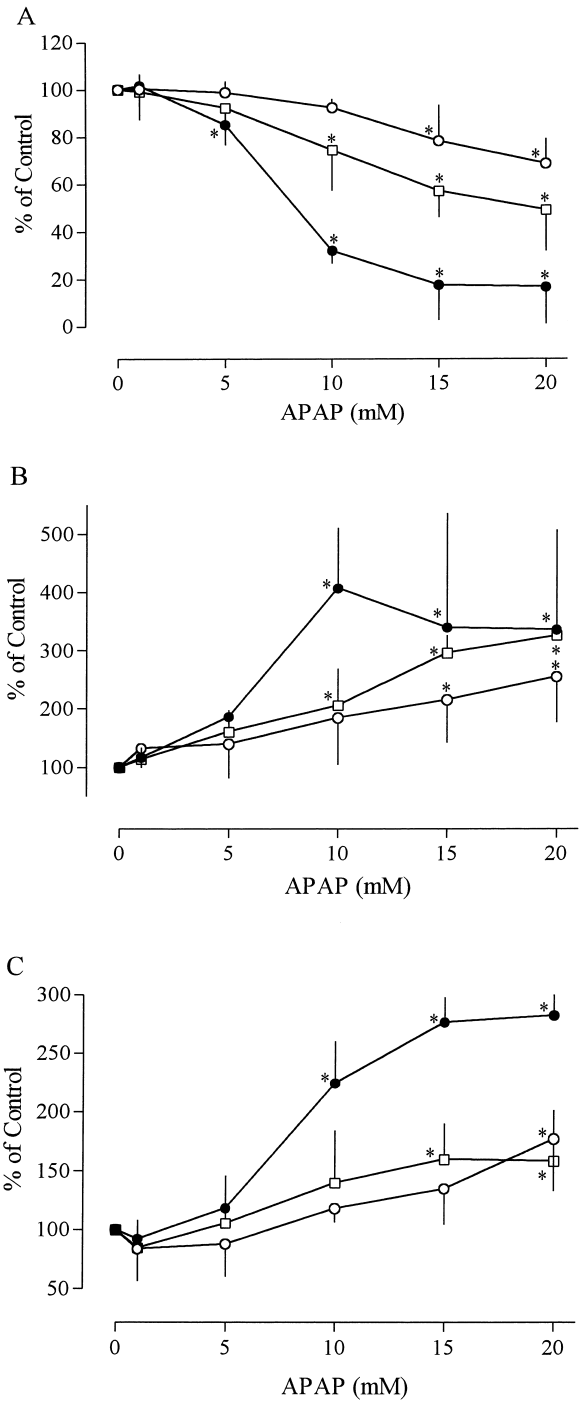

FIG. 2. LDH activity in the fraction of attached cells (A), non-attached cells (B), and in the medium (C) after 20-hr incubation of freshly isolated type II pneumocytes (-●-), 24-hr-old type II pneumocytes (-○-), and alveolar macrophages (-□-) with APAP (1–20 mM). The results are expressed as a percentage of control. Data are the means ± SD; N = 4; repeated measures ANOVA followed by Dunnett's comparison test; *P ≤ 0.05 significant vs corresponding control.

occurred in freshly isolated type II pneumocytes and alveolar macrophages after 2-hr exposure to APAP (range 0.01–1 mM). In 24-hr-old type II cells, there were no significant changes in the intracellular GSH level during

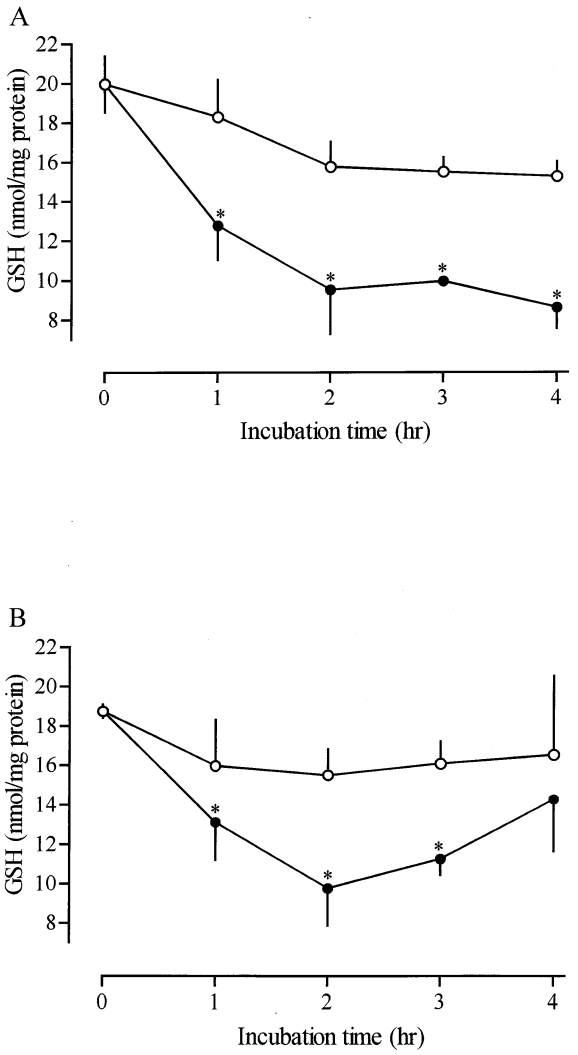

FIG. 3. Time–course of APAP-induced intracellular GSH depletion in freshly isolated type II pneumocytes (A) and alveolar macrophages (B). Type II pneumocytes and alveolar macrophages were incubated from 0 to 4 hr with 1 mM APAP (-●-) or without APAP (-○-). Data are the means ± SD; N = 3–4; paired Student's t-test; *P ≤ 0.05 significant vs corresponding control.

2-hr exposure to APAP in concentrations from 0.01 to 1 mM. Cellular protein content was not affected at the APAP concentrations used (data not shown).

Effect of P450 and PGHS Inhibitors on APAP-Induced GSH Depletion

Freshly isolated type II pneumocytes and alveolar macrophages were preincubated for 10 min with PEX (50 or 100 μM), indomethacin (1 or 10 μM), or 100 μM PEX and 10 μM indomethacin. After this short preincubation, the cells were exposed for 2 hr to 1 mM APAP in the presence of PEX and/or indomethacin. Neither PEX nor indomethacin per se affected the intracellular GSH level in freshly isolated type II pneumocytes and alveolar macrophages, but they did decrease the fall in GSH content induced by

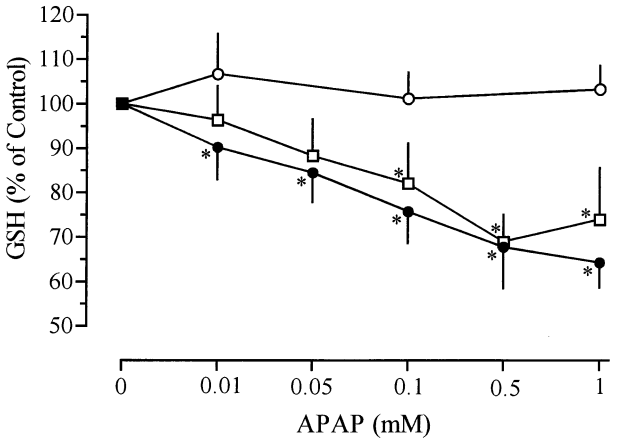

FIG. 4. Effect of APAP concentration on the intracellular GSH level in freshly isolated type II pneumocytes (-●-), 24-hr-old type II pneumocytes (-○-), and alveolar macrophages (-□-) after 2-hr incubation. The results were expressed as a percentage of corresponding control. Controls (nmol/mg protein): freshly isolated type II pneumocytes, 15.8 ± 1.2; 24-hr-old type II cells, 21.9 ± 2.9; alveolar macrophages, 19.1 ± 3.8. Data are the means ± SD; N = 4 for freshly isolated and 24-hr-old type II pneumocytes; N = 3 for alveolar macrophages; repeated measures ANOVA followed by Dunnett's comparison test; *P ≤ 0.05 significant vs corresponding control.

APAP (Fig. 5). In freshly isolated type II pneumocytes, the effect of PEX was concentration-related and more pronounced than that of indomethacin (Fig. 5A). The protective effect of 100 μM PEX was significantly higher than that of indomethacin (1 and 10 μM). Indomethacin (1 μM) significantly decreased the depletion of GSH induced by APAP. When the cells were incubated together with PEX and indomethacin, the protective effect was additive. In alveolar macrophages, the effect of PEX and indomethacin on APAP-induced GSH depletion was concentration-dependent and significant at 100 and 10 μM, respectively. In these cells, the effect of PEX and indomethacin was not additive (Fig. 5B). In the concentrations and exposure time used, APAP, PEX, indomethacin, and their combinations did not cause cell toxicity, as assessed by cell protein content (data not shown).

DISCUSSION

These studies demonstrated that rat type II pneumocytes and alveolar macrophages are sensitive to APAP-induced toxicity *in vitro* in a concentration-dependent manner, with the order of susceptibility being as follows: freshly isolated type II pneumocytes > alveolar macrophages > 24-hr-old type II pneumocytes. The concentrations that caused cytotoxicity may appear quite high, since APAP concentrations of ≥ 5 mM were needed to produce cytotoxicity in freshly isolated type II pneumocytes and alveolar macrophages, and even higher concentrations (≥ 10 mM) were required in 24-hr-old type II pneumocytes. However, this is not much different from the situation observed in cultured rodent hepatocytes [5, 37, 38].

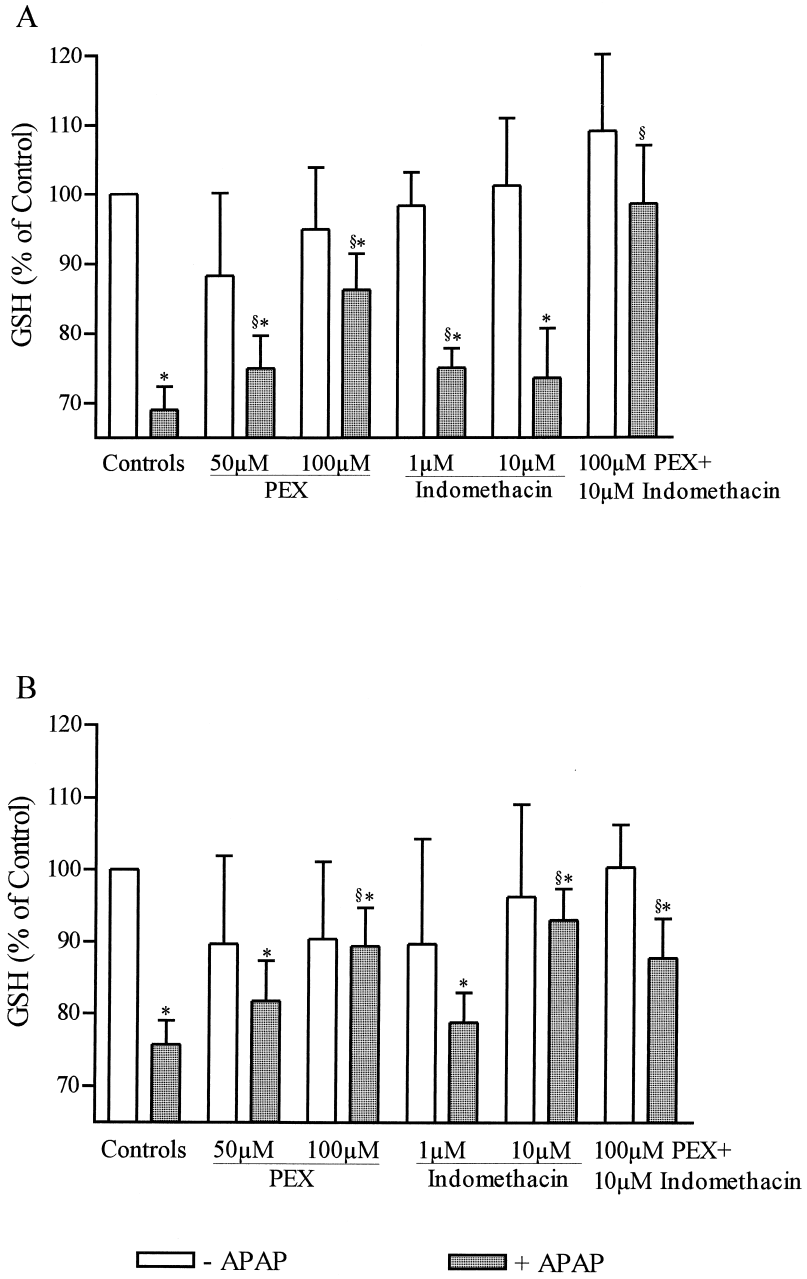

FIG. 5. Effect of PEX and indomethacin on APAP-induced intracellular GSH depletion in freshly isolated type II pneumocytes (A) and alveolar macrophages (B). The cells were preincubated for 10 min with PEX and/or indomethacin, followed by 2-hr exposure to 1 mM APAP. The results are expressed as a percentage of corresponding time-point untreated control. Controls (nmol/mg protein): freshly isolated type II pneumocytes, 14.3 ± 1.6; alveolar macrophages, 22.2 ± 3.3. Data are the means ± SD; N = 4–6; paired Student's t-test; §P ≤ 0.05 significant vs APAP control; *P ≤ 0.05 significant vs untreated control.

MTT reductive capacity was more markedly affected by APAP than LDH retention in the fraction of attached cells, which suggests a toxic action of APAP on mitochondrial function and is in agreement with data on hepatocytes [39]. A concentration-related increase in LDH activity in the fraction of non-attached cells after APAP exposure is indicative of reduced attachment efficiency (freshly isolated type II pneumocytes) or increased detachment (24-hr-old type II pneumocytes and alveolar macrophages). Cell detachment after APAP has been observed in PC12 pheochromocytoma cells [40], and reduced attachment efficiency has been shown with various pulmonary toxins (paraquat, 4-ipomeanol, 3-methylindole, etc.) in mouse Clara cells [41].

Many studies have shown that APAP depletes GSH in the liver *in vivo* and *in vitro* in hepatocytes. This effect is a consequence of APAP activation to a reactive metabolite, which is detoxified by GSH [3]. In chemical systems and isolated hepatocytes, APAP reactive metabolite reacts with GSH in at least two ways: it forms a GSH conjugate, on the

one hand, and it oxidises GSH to its disulphide, GSSG, with the concomitant production of APAP, on the other [2]. Thus, the decrease in intracellular GSH may reflect the formation of conjugate as well as the oxidation of GSH. Our results showed a time- and concentration-dependent decrease in intracellular GSH levels at subtoxic APAP concentrations in freshly isolated type II pneumocytes and alveolar macrophages, suggesting the formation of APAP reactive metabolite in these cell preparations. These results are in agreement with the *in vivo* studies showing that hepatotoxic APAP doses significantly decrease the lung GSH level in rats [21] and in mice [20]. In A549 human lung adenocarcinoma cells, which have some characteristic features of type II pneumocytes and possess functional CYP1A1 and CYP2B6 [42], Schønberg and Skorpen [43] found a decrease in intracellular GSH level after APAP exposure. Our results indicate that APAP in concentrations that may be achieved in human plasma after a therapeutic dose (0.1–0.2 mM) [44] decreased intracellular GSH in freshly isolated rat type II pneumocytes and alveolar macrophages. This could not only cause toxicity, but also predispose the cells to the toxic action of specific pulmonary toxic compounds or air pollutants.

In 24-hr-old type II pneumocytes, APAP did not affect the intracellular GSH level, suggesting no APAP reactive metabolite formation in these cell preparations. The lower APAP toxicity in 24-hr-old type II pneumocytes compared to freshly isolated cells is presumably related to loss of P450 activity with time in culture. Lag *et al.* [45] found an approx. 50% decrease in CYP2B1 apoenzyme expression in rat type II pneumocytes after 24 hr in culture. We speculate that the observed APAP cytotoxicity in 24-hr-old type II pneumocytes is due to the unmetabolised APAP. Recent *in vitro* studies suggest that some of the APAP effects are indeed independent of P450 activation [6, 46].

To test which enzyme(s) are involved in APAP bioactivation in freshly isolated type II pneumocytes and alveolar macrophages, we used an inhibitor of P450 (PEX) and an inhibitor of the cyclooxygenase activity of PGHS (indomethacin). Various derivatives of dithiocarbonic acid, known as xanthates, have been found to be specific mechanism-based inactivators of P450 [31, 32]. Recent data have shown that PEX inactivates CYP2B1 and CYP2E1, inhibits the enzymatic activity of CYP1A1 and has no effect on the activities of CYP3A2 or CYP3A4 [32]. Our previous investigations, in mice, have shown that PEX reduces the mortality rate and severity of hepatic necrosis as well as the depletion of liver GSH caused by toxic APAP doses [47, 48]. PEX also reduces APAP-induced intracellular GSH depletion *in vitro* in hepatocytes.* Our present results show that PEX significantly decreases APAP-induced GSH depletion in freshly isolated type II pneumocytes and alveolar macrophages *in vitro*, thus suggesting a role of P450 in APAP bioactivation to a reactive metabolite which depletes GSH in these cells. This is in line with the results of Bartolone *et al.* [15], who have found that pretreatment of mice with another P450 inhibitor, piperonyl butoxide, significantly alleviates the severity of the APAP-induced necrosis not only in the liver but also in the lung.

Indomethacin, an inhibitor of the cyclooxygenase activity of PGHS, inhibits the formation of one- and two-electron oxidized APAP products and the covalent binding of APAP reactive metabolite(s) to rabbit and human kidney medullary microsomal protein [13]. Here, indomethacin reduced the decrease in intracellular GSH caused by APAP in freshly isolated type II pneumocytes and alveolar macrophages. This suggests that, similar to the kidney medulla, the lung PGHS can take part in APAP bioactivation. The involvement of PGHS in the bioactivation of aflatoxin B1 and of (±)benzo(*a*)pyrene 7,8-dihydrodiol has been suggested for human alveolar macrophages and rat type II pneumocytes, respectively [24, 26]. In alveolar macrophages, the effect of indomethacin was more pronounced than in freshly isolated type II pneumocytes and was similar to that of PEX. Based on this, we suggest that in alveolar macrophages P450 and PGHS have an equal role in APAP bioactivation. In freshly isolated type II pneumocytes, the effect of PEX was more pronounced than that of indomethacin, suggesting that in these cells the observed GSH depletion is mainly due to P450-dependent APAP bioactivation. When the type II cells were incubated together with PEX and indomethacin, the protective effect was additive, showing that not only P450 but also PGHS can be involved in APAP oxidation in freshly isolated type II pneumocytes. We can summarise that P450 and/or PGHS are possibly involved in the formation of APAP reactive metabolite(s), which deplete(s) GSH in freshly isolated type II pneumocytes and alveolar macrophages *in vitro*.

In conclusion, the novel finding of our study is that rat type II pneumocytes and alveolar macrophages are sensitive to APAP-induced toxicity *in vitro*. In freshly isolated type II pneumocytes and alveolar macrophages, APAP decreased the intracellular GSH level, and our results indicate that P450 and/or prostaglandin synthetase are involved in this process.

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