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THE MECHANISM OF PREVENTION OF PARACETAMOL-INDUCED HEPATOTOXICITY BY 3,5-DIALKYL SUBSTITUTION

THE ROLES OF GLUTATHIONE DEPLETION AND OXIDATIVE STRESS

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Abstract—Recently, we have reported that 3,5-dialkyl substitution of paracetamol, in contrast to 3-monoalkyl substitution, prevented the paracetamol-induced toxicity in freshly isolated rat hepatocytes without having any effect on its cytochrome P-450 mediated bioactivation to reactive *N*-acetyl-*p*-benzoquinone imines (NAPQI). In the present study the mechanism of this prevention of toxicity, with special emphasis on oxidative stress, was studied in more detail in freshly isolated rat hepatocytes, using paracetamol, 3-methyl-, 3,5-dimethyl-paracetamol, synthetic NAPQI and 3,5-dimethyl-NAPQI.

3-Methyl-paracetamol was found to induce glutathione (GSH) depletion, lipid-peroxidation and cytotoxicity in hepatocytes to the same extent as paracetamol. 3,5-Dimethyl-paracetamol, however, even when added in a ten-fold higher concentration when compared to paracetamol, did not induce any of these effects. Similar differences of toxicity were observed between NAPQI and 3,5-dimethyl-NAPQI; 3,5-dimethyl-NAPQI, in contrast to NAPQI, did not reduce protein thiol levels, did not induce GSH depletion, lipid-peroxidation nor cytotoxicity. Only after artificial depletion of GSH levels in the hepatocytes by DEM or BCNU, 3,5-dimethyl-NAPQI was cytotoxic. This effect was accompanied by depletion of protein thiol levels, but not by lipid-peroxidation. Addition of the disulfide reducing agent, dithiothreitol, prevented the artificially created cytotoxicity of 3,5-dimethyl-NAPQI.

It is concluded that prevention of paracetamol-induced toxicity by 3,5-dialkyl substitution is primarily due to prevention of irreversible GSH-depletion, presumably caused by the inability of 3,5-dialkyl-NAPQI to conjugate with thiols. As a result, the GSH-dependent cellular defense mechanism against potential oxidative cellular injury by 3,5-dialkyl-NAPQI is left unimpaired. Our observations indicate that a compound, not capable of covalent binding to thiol groups of proteins, can induce toxicity solely as a result of protein thiol oxidation without inducing lipid-peroxidation.

Paracetamol (4-hydroxyacetanilide) is a commonly used analgesic drug, which upon overdosage is known to cause centrilobular hepatic necrosis [1, 2]. At normal dose levels the drug mainly undergoes sulfation and glucuronidation in most species. At higher doses, however, these metabolic routes may become saturated and bioactivation of the drug by the hepatic microsomal cytochrome P-450 mixed-function oxidase system is increasingly occurring under the formation of a toxic metabolite, presumably *N*-acetyl-*p*-benzoquinone imine (NAPQI‡) [3, 4]. This highly reactive compound possesses both electrophilic and oxidans characteristics and can, in principle, be detoxified either by Michael-1,4-addition to GSH under formation of a 3-glutathionyl conjugate, or by reduction by GSH under formation of paracetamol and GSSG [6, 17]. Upon overdosage with paracetamol, depletion of the protective cellular GSH, followed by oxidation and/or arylation of thiol groups of cysteinyl residues in cellular proteins has been shown to occur [7].

The precise molecular mechanisms underlying the hepatotoxic effects of paracetamol are still subject of investigation. Initially, covalent binding of the reactive metabolite of paracetamol to protein thiol groups was thought to initiate the paracetamol-induced hepatotoxicity [8]. However, more recently, several investigators reported a dissociation between paracetamol-induced covalent binding and hepatotoxicity [9–13]. Recent studies strongly suggest that the paracetamol-induced hepatotoxicity more probably is due to oxidative stress, resulting in lipid-peroxidation [12, 14] and depletion of protein thiols [5, 15]. Both processes, which have been implicated as alternative mechanisms of paracetamol-induced hepatotoxicity, are believed to occur after depletion of GSH. GSH is normally used in the detoxication of active oxygen species and in the protection against oxidation of protein thiol groups which are critical in maintaining of a number of cellular functions [16].

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‡ Abbreviations used: NAPQI, *N*-acetyl-*p*-benzoquinone imine; GSH, glutathione; GSSG, glutathione disulfide; 3,5-dimethyl-NAPQI, 3,5-dimethyl-*N*-acetyl-*p*-benzoquinone imine; DEM, diethylmaleate; BCNU, *N,N*-bis(2-chloroethyl)-*N*-nitrosourea; LDH, lactate dehydrogenase.

Recently, we have reported that 3,5-dialkyl substitution of paracetamol (R = CH_3, C_2H_5 or i-C_3H_7), in contrast to the corresponding 3-monoalkyl substitution, prevented its cytotoxicity in freshly isolated hepatocytes of rat [17]. The precise mechanism of prevention of the toxicity of paracetamol by 3,5-dialkyl substitution is as yet unknown. We have shown that the microsomal cytochrome P-450-mediated oxidation of paracetamol to the corresponding quinone imine was not affected by 3-mono- nor 3,5-dialkyl substitution. Only 3,5-dialkyl substitution of paracetamol efficiently prevented conjugation of the 3,5-dialkyl-NAPQI's enzymatically formed with GSH, which probably implies that covalent binding to protein thiols can neither occur; however, since the quinone imines are reducible by GSH under formation of GSSG [6, 17], in principle, oxidation of protein thiols by 3,5-dialkyl-NAPQI's might still occur.

The aim of the present study was to investigate in more detail the effect of 3-monoalkyl and 3,5-dialkyl substitution of paracetamol on paracetamol-induced GSH depletion and subsequent oxidative stress, as possible mechanisms underlying the hepatotoxic action. For this purpose the effects of paracetamol, 3-methyl-paracetamol, 3,5-dimethyl-paracetamol, NAPQI and 3,5-dimethyl-NAPQI on GSH and protein thiol levels, lipid-peroxidation and cytotoxicity in freshly isolated rat hepatocytes were studied.

MATERIALS AND METHODS

Materials. 3-Methyl-paracetamol and 3,5-dimethyl-paracetamol were synthesised from 2-methylphenol and 2,6-dimethylphenol, respectively, as described by Dearden and O'Hara [18]. NAPQI and 3,5-dimethyl-NAPQI were prepared by oxidation of paracetamol [19] and 3,5-dimethyl-paracetamol [20], respectively. Collagenase, pyruvate and lactate were obtained from Boehringer (Mannheim, F.R.G.). 3-Methylcholanthrene, bovine serum albumine, DEM and dithiothreitol were purchased from Sigma (St. Louis, MO). BCNU was obtained from Bristol-Meyer (New York).

Animals and isolation of hepatocytes. Male albino Wistar rats (180–200 g) were used. Since hepatocytes from 3-methylcholanthrene-induced rats are more susceptible to paracetamol-induced toxicity [17, 21], the rats were pretreated with 3-methylcholanthrene (40 mg/kg, dissolved in archides oil), once injected intraperitoneally 48 hr before use. Rats were fasted overnight before the isolation of hepatocytes. Hepatocytes were isolated by collagenase perfusion according to the procedure described by Seglen [22] with some modifications [17]. Cells isolated in this way usually contained more than 90% viable cells as judged by trypan blue exclusion.

Incubation of hepatocytes. Freshly isolated hepatocytes (1.5-2 × 10^6 cells/ml) were incubated in 3 ml volumes of Krebs–Henseleit buffer (pH 7.4) containing 2% bovine serum albumine, 10 mM lactate and 1 mM pyruvate. The cells were equilibrated at 37° with 95% oxygen/5% carbon dioxide prior to addition of either paracetamol, 3-methyl-paracetamol or 3,5-dimethyl-paracetamol dissolved in incubation medium, or NAPQI or 3,5-dimethyl-NAPQI freshly dissolved in dry dimethylsulfoxide. In the case of pretreatment of cells with BCNU or DEM, BCNU (50 μM) and DEM (0.75 mM) dissolved in dimethylsulphoxide were added; after 30 min of preincubation, either NAPQI or 3,5-dimethyl-NAPQI were added. Dithiothreitol (5 mM), dissolved in incubation medium, was added 15 min after addition of NAPQI or 3,5-dimethyl-NAPQI. In experiments in which dimethylsulfoxide was used as a solvent, an equal amount of dimethylsulfoxide, maximally 20 μl, was added to the control incubations.

Assays. Hepatocyte GSH levels were measured as acid-soluble thiols using Ellmans reagent [23]. Protein thiol levels were determined, after resuspending of cells in Krebs-Henseleit buffer (pH 7.4), as described by Albano *et al.* [5] using Ellmans reagent [23]. Protein was determined according to the method of Bradford [24]. Lipid-peroxidation was monitored by measuring the formation of products reacting with 2-thiobarbituric acid [25]. Cell destruction was determined by measurement of LDH-leakage from cells [26].

RESULTS

Paracetamol, 3-methyl- and 3,5-dimethyl-paracetamol

Addition of either paracetamol (0.5 mM) or 3-methyl-paracetamol (0.5 mM) to isolated hepatocytes from 3-methylcholanthrene treated rats within 1 hr resulted in depletion of GSH levels to approximately 20% of the control levels and subsequently to 5% within 3 hr of incubation. In contrast, the same concentration of 3,5-dimethyl-paracetamol (0.5 mM) did not significantly deplete GSH (Fig. 1A). Increasing the concentration of 3,5-dimethyl-paracetamol upto 5 mM resulted only in a small depletion of GSH after 3-hr of incubation, viz. to about 70% of the control level of GSH (Fig. 1A).

As far as lipid-peroxidation is concerned, both paracetamol and 3-methyl-paracetamol (0.5 mM) strongly induced the formation of thiobarbituric acid-reactive material in the hepatocytes (Fig. 1B). In the case of 3,5-dimethyl-paracetamol, no lipid-peroxidation was observed when added at concentrations of 0.5 mM or 5 mM (Fig. 1B). On the contrary, 3,5-dimethyl-paracetamol appeared to inhibit the slight spontaneous lipid-peroxidation normally observed in control incubations.

Similarly to what we reported earlier, paracetamol and 3-methyl-paracetamol (0.5 mM) were found to be equally toxic in hepatocytes isolated from 3-methylcholanthrene-treated rats (Fig. 1C). 3,5-Dimethyl substitution of paracetamol again resulted in a prevention of toxicity, though a tenfold higher concentration (5 mM) of the compound caused a small increase in LDH leakage (Fig. 1C).

NAPQI and 3,5-dimethyl-NAPQI

Using synthetic NAPQI and 3,5-dimethyl-NAPQI, further studies were performed to evaluate the difference in toxicity between paracetamol and 3,5-dimethyl-paracetamol. Addition of NAPQI (0.5 mM) to the hepatocytes depleted GSH, in contrast to 3,5-dimethyl-NAPQI (0.5 mM) (Fig. 2A).

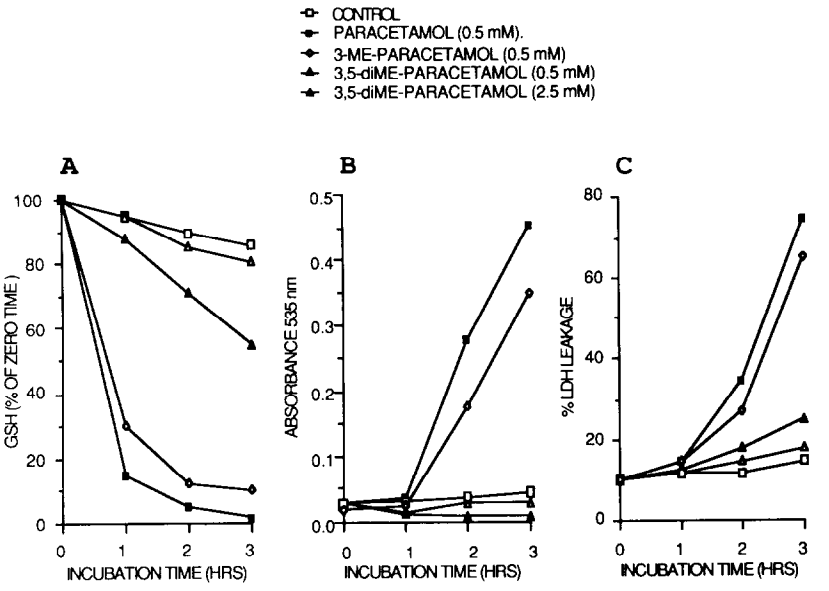

Fig. 1. Effects of paracetamol, 3-methyl-paracetamol and 3,5-dimethyl-paracetamol on (A) GSH-depletion, (B) lipid-peroxidation and (C) LDH leakage in isolated hepatocytes from 3-methyl-cholanthrene treated rats. Results of one typical experiment out of four.

Furthermore, NAPQI depleted protein thiol levels to 50% of control values, whereas 3,5-dimethyl-NAPQI did not affect protein thiol levels (Table 1). Lipid-peroxidation was induced by NAPQI, though less extensively when compared to paracetamol. 3,5-Dimethyl-NAPQI, however, tended to inhibit the small spontaneous lipid-peroxidation in control incubations (Fig. 2B). NAPQI induced 95% LDH leakage after 3 hr of incubation, whereas 3,5-dimethyl-NAPQI only induced a slight increase in LDH leakage (Fig. 2C).

3,5-Dimethyl-NAPQI in hepatocytes treated with DEM or BCNU

3,5-Dimethyl-NAPQI was found not to deplete GSH, presumably by the fact that no conjugation to GSH can occur and that the GSSG formed by oxidation of GSH through the action of 3,5-dimethyl-NAPQI, is quickly reduced again by GSH-reductase. To support this hypothesis, the effects of GSH depletion by DEM and GSH depletion and inhibition of GSH-reductase by BCNU on the cytotoxicity of 3,5-

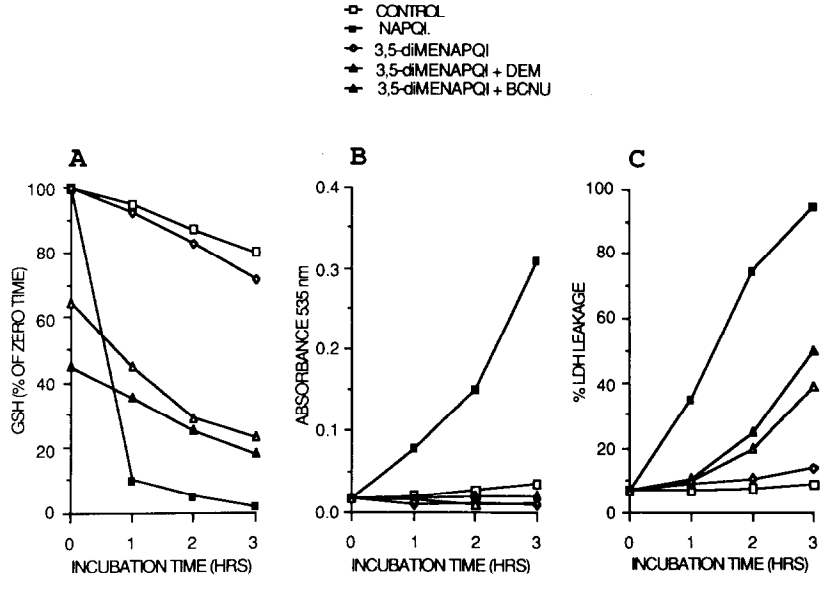

Fig. 2. Effects of NAPQI and 3,5-dimethyl-NAPQI on (A) GSH-depletion, (B) lipid-peroxidation and (C) LDH leakage in DEM, BCNU and untreated hepatocytes. DEM and BCNU were added 30 min prior to the start of the experiments. Results of one typical experiment out of four.

Table 1. Effects of NAPQI, 3,5-dimethyl-NAPQI, DEM and BCNU on protein thiol levels of hepatocytes after 3 hr of incubation

Experiment	% Protein thiols*
Control	100 ± 8
DEM (0.75 mM)	98 ± 4
BCNU (50 μM)	96 ± 5
NAPQI (0.5 mM)	51 ± 8
3,5-dimethyl-NAPQI (0.5 mM)	105 ± 5
3,5-dimethyl-NAPQI (0.5 mM) + DEM (0.75 mM)	70 ± 8
3,5-dimethyl-NAPQI (0.5 mM) + BCNU (50 μM)	76 ± 7

* Values represent means ± SD of four experiments. The 100% value was 85 nmol/mg protein.

dimethyl-NAPQI were studied. Pretreatment of hepatocytes with these compounds has been shown to enhance the toxicity of NAPQI [5]. In hepatocytes with DEM (0.75 mM), the GSH levels were depleted to 45% of the control level. DEM did not deplete protein thiols and, furthermore, this pretreatment did not induce lipid-peroxidation nor LDH leakage (data not shown). Addition of 3,5-dimethyl-NAPQI (0.5 mM) to DEM-treated hepatocytes led to a slightly increased depletion of GSH (Fig. 2A) and, interestingly, to a decrease in protein thiol levels to 70% of the control value (Table 1). Although no increase in lipid-peroxidation was observed, 3,5-dimethyl-NAPQI increased the LDH leakage fivefold when compared to DEM-pretreated control hepatocytes (Figs. 2B and 2C). The disulfide reducing agent dithiothreitol (5 mM), which has been shown to protect hepatocytes against paracetamol [27] and NAPQI-induced toxicity [5], was found to protect also against the toxic action of 3,5-dimethyl-NAPQI in DEM-pretreated hepatocytes (data not shown).

Pretreatment of hepatocytes with BCNU (50 μM) depleted GSH levels to 65% of the control values, but it did not affect either protein thiol levels, nor lipid-peroxidation or LDH leakage of the hepatocytes (data not shown). In BCNU-pretreated hepatocytes, 3,5-dimethyl-NAPQI further depleted GSH levels down to 30% (Fig. 2A) and decreased the protein thiol levels to 75% of the control values (Table 1). Lipid-peroxidation was not induced under these conditions, whereas LDH leakage was increased fourfold when compared to BCNU-pretreated controls (Figs. 2B and 2C). Also in the case of BCNU-pretreated cells the cytotoxicity of 3,5-dimethyl-NAPQI could be prevented by the addition of dithiothreitol (5 mM) (data not shown).

DISCUSSION

NAPQI, the presumed toxic metabolite of paracetamol, has been shown to deplete irreversibly cellular GSH, to bind covalently to protein thiol groups [7] and to induce an oxidative stress in hepatocytes manifesting itself in the form of lipid-peroxidation and oxidative depletion of protein thiol levels [5, 8–15]. Lipid-peroxidation has been shown to be an important event in the development of cellular injury induced by various chemicals [28]. Depletion of critical protein thiol levels also can result in cytotoxic effects, as they play an essential role in a number of cellular functions. For instance, inactivation of Ca^{2+}-dependent plasma membrane ATPase, resulting in a disturbance of the cellular Ca^{2+}-homeostasis, has been associated with protein thiol depletion induced by menadione [29], t-butylhydroperoxide [30], formaldehyde [31] and paracetamol [15]. We recently reported on the prevention of toxicity of paracetamol in rat hepatocytes by 3,5-dialkyl substitution of the drug [17]. This type of substitution did not affect the cytochrome P-450 mediated oxidation of paracetamol. As 3,5-dialkyl-NAPQI's formed do not conjugate with GSH anymore, it most likely also prevents irreversible conjugation to cellular thiols. The aim of the present study was to further investigate the molecular mechanism of this type of prevention.

GSH, which for 95% is present in its reduced form in the cell, plays a major role in the protection against chemically induced oxidative cellular injury. Depletion of hepatic GSH levels has been shown to enhance the susceptibility to cytotoxicity, whereas the reverse is true for agents which promote GSH synthesis [16]. 3-Alkyl substitution of paracetamol was still found to induce GSH depletion and LDH-leakage in hepatocytes from 3-methylcholanthrene treated rats to the same extent as paracetamol (Figs. 1A and 1C). However, 3,5-dimethyl-paracetamol did not deplete GSH levels in hepatocytes anymore, unless the concentration was very high (5 mM) (Fig. 1A). Only at the high concentration a slight increase in LDH leakage from the hepatocytes was observed when compared to controls (Fig. 1C). From these results, it can be concluded that only a complete blocking of the electrophilic 3- and 5-positions of paracetamol with alkyl groups, such as is the case in 3,5-dimethyl-paracetamol, efficiently prevents both GSH depletion and toxicity. As the cytochrome P-450 mediated oxidation of paracetamol to the reactive quinone imine is not affected by 3,5-dialkyl substitution [17], the prevention of GSH depletion in the case of 3,5-dimethyl-paracetamol most probably is a consequence of the inability of 3,5-dimethyl-NAPQI formed to conjugate with GSH. In conformity with this suggestion, synthetic 3,5-dimethyl-NAPQI neither depleted GSH when added directly to hepatocytes (Fig. 2A). Since 3,5-dimethyl-NAPQI, in principle, has been shown to be capable of oxidising GSH to GSSG [6, 17], presumably the GSSG once formed in the cells is reduced rapidly

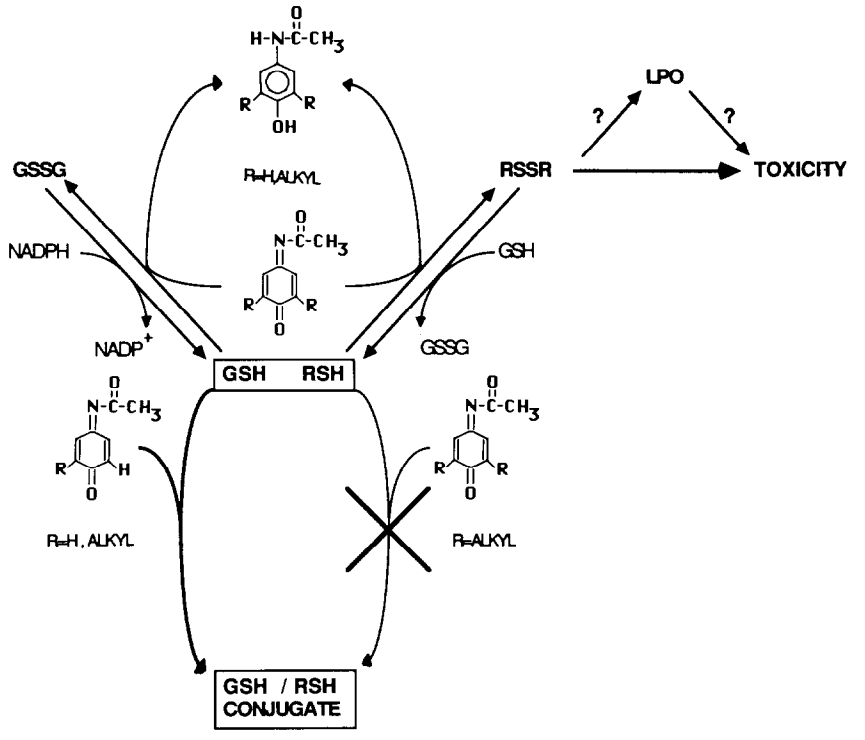

Fig. 3. Proposed effects of NAPQI's formed from paracetamol, its 3-alkyl and 3,5-dialkyl substituted derivatives on the cellular thiol status and the cell viability. RSH denotes protein thiol groups; RSSR protein disulfide groups and LPO lipid-peroxidation.

again by GSH-reductase, thus maintaining the critical cellular equilibrium between GSH and GSSG [16]. This hypothesis was further supported by our observations that 3,5-dimethyl-NAPQI was only cytotoxic when the cellular GSH pool was partially depleted by pretreatment of the hepatocytes with either DEM or BCNU and when GSH-reductase was inhibited by the latter compound (Fig. 2C). These observations illustrate that efficient prevention of irreversible GSH depletion by 3,5-dimethyl substitution of paracetamol might be the primary reason why its toxicity is precluded.

As far as the precise molecular mechanism of hepatotoxicity of paracetamol and its alkyl-substituted derivatives is concerned, the present study adds further information to what is known as yet. Paracetamol- and NAPQI-induced toxicities have been related to oxidative stress in hepatocytes leading to lipid-peroxidation and depletion of protein thiols [5, 8–15]. We found 3,5-dimethyl-NAPQI only to be toxic in hepatocytes after artificial depletion of GSH and/or inhibition of GSH-reductase. Under these circumstances the toxicity of 3,5-dimethyl-NAPQI was accompanied by depletion of protein thiols (Table 1), but not by lipid-peroxidation (Fig. 2B). Since 3,5-dimethyl-NAPQI is an oxidans of thiols, this strongly suggests that protein thiol depletion due to oxidation by itself can be a cause of cytotoxicity. NAPQI, similar to what has earlier been shown [5, 15], also strongly depleted thiol levels (Table 1). The observation that reduction of protein disulfides by dithiothreitol, as in the case of paracetamol [27] and NAPQI [5], prevented the toxicity of 3,5-dimethyl-NAPQI in DEM- or BCNU-pretreated hepatocytes further substantiates the proposed independent role of oxidative protein thiol depletion in the development of cytotoxicity of paracetamol and its derivatives.

Under the conditions in which 3,5-dimethyl-NAPQI induced cytotoxicity, no lipid-peroxidation was observed. In contrast, spontaneous lipid-peroxidation tended to be inhibited by 3,5-dimethyl-paracetamol and 3,5-dimethyl-NAPQI, presumably by an antioxidant character of the *ortho*-dimethyl-phenol group. This observation tends to rule out a causal role of lipid-peroxidation in the development of cytotoxic effects of 3,5-dimethyl-NAPQI. It should be realized, however, that the thiobarbituric acid assay for lipid-peroxidation, in principle, does not detect all reactive species involved in lipid-peroxidation. In analogy, a causal role of lipid-peroxidation in the induction of cytotoxicity by paracetamol and NAPQI might be questioned. In recent *in vivo* studies it has been shown that paracetamol-induced lipid-peroxidation was stimulated twenty-fold [32] or prevented totally [33], without any effect on the hepatotoxicity of the drug. Several antioxidants, such as ascorbic acid [34], promethazine [10], α-tocopherol [12] and (+)catechin [10], however, have been reported to prevent paracetamol-mediated hepatotoxicity. It remains to be established in what way antioxidants protect against hepatotoxicity of paracetamol.

In view of the fact that 3,5-dialkyl substitution of

paracetamol does not diminish its analgesic activity, it is of importance to determine whether the prevention of toxicity by 3,5-dialkyl substitution, observed in hepatocytes, also occurs *in vivo*. After oral treatment of mice with 3,5-dialkyl derivatives of paracetamol (R=CH_3, C_2H_5 and C_3H_7) recently, we observed no hepatotoxicity, even at dosages higher than the hepatotoxic dose of paracetamol [35]. These findings, however, are in contrast to equitoxicity of paracetamol and 3,5-dimethyl-paracetamol reported previously [20]. The reason for this discrepancy is as yet unclear.

In summary, the present findings on the mechanism of toxicity of NAPQI's, from paracetamol and its alkyl substituted derivatives formed by the cytochrome P-450 mixed-function oxidase system, are illustrated in Fig. 3. NAPQI and 3-alkyl-NAPQI's conjugate with GSH and, after depletion of GSH, with protein thiols. 3,5-Dialkyl-NAPQI's, however, are unable to conjugate with the corresponding thiols. Quinone imines, formed from paracetamol, 3-alkyl and 3,5-dialkyl derivatives, are all capable of oxidising thiols from GSH and proteins to the corresponding disulfides. The GSSG formed, however, is quickly reduced again by NADPH-dependent GSH-reductase. Protein disulfides formed have been shown to be reducible both enzymatically and non-enzymatically by GSH [36]. In the case of NAPQI and 3-alkyl-NAPQI's both reversible and irreversible depletion of GSH is taking place, whereas 3,5-dialkyl-NAPQI's only cause reversible GSH depletion during the reductive detoxication of the quinone imines. Reversible depletion of GSH to a certain extent apparently is not detrimental to the hepatocyte. As far as the mechanism of toxicity is concerned, the facts that the cytotoxicity of NAPQI and 3,5-dimethyl-NAPQI (the latter only after artificial depletion of GSH) is accompanied by oxidative depletion of protein thiol levels and that the cytotoxicity can be prevented by the disulfide reducing agent dithiothreitol, suggest that oxidation of protein thiols plays a major role in the development of cytotoxicity of these compounds. Lipid peroxidation is clearly not necessarily involved in the development of cytotoxicity of 3,5-dimethyl-NAPQI. However, a possible role of this process, in the cytotoxic action of NAPQI cannot be completely ruled out on the basis of the present data.

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