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MECHANISM OF PROTECTION OF EBSELEN AGAINST PARACETAMOL-INDUCED TOXICITY IN RAT HEPATOCYTES

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Abstract—The protective effect of ebselen (PZ 51), an anti-inflammatory agent, on paracetamol-induced (1 mM) cytotoxicity in hepatocytes freshly isolated from β-naphthoflavone-pretreated rats was studied. At a concentration of 50 μM added simultaneously with paracetamol, ebselen prevented paracetamol-induced leakage of lactate dehydrogenase (LDH) almost completely and lipid peroxidation (LPO) and depletion of glutathione (GSH) substantially. These protective effects were even more pronounced at 100 μM concentration of ebselen. When added to the hepatocytes 1 hr before paracetamol, 50 μM of ebselen also prevented LDH leakage, LPO and GSH depletion. Reverse addition of paracetamol and ebselen did not result in protection. Simultaneous incubation of 100 μM ebselen and paracetamol inhibited GSH conjugation of paracetamol by more than 50%, however, without any effect on glucuronidation and sulfation of paracetamol. Ebselen was shown not to react directly with paracetamol nor to inhibit cytochrome P450 activity measured as 7-ethoxycoumarin *O*-deethylase (ECD) activity in the hepatocytes. At mixing, synthetic ebselen selenol and synthetic *N*-acetyl-*p*-benzoquinone imine (NAPQI) were shown to form paracetamol and ebselen diselenide. No indication was found for the formation of an ebselen–paracetamol conjugate upon reacting synthetic NAPQI and synthetic ebselen selenol. Reduction of NAPQI, the reactive metabolite of paracetamol, by ebselen selenol is discussed in terms of the mechanism of cytoprotection.

Key words: ebselen; anti-inflammatory agent; paracetamol; cytotoxicity; mechanism of protection; reactive intermediate; ebselen selenol

Paracetamol (acetaminophen, 4′-hydroxyacetanilide) is a commonly used and safe analgesic drug, which upon overdose is known to cause centrilobular hepatic necrosis [1–3]. At normal dose levels the drug mainly undergoes sulfation and glucuronidation in man and most other mammalian species. At higher doses, however, paracetamol is increasingly metabolized into a reactive metabolite, NAPQI‡ [4], by rat liver cytochrome P450. In *in vitro* studies it had been shown that the β-NF-inducible cytochrome P450 form 1A1 is mainly involved [5]. Recently, also the P450 enzymes 1A2 and 2E1 in man [6], 3A1 and 3A2 in rat liver microsomal incubations and 3A4 in Hep G2 cells expressing human cytochrome P450 [7] were reported to be importantly involved in bioactivation of paracetamol. NAPQI possesses both electrophilic and oxidant characteristics. As a consequence, it can deplete intracellular GSH and protein thiol groups by alkylation [8] and oxidation [9] which can lead to the formation of mixed disulfides [10, 11]. These events are subsequently giving rise to changes in the cellular calcium homeostasis [12–14], LPO [15, 16], loss of mitochondrial respiratory function [17], and finally to cell death. Taking these mechanisms into consideration there have been many efforts to prevent paracetamol-induced hepatotoxicity either by interference with biochemical processes involved [18] or by modification of the structure of paracetamol [15]. Extensive knowledge of the molecular mechanisms of paracetamol-induced cytotoxicity makes paracetamol an interesting and useful model toxin to study effects and mechanisms of cytoprotective agents [3].

Ebselen (PZ 51; 2-phenyl-1,2-benzisoselenazol-3(2*H*)-one), is a seleno-organic compound with low reported toxicity [19, 20], presumably because elemental selenium is not bioavailable as such. Ebselen is undergoing clinical trials for the treatment of various liver diseases and as an anti-inflammatory drug [21]. Ebselen has been demonstrated to have direct antioxidant [22] and thiol-dependent peroxidase-like activity [23, 24]. During the latter activity peroxides are inactivated by a metabolic reaction cycle in which a selenenyl sulfide, a hypothetical intermediate ebselen selenol, diselenide and selenenic acid anhydride intermediates are

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‡ Abbreviations: α-NF, α-naphthoflavone; β-NF, β-naphthoflavone; CDNB, 1-chloro-2,4-dinitrobenzene; EC, 7-ethoxycoumarin; ECD, ethoxycoumarin *O*-deethylase; GSH, glutathione; GSSG, disulfide form of glutathione; LDH, lactate dehydrogenase; LPO, lipid peroxidation; NAPQI, *N*-acetyl-*p*-benzoquinone imine.

formed through chemical reactions with GSH [25, 26] and synthetic thiols such as dithioerythreitol [27] and *N*-acetyl-L-cysteine [28]. Recently, the formation of a selenol derivative of ebselen in reaction with thiols was proven [29] by trapping the ebselen selenol with CDNB. Ebselen was shown to inhibit adenosinediphosphate-$FeSO_4$ induced LPO in isolated hepatocytes [27] and to prevent diquat induced toxicity in freshly isolated hepatocytes, which is thought to be mediated by oxygen radicals formed by redox-cycling [28].

Sodium selenite, another selenium-containing compound, has recently been shown to protect against nephrotoxicity of cisplatin [30] as well as against the toxicity of paracetamol induced in rat hepatocytes [31]. Earlier, protective effects of sodium selenite consumption against LPO after paracetamol intoxication in mice and rats, respectively, were demonstrated [16]. Recently, the nephrotoxicity of cisplatin was reported to be alleviated in mice and rats also by ebselen [32]. Baldew *et al.* [32] assumed the protective effects of ebselen and sodium selenite on cisplatin toxicity occur via ebselen selenol, a product of a reaction between ebselen and thiols for which only recently solid experimental evidence has been presented [29].

This led us to investigate whether ebselen, like sodium selenite, could protect against the various toxicity mechanisms induced by paracetamol and, moreover, to investigate the possible role of a selenol in such a protection. For reasons indicated above, hepatocytes freshly isolated from β-naphthoflavone-induced rats were selected as a test system.

MATERIALS AND METHODS

Biological materials. Collagenase B, β-glucuronidase, arylsulfatase and pyruvate were obtained from Boehringer (Mannheim, Germany). BSA, GSH and GSSG reductase, NADPH and γ-glutamyltranspeptidase (γ-GT) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Bio-Rad Protein Assay kit was obtained from Bio-Rad Laboratories GmbH (München, Germany).

Chemicals. Paracetamol was purchased from Brocacef (Maarssen, The Netherlands), CDNB and EC were from Sigma. 7-Hydroxycoumarin and β-NF were from Janssen Chimica (Beerse, Belgium) and α-NF was from Aldrich-Europe (Beerse, Belgium). Ebselen was a generous gift of Rhône-Poulenc Nattermann (Cologne, Germany).

Ebselen diselenide [2,2′-diselenobis(benzanilide)] was synthesized, recrystallized and analysed by ^{1}H-NMR according to Engman and Hallberg [33]. Ebselen selenol (*N*-phenyl-2-carboxamidobenzeneselenol) was prepared by reduction of ebselen diselenide as described by Cotgreave *et al.* [29]. Synthesis of ebselen selenol was confirmed by GC–MS after derivatization by diazomethane. 2-Methylselenobenzanilide (characteristic selenium isotopic distribution; molecular ion at m/z 291 with fragment ions at m/z 276 and m/z 199) was formed [34].

NAPQI was prepared in dichloromethane with silver oxide as described by Huggett and Blair [35]. The stability and the purity of NAPQI was checked by ^{1}H-NMR and GC–MS.

Reactions between ebselen, paracetamol and their metabolites. Routinely, the stability of ebselen selenol was assessed by aromatic S_N2-substitution with CDNB as described by Cotgreave *et al.* [29]. Furthermore, this reaction between CDNB and the selenol was used to determine remaining quantities of ebselen selenol after various reactions. For the ebselen-dinitrobenzene conjugate we developed an HPLC analysis method based on reversed phase chromatography. Two RP-C_{18} cartridge columns in series (3 × 100 mm each; particle size 5 μm; Chrompack, Middelburg, The Netherlands) were used and were eluted with 1% H_3PO_4/acetonitrile (60/40) at 0.4 mL/min.

To investigate possible direct reactions between ebselen (50 μM) and paracetamol (50–350 μM) both compounds were incubated in potassium phosphate buffer (pH 7.4). UV/vis absorption between 280 and 380 nm was followed spectrophotometrically. The reactions between NAPQI and ebselen selenol (respectively metabolites of the parent compounds paracetamol and ebselen) were studied by mixing ethyl acetate solutions of NAPQI (50 and 100 μM) and selenol (50 μM).

Animals and isolation of hepatocytes. Male Wistar rats (220–240 g) from Harlan CPB (Zeist, The Netherlands) were used in the present study. They were housed in humidity (50%) and temperature (22°) controlled rooms with a 12 hr lighting cycle. Food and water were provided *ad libitum*. The rats were pretreated twice intraperitoneally with β-NF (80 mg/kg dissolved in arachides oil) at 48 and 24 hr before isolation of the hepatocytes, to induce cytochrome P450 1A1 thus making the hepatocytes more susceptible to paracetamol-induced cytotoxicity [13]. Rats were fasted overnight before isolation of the hepatocytes. The liver parenchymal cells were isolated by a two-step collagenase perfusion method essentially according to the procedure described by Seglen [36] as modified by Nagelkerke *et al.* [37]. The viability of the hepatocytes obtained was usually between 94 and 98% as judged by trypan blue exclusion.

Incubation of hepatocytes. Freshly isolated hepatocytes (1.5–2 × 10^6 cells/mL) were incubated in 7.0 mL of Hanks' HEPES buffer (pH 7.6), containing 1.5% BSA. The cells were incubated in plastic scintillation vials in a rotary shaker (140 rpm) equilibrated at 37° under a 95% oxygen and 5% carbon dioxide atmosphere for 15 min. Then paracetamol, dissolved in physiological saline, or ebselen, dissolved in DMSO were added (concentrations of paracetamol and ebselen are given in the legends). The maximal concentration of DMSO in incubation medium was 0.3% (v/v) and equal amounts of DMSO were added to the control incubations. At the start of the incubations and during 3 hr thereafter, 0.4 mL samples were taken for measuring LDH leakage and cellular GSH levels and 0.5 mL samples for measuring LPO.

At time 0 and 3 hr, 0.5 mL cell suspensions were taken for analysis of paracetamol metabolites by HPLC as described by Howie *et al.* [38]. When measuring ECD activity in the hepatocytes [39],

2 mL cell suspensions were preincubated with 1 mM paracetamol and/or 50 μM ebselen. Then ECD assays were initiated by adding EC dissolved in Hanks' HEPES buffer to a final concentration of 100 μM. After 30 min, incubations were stopped by placing the vials into liquid nitrogen.

Assays. The cytotoxicity of paracetamol was determined as LDH leakage from the hepatocytes into the medium [40]. Samples of cell suspensions (0.4 mL) were centrifuged at 100 *g* for 3 min. Of the supernatant, 0.2 mL samples were taken for the assay of LDH leakage, whereas the cell pellet was used for measuring intracellular GSH levels using the method described by Redegeld *et al.* [41]. Also lipid peroxidation (LPO) was determined in the cell pellet by measuring the formation of products reacting with 2-thiobarbituric acid essentially as described by Haenen and Bast [42], without the addition of butylated hydroxytoluene.

ECD activities were determined by measuring the formation of 7-hydroxycoumarin during 30 min of incubation with 7-ethoxycoumarin using a Perkin–Elmer fluorescence spectrophotometer with excitation and emission wavelengths of 368 and 456 nm, respectively, as described by Edwards *et al.* [39]. Protein contents were measured by a Bio-Rad protein assay.

Analysis of paracetamol metabolites. Metabolites of paracetamol were analysed by an HPLC system consisting of a Millipore Waters Model 510 pump, a Kontron Uvikon 725 ultraviolet detector (set at 250 nm), a Kipp & Zonen BD 40 recorder, and two RP-C_{18} glass tube cartridge columns (3 × 100 mm each; particle size 5 μm; Chrompack) in series. The mobile phase consisted of 1% aqueous acetic acid–methanol–ethyl acetate (90:15:0.1) and the flow rate was 0.4 mL/min. All samples were deproteinized by 12.5% trichloroacetic acid. For peak identification and quantification, samples were incubated with β-glucuronidase or arylsulfatase for 16 hr [43] or with γ-GT for 24 hr [44].

All results were expressed as means ± SD. Statistical significance was tested by Student's *t*-test.

RESULTS

Cytotoxicity of paracetamol

Using LDH leakage as a parameter, 1 mM of paracetamol was found to be cytotoxic in freshly isolated hepatocytes from β-NF-pretreated rats (Fig. 1). Paracetamol strongly and consistently induced LDH leakage from the hepatocytes, namely from 8.8 ± 3.3% before to 59.6 ± 6.9% after 3 hr incubation (Fig. 1a). Paracetamol also induced extensive LPO, measured as 2-thiobarbituric acid reactive materials in the hepatocytes over the same time period (Fig. 1b). Addition of paracetamol resulted in depletion of intracellular GSH levels to 19.2 ± 1.1% of the zero time levels after 1 hr of incubation, and subsequently to 5.2 ± 1.5% after 3 hr of incubation (Fig. 1c). The paracetamol-induced increase of LDH leakage and LPO in the hepatocytes consistently appeared 1 hr later than the decrease of intracellular GSH levels.

Cytotoxicity of ebselen

Ebselen has a maximum absorbance at 324 nm. Therefore, it was firstly examined whether ebselen itself might interfere with the assay of LDH leakage involving measurement of changes of NADH concentrations at 340 nm. It appeared that ebselen did not disturb the assay of LDH leakage in the concentration range between 50 and 200 μM.

As shown in Fig. 2a, up to a concentration of 100 μM, ebselen was not toxic to hepatocytes. However, at concentrations of 150 and 200 μM, ebselen caused a slight increase of LDH leakage from the hepatocytes after 1 hr incubation. At 50 and 100 μM concentrations ebselen did not significantly influence spontaneous LPO (Fig. 2b) and GSH depletion (Fig. 2c) as seen in control incubations with hepatocytes from β-NF-pretreated rats. Therefore and because of the limited solubility of ebselen in aqueous suspensions, 50 and 100 μM concentrations of ebselen were chosen for testing protective effects against paracetamol-induced cytotoxicity in the hepatocytes.

Protective effects of ebselen against paracetamol-induced cytotoxicity in hepatocytes

When added simultaneously with paracetamol to hepatocytes from β-NF-pretreated rats, 50 μM ebselen significantly prevented the paracetamol-induced LDH leakage from the hepatocytes (Fig. 1a). Ebselen also significantly protected against the paracetamol-induced LPO (Fig. 1b) and it delayed the paracetamol-induced GSH depletion (Fig. 1c). After 1 and 3 hr of incubation of the hepatocytes with 1 mM paracetamol and 50 μM ebselen, intracellular GSH levels decreased to 49.4 ± 9.2% and 11.1 ± 2.6% of zero time levels, respectively. Increasing the concentration of ebselen to 100 μM almost completely prevented cellular LDH leakage and LPO induced by paracetamol. GSH levels only decreased to 81.2 ± 13.9% of zero time levels after 1 hr of incubation and to 30.7 ± 10% after 3 hr of incubation (Fig. 1c).

In order to find out whether there exists a time dependency of the protection by ebselen against the paracetamol-induced toxicity, the protection of ebselen was also examined when added 1 hr before or after paracetamol (Table 1). Added 1 hr before paracetamol, 50 μM ebselen still alleviated paracetamol-induced LDH leakage, LPO and GSH depletion in the hepatocytes (Table 1). However, when ebselen (50 μM) was added 1 hr later than paracetamol, it did not protect anymore against LDH leakage, LPO and intracellular GSH depletion due to paracetamol (Table 1).

Effects of ebselen on paracetamol metabolism

During 3 hr of incubation, approximately 35% of 1 mM paracetamol was metabolized by the hepatocytes to only three well-known metabolites which were detected by HPLC, i.e. the glucuronide, the sulfate and the GSH conjugate. As shown in Table 2, 100 μM ebselen added simultaneously with 1 mM paracetamol to hepatocytes had no statistically significant effect on the formation of the glucuronide and sulfate conjugates of paracetamol. However, it significantly decreased the formation of the GSH conjugate of paracetamol (which forms about 5% of total paracetamol metabolism) to less than about

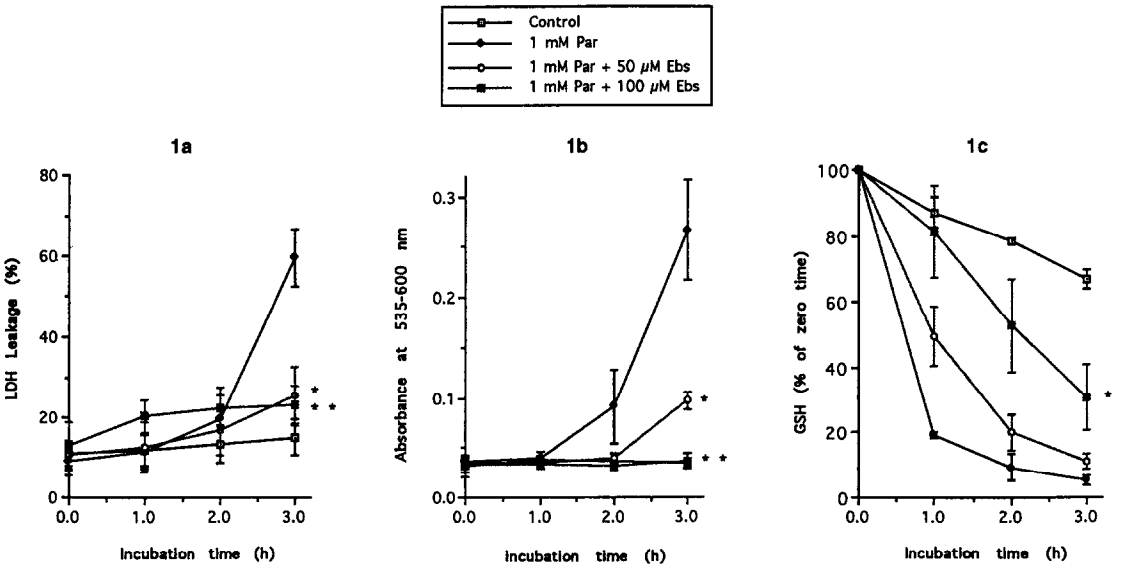

Fig. 1. Effects of paracetamol (1 mM) and paracetamol plus concomitantly added ebselen (50 or 100 μM) on LDH leakage (a), LPO (b) and GSH depletion (c) in freshly isolated hepatocytes from β-NF-pretreated rats during incubation. Par: paracetamol; Ebs: ebselen. Results are presented as mean values ± SD of four experiments. *$P < 0.05$, **$P < 0.01$.

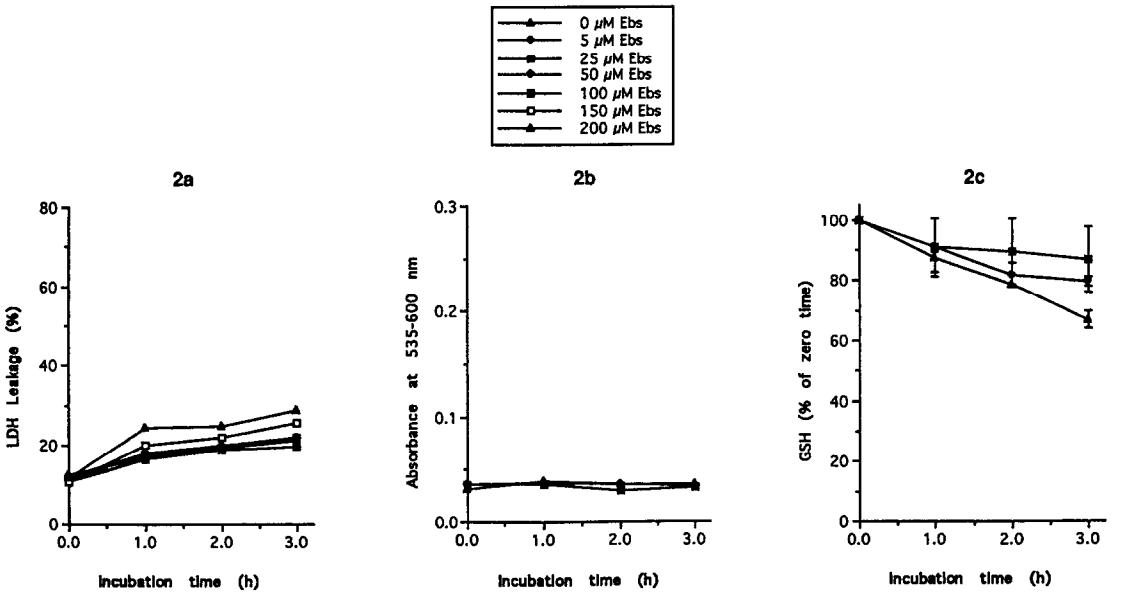

Fig. 2. Cytotoxicity of ebselen measured as LDH leakage (a) and effects of ebselen on LPO (b) and GSH levels (c) in hepatocytes from rats pretreated with β-NF. During the incubation, 0.2 mL samples of the cell suspensions were taken at every hour for measuring LDH leakage from the hepatocytes, 0.4 and 0.5 mL for GSH levels and LPO, respectively. LDH leakage is expressed as % of total activity in the cells. LPO was measured as TBA-reactive material at 535–600 nm. The results are presented as mean values ± SD of four separate experiments.

Table 1. Time dependency of the effect of ebselen on paracetamol-induced toxicity in freshly isolated hepatocytes

Treatment	LDH leakage (%)		Lipid peroxidation		GSH levels (%)	
	t = 0 hr	t = 3 hr	t = 0 hr	t = 3 hr	t = 0 hr	t = 3 hr
Control	12.9 ± 3.2	19.3 ± 7.1	7.0 ± 7.5	9.3 ± 5.4	100	93.4 ± 3.2
Par (1 mM)	12.2 ± 5.2	63.3 ± 19.4	5.0 ± 6.0	208.3 ± 72.7	100	2.6 ± 0.7
Par + Ebs*	13.0 ± 2.4	30.0 ± 13.5§	5.3 ± 3.8	65.5 ± 45.8§	100	7.7 ± 5.1
Par + Ebs†	12.1 ± 2.3	47.3 ± 8.0§	5.7 ± 5.0	83.7 ± 72.7	100	6.2 ± 2.0
Par + Ebs‡	11.8 ± 4.6	75.5 ± 18.4	4.0 ± 4.6	134.3 ± 81.9	100	3.1 ± 1.6

The final concentrations of paracetamol (Par) and ebselen (Ebs) in incubation mixtures were 1 mM and 50 μM, respectively. Rats were pretreated with β-NF as described in Materials and Methods. LPO was measured as absorbance at 535–600 nm $\times 10^{-3}$. The results are presented as means ± SD (N = 3).

* Paracetamol and ebselen were added concomitantly; †ebselen was added 1 hr before paracetamol; ‡ ebselen was added 1 hr later than paracetamol.

§ $P < 0.05$ as compared with paracetamol alone.

Table 2. The effect of ebselen on paracetamol metabolism in hepatocytes freshly isolated from β-NF-pretreated rats during 3 hr incubation

Treatment	Paracetamol	Paracetamol conjugates		
		Glucuronide	Sulfate	Glutathionyl
Par	100 ± 9	100 ± 37	100 ± 20	100 ± 22
Par + Ebs	108 ± 8	81 ± 11	103 ± 29	41 ± 20*

Par: 1 mM paracetamol; Par + Ebs: 1 mM paracetamol + 100 μM ebselen. Individual values are given as percentage of the quantity recovered in the reference incubation with paracetamol solely. All data are presented as means ± SD (N = 4).

* $P < 0.001$.

50% of the original amount. In the presence of 100 μM ebselen, total biotransformation of paracetamol was not changed significantly, since still some 30% of 1 mM paracetamol was metabolized by the hepatocytes (Table 2).

Effect of ebselen on ECD activity

The cytotoxicity of paracetamol is thought to be mediated by NAPQI, the formation of which is catalysed by various forms of cytochrome P450. ECD activity reflects the activity of a number of cytochromes P450, some of which (e.g. 1A1) are responsible for the conversion of paracetamol to NAPQI [5]. Therefore, the effect of ebselen on the *in situ* ECD activity in hepatocytes was examined. Firstly, the effect of DMSO (solvent for ebselen) was checked. ECD activities in the hepatocytes were 26.5 ± 4.8 (no preincubation), 26.9 ± 6.8 (preincubated with DMSO for 1.5 hr) and 29.0 ± 13.3 (preincubated with DMSO for 2.5 hr) nmol/min/mg protein (Fig. 3; control bars). This indicates no significant influence of DMSO during preincubation on the ECD activity. ECD activity in hepatocytes was completely inhibited by the presence of α-NF (50 μM) upon 30 min of incubation (results not shown), indicating that ECD activity as assayed can be inhibited (positive control).

Neither ebselen (50 μM) nor paracetamol (1 mM) significantly influenced ECD activity in the hepatocytes, even when incubated with hepatocytes for

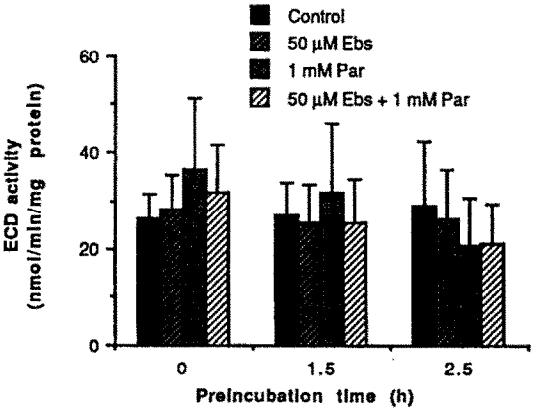

Fig. 3. Effects of ebselen and paracetamol on ECD activity in freshly isolated hepatocytes from β-NF-pretreated rats. Before the ECD assays started the hepatocytes were preincubated with 0.5% DMSO (control), 50 μM ebselen and/or 1 mM paracetamol for 0, 1.5 and 2.5 hr. ECD assays were started by adding EC (100 μM) to the hepatocytes and the incubation continued for another 30 min. The formation of EC was measured fluorimetrically with excitation and emission wavelengths set at 368 and 456 nm, respectively. The results are presented as mean values ± SD of six experiments.

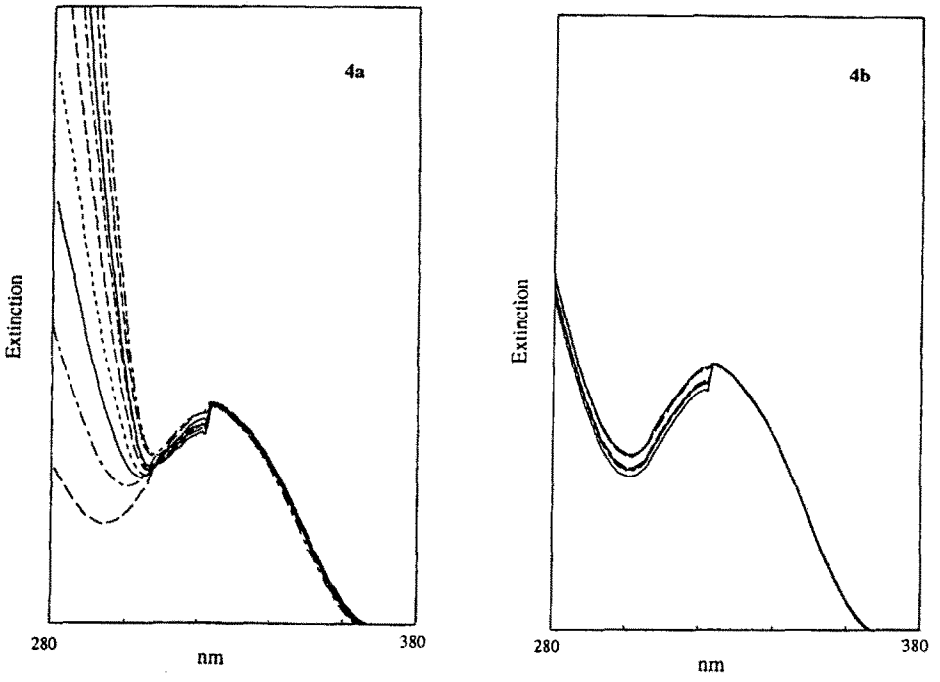

Fig. 4. UV/vis spectra of ebselen in the presence or absence of paracetamol. Incubations were carried out at 37° in potassium phosphate buffer (pH 7.4). Time dependency (a): ebselen (50 μM) and paracetamol (50 μM) were incubated for 30 min and during the incubation the UV/vis spectra were recorded every 2 min. Concentration dependency (b): ebselen (50 μM) was incubated with 50, 100, 150, 200, 250, 300 and 350 μM paracetamol, respectively. After incubation for 2 min the UV/vis spectra were recorded.

1.5 and 2.5 hr before the ECD assay was started (Fig. 3). Even when ebselen (50 μM) and paracetamol (1 mM) were added simultaneously, the ECD activity was not significantly different from incubations with paracetamol alone.

Reactions between ebselen, paracetamol and metabolites

Ebselen is known to react rapidly with the thiol group of GSH and other biological thiols to give a selenenyl sulfide, which in the presence of excess thiols is rapidly converted into the diselenide of ebselen [25, 26]. With UV/vis spectroscopy and HPLC it was investigated whether ebselen reacts directly with paracetamol in buffer. Besides an aromatic absorption at 280 nm, ebselen has a maximum absorption at 324 nm representing the five-membered isoselenazol ring. Addition of paracetamol in concentrations varying from 50 to 350 μM to 50 μM ebselen, increased the absorption at 280 nm without any change at 324 nm (Fig. 4a). Incubation of 50 μM paracetamol and 50 μM ebselen for 30 min did not change the absorption of ebselen at 324 nm (Fig. 4b). HPLC analysis of the latter reaction mixture revealed only two peaks with retention times of 2.7 and 4.9 min, respectively, which were identical to those of separately injected paracetamol and ebselen, indicating that ebselen did not react directly with paracetamol.

When NAPQI (50 μM) and ebselen selenol (50 or 100 μM), both in ethyl acetate, were mixed, HPLC analysis revealed four peaks after "trapping" of unreacted ebselen selenol with CDNB (Fig. 5). One peak coeluted with authentic paracetamol (2.7 min), the second with unreacted CDNB (4.9 min), the third (7.1 min) with authentic ebselen dinitrobenzene conjugate (reflecting trapped ebselen selenol) and the last peak with authentic ebselen diselenide (13.0 min). No peak of a conjugated product of NAPQI and ebselen selenol was detected.

DISCUSSION

The aim of the present study was to investigate whether ebselen possesses protective activities against paracetamol-induced cytotoxicity in freshly isolated rat hepatocytes and furthermore to study the underlying mechanisms of protection. The mechanisms by which paracetamol causes hepatic necrosis upon overdose involve metabolic conversion of the drug into a reactive metabolite, NAPQI, primarily by 3-methylcholanthrene and β-NF-inducible forms of cytochrome P450. NAPQI, the presumed toxic metabolite of paracetamol, has been shown to irreversibly deplete cellular GSH, to bind covalently to protein thiol groups and to induce an oxidative stress in hepatocytes manifesting itself in oxidative depletion of protein thiol levels, LPO and

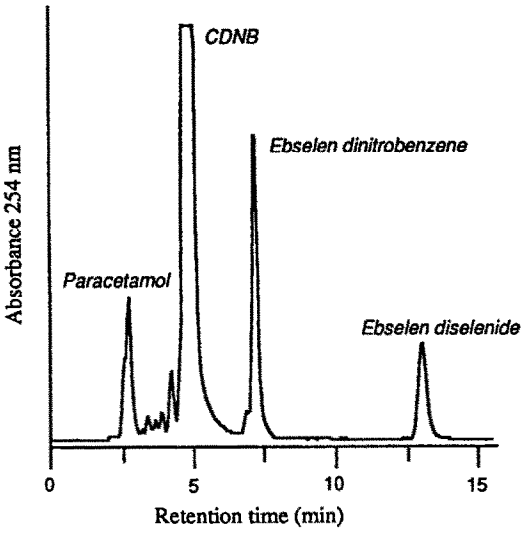

Fig. 5. HPLC analysis at reaction of ebselen selenol and NAPQI. Paracetamol (2.7 min), CDNB (4.9 min), ebselen dinitrobenzene (7.1 min), ebselen diselenide (13.0 min).

disturbances of e.g. the cellular Ca^{2+}-homeostasis [3, 13, 45].

The present results show that cell death caused by paracetamol occurred 2–3 hr after addition of paracetamol (1 mM) to hepatocytes isolated from β-NF-pretreated rats (Fig. 1). The cytotoxicity of paracetamol observed in this study corresponds well with that previously reported [13, 15], and indicates that this system under the applied circumstances is useful as a test system for cytoprotective effects. During the first 2 hr paracetamol was apparently converted into NAPQI, immediately causing GSH depletion and subsequently LPO and cell death (Fig. 1). GSH plays an important role in the detoxification of electrophilic reactive intermediates, active oxygen species and other radicals. Hydrogen peroxide and lipid hydroperoxides are reduced by GSH peroxidase, in a process in which GSH is oxidized to GSSG. Furthermore, GSH plays a role in the reduction of protein disulfides and protein mixed disulfides under the formation of GSSG [11].

Ebselen has been shown to be a potential anti-inflammatory drug [19] and to possess antioxidant activity [23]. In addition, ebselen possesses GSH peroxidase-like activity, using hydrogen peroxide and lipid hydroperoxides as substrates [23, 25]. In the present study, at concentrations of 50 and 100 μM, ebselen showed no toxicity to freshly isolated hepatocytes from β-NF-pretreated rats. When added concomitantly with paracetamol to the hepatocytes, ebselen at 100 μM completely prevented paracetamol-induced cytotoxicity, as indicated by LDH leakage (Fig. 1a) and LPO (Fig. 1b), yet, it only partly inhibited GSH depletion induced by paracetamol (Fig. 1c). These results support the concept that a moderate GSH depletion by paracetamol does not necessarily cause cell death [13, 15]. Ebselen (50 μM) added 1 hr before paracetamol also could prevent paracetamol-induced cytotoxicity. However, added 1 hr after paracetamol 50 μM of ebselen could not protect the hepatocytes anymore. A concentration of 100 μM ebselen decreased paracetamol GSH conjugate formation in the hepatocytes more than 50% (Table 2). In contrast, no statistically significant effect on formation of paracetamol glucuronide and sulfate conjugates was seen. This suggests that ebselen prevents the paracetamol-induced depletion of cellular GSH, and afterwards, LPO and cell death, by inhibiting the conjugation of paracetamol to GSH.

The mechanisms for the inhibition of the formation of a paracetamol–GSH conjugate by ebselen may be at least 5-fold:

(1) Ebselen inhibits cytochrome P450 and hence decreases the formation of NAPQI from paracetamol. Ebselen has been shown to disrupt rat hepatic microsomal electron transport chains, e.g. the one catalysed by NADPH-dependent cytochrome P450 reductase [46]. Furthermore, ebselen has been shown to convert microsomal cytochrome P450 to cytochrome P420 [47], presumably by interaction with a sulfhydryl group. In this study the effect of ebselen on ECD activity of cytochrome P450 was investigated in hepatocytes from β-NF-pretreated rats. The ECD activity in control hepatocyte incubations (containing 0.5% DMSO) was in the same order of magnitude as the activity shown by Edwards *et al.* [39]. The ECD activity in our hepatocytes could be completely inhibited by 50 μM α-NF. Ebselen however, at 50 μM, did not significantly influence the ECD activity in the freshly isolated hepatocytes (Fig. 3). The aforementioned inhibitory effects of ebselen on the microsomal mixed function oxidase system and/or purified NADPH-dependent cytochrome P450 reductase obviously are not relevant in a more integrated test system such as the hepatocyte which contains GSH. Protection mechanism (1) thus does not seem to be relevant in rat hepatocytes.

(2) Ebselen protects as a direct, thiol-independent antioxidant or radical scavenger. Our results show that ebselen significantly reduced paracetamol-induced LPO. However, it is still questionable, whether radical-mediated LPO is causally involved in cell death by paracetamol. The antioxidant curcumin for example has been published to protect against paracetamol-induced LPO but not cell death [48]. On the other hand, flavones can protect hepatocytes against the toxicity of paracetamol by antioxidant activity [18]. A direct, radical scavenging antioxidant activity of ebselen as observed e.g. for halogenated peroxyl radicals [22] cannot be excluded beforehand as a mechanism for the observed prevention of paracetamol-induced cell death.

(3) Ebselen protects via thiol-dependent GSH peroxidase-like activity. Since in this study ebselen showed a significant protection against paracetamol-induced LPO (Fig. 1), it is conceivable that GSH peroxidase-like activity may also be responsible for this phenomenon. The inhibitory effect of ebselen on paracetamol-induced LPO could be based on removal of hydrogen peroxide which was assumed by several authors to be involved in this form of

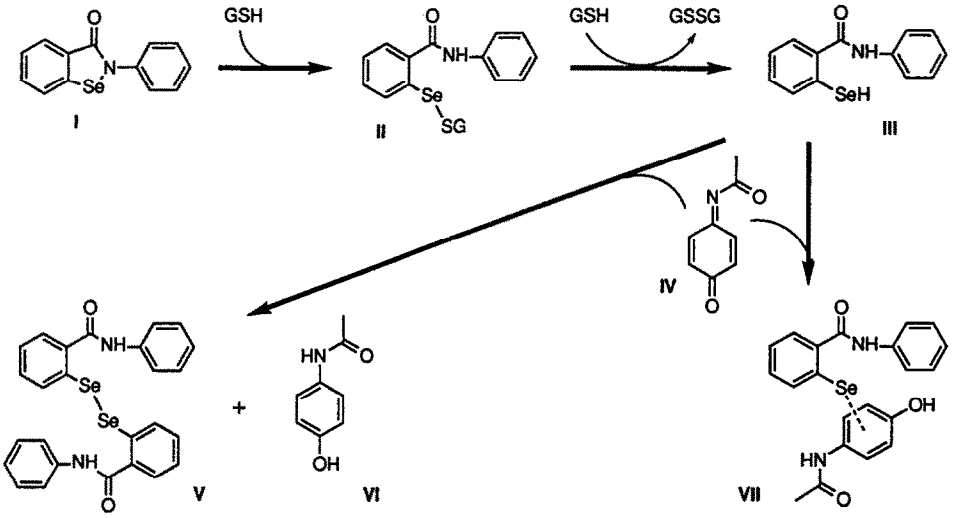

Fig. 6. Proposed mechanism of protection against paracetamol-induced cytotoxicity by ebselen. The route to compound VII is very unlikely, based on the results of this study. I, ebselen; II, selenenylsulfide; III, ebselen selenol; IV, NAPQI; V, ebselen diselenide; VI, paracetamol; VII, ebselen–paracetamol conjugate.

LPO [45, 49]. At investigating the protective effects of ebselen on paracetamol-induced LPO, Harman *et al.* [49] suggested the GSH peroxidase-like activity of ebselen to be mainly involved. However, care should be taken when comparing their results with our data because freshly isolated mouse hepatocytes were used by these authors. The fact that ebselen does not exhibit GSH peroxidase-like activity in GSH depleted hepatocytes [27] favors a contribution of the latter mechanism (3) over the GSH-independent protection mechanism (2). When adding ebselen 1 hr after paracetamol, ebselen did not protect the hepatocytes anymore against paracetamol-induced cytotoxicity (Table 1). The reason for this may be the fact that insufficient GSH remained to bioactivate ebselen to ebselen selenol. This ebselen selenol is assumed to be an intermediate in the formation of ebselen diselenide, which has been published to be responsible for the GSH peroxidase-like action of ebselen [25, 28].

(4) Ebselen or an ebselen metabolite reacts directly with the remaining paracetamol (after glucuronidation and sulfation), thus decreasing the formation of NAPQI from paracetamol. In the present study we showed however, that ebselen does not react with paracetamol directly (Fig. 4). Furthermore, HPLC analysis revealed no reaction products upon mixing paracetamol and synthetic ebselen selenol.

(5) Ebselen or a metabolite of ebselen reacts with NAPQI. Two possible reactions as depicted in Fig. 6 may occur. An important metabolite of ebselen *in vitro* in the presence of thiols is ebselen selenol as has been postulated [25] and recently demonstrated [29]. Also, *in vivo* experiments (the urinary excretion of an Se-glucuronide of ebselen) support the formation of ebselen selenol [34]. In our experiments in which synthetic ebselen selenol and NAPQI were reacted in ethyl acetate in the absence of GSH, it was shown that only ebselen diselenide and paracetamol were formed. This indicates a simple reduction of NAPQI by ebselen selenol (Fig. 6). Moreover, we have shown in an aqueous system (unpublished results) that ebselen itself reduced NAPQI to paracetamol only in the presence of GSH which is necessary to activate ebselen to ebselen selenol. Importantly, in a direct reaction between NAPQI and ebselen selenol no selenium-containing conjugate of paracetamol could be identified (Fig. 6). Similar analysis of hepatocyte incubations failed to detect any such conjugate (results not shown). Apparently, reduction of NAPQI by ebselen selenol is much more efficient than conjugation between the nucleophilic selenol and NAPQI. In line with a decrease of the NAPQI concentration in the hepatocyte due to reduction by ebselen selenol, less paracetamol GSH conjugate was found to be formed (Table 2).

Summarizing, we have shown that at a concentration of 50 μM, ebselen protects rat hepatocytes almost completely against cell death and partly against LPO and GSH depletion induced by paracetamol. At a concentration of 100 μM, the protective effect of ebselen was even more complete and highly significant for GSH depletion. The mechanism of protection may be the GSH peroxidase-like activity of ebselen, as postulated by Harman *et al.* [49] recently, assuming hydrogen peroxide to be involved in paracetamol-induced cytotoxicity. The results of this study however, strongly indicated that a reaction between ebselen selenol and NAPQI may also be responsible. In contrast to the reaction between CDNB and ebselen selenol recently described [29], we were not able to detect any nucleophilic substitution product between NAPQI and ebselen selenol in a chemical system. Therefore

conjugation between these intermediates is not a likely mechanism of protection in the hepatocytes. Ebselen selenol obviously more efficiently reduces NAPQI back to paracetamol instead of forming a substitution product. We are currently working on the kinetics of this unexpected mechanism of protection against paracetamol-induced toxicity. Further studies have to be carried out as to the relevance of this protection mechanism for *in vivo* toxicity of paracetamol.

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