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N-ACETYLCYSTEINE AND GLUTATHIONE-DEPENDENT PROTECTIVE EFFECT OF PZ51 (EBSELEN) AGAINST DIQUAT-INDUCED CYTOTOXICITY IN ISOLATED HEPATOCYTES

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Abstract—The glutathione peroxidase (GSH-Px)-like reduction of H_2O_2 by the selenoorganic compound 2-phenyl-1,2-benzoisoselenazol-3(H)-one (PZ51: Ebselen) was studied using glutathione (GSH) and the therapeutic agent *N*-acetylcysteine (NAC) to provide reducing equivalents. In a purely chemical system containing H_2O_2 and in an enzymatic system of glucose/glucose oxidase-generated H_2O_2 Ebselen alone did not reduce H_2O_2. Ebselen in combination with either GSH (1 mM) or NAC (1 mM) was capable of reducing H_2O_2 in both systems. In these non-cellular systems GSH was a more effective source of reducing equivalents than NAC. The GSH-Px-like activity of Ebselen was further investigated in a cellular system. The redox-cycling bipyridylium compound diquat generates active oxygen species, depletes intracellular glutathione, and is cytotoxic in isolated hepatocytes pretreated with the glutathione reductase inhibitor 1,3-bis(Z-chloro-ethyl)-1-nitrosourea (BCNU). Ebselen alone did not ameliorate diquat cytotoxicity, but in combination with either GSH (1 mM) or NAC (1 mM) it produced a significant delay in diquat-induced cytotoxicity. Further additions of either GSH (0.5 mM) or NAC (0.5 mM) at 30 min intervals provided significantly more protection against diquat-induced cytotoxicity and intracellular GSH depletion than the single 1 mM addition. Thus, the combination of Ebselen and NAC may provide an effective antidote in cases of overexposure to bipyridylium herbicides, such as diquat and paraquat.

The association between the localised production of reactive oxygen species and lipid hydroperoxides during the inflammatory process and irreversible tissue damage is becoming increasingly clear [1–3]. Similarly, it is clear that cells have developed defence mechanisms to cope with the normal "oxidative load" of the intracellular milieu, which may become overwhelmed during inflammation. Of these, the selenium-dependent enzyme glutathione peroxidase (GSH-Px) has been shown to play a central role in detoxifying both organic hydroperoxides (ROOH) and hydrogen peroxide (H_2O_2). Oxidized glutathione (GSSG) which results from these reactions is then reduced by NADPH through the coupled activity of glutathione reductase [4].

In view of this, it would be of considerable therapeutic significance if the resident GSH-Px activity of tissues undergoing inflammation could be therapeutically amplified. Recently, a novel series of selenoorganic compounds was synthesised [5], one of which; 2-phenyl-1,2-benzoisoselenazol-3(H)-one (PZ51: Ebselen), has shown encouraging anti-inflammatory activities. Ebselen has demonstrated both antioxidant activity *per se* [6] and importantly, thiol-dependent GSH-Px-like activity [7]. It also inhibits lipid peroxide accumulation in both microsomal membrane preparations [7] and isolated hepatocytes [8].

Hydrogen peroxide is produced intracellularly during endogenous metabolism [4]. Elevated quantities of reactive oxygen species, including H_2O_2, are also produced during the metabolism of certain xenobiotics and are often associated with the initiation of lipid peroxidation and acute toxicity [9, 10]. Ebelsen has demonstrated GSH-Px-like reduction of H_2O_2 [7] and may therefore ameliorate cellular toxicity associated with elevated intracellular production of H_2O_2.

We have therefore investigated more closely the GSH-Px-like reduction of H_2O_2 by Ebselen in a purely chemical system and also in a system where H_2O_2 was continuously generated by the activity of glucose oxidase on glucose. In addition, we have assessed the ability of the therapeutic agent *N*-acetylcysteine (NAC) to provide reducing equivalents in this reaction, since Ebselen, unlike the endogenous enzyme, shows no specific requirements for GSH as a co-substrate [8].

Furthermore, we have studied the potential GSH-Px-like protective effect of Ebselen against cytotoxicity caused by the bipyridyl herbicide diquat. Diquat undergoes rapid redox cycling in cells thereby producing active oxygen species [12, 13], and is toxic *in vitro* to isolated hepatocytes pretreated with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), as well as *in vivo* [13, 14]. In these studies we have used BCNU-treated hepatocytes to determine if Ebselen alone and in combination with GSH or NAC can ameliorate diquat cytotoxicity.

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MATERIALS AND METHODS

Chemicals. Highly purified diquat (>99%) was a kind gift of Dr L. L. Smith, ICI (U.K.) plc. BCNU was the kind gift of Bristol-Meyers Pharmaceuticals (Sweden) or obtained from the National Cancer Institute, U.S.A. Ebselen was a gift of A. Nattermann & Co. GmbH (Cologne, F.R.G.). NAC and GSH and materials for their analysis were obtained as previously described [15, 16]. Glucose oxidase (19,000 U/ml), glucose and H_2O_2 (30% v/v aqueous solution) were obtained from Sigma Chemical Co. (St. Louis, MO). Materials for the isolation of rat hepatocytes were obtained as previously described [17]. All other chemicals were of the highest grade available from local suppliers.

Chemical incubations. Chemical incubations were performed in a volume of 10 ml Krebs–Henseleit buffer, pH 7.4, containing HEPES (12.5 mM) at 37° under air. Ebselen was added in DMSO to a final concentration of 50 μM. H_2O_2 was added to a concentration of 250 μM and thiols (in aqueous solution) added to a concentration of 1 mM. Enzyme incubations were performed under similar conditions but in oxygen-saturated buffer and contained 0.25 U/ml glucose oxidase and 2 mM glucose as a source of H_2O_2. Ebselen and thiols were added in similar proportions to those in the incubations with H_2O_2.

Preparation of BCNU-treated hepatocytes from rat liver. Hepatocytes were prepared from male Sprague–Dawley rats (250 g) by the method of Moldéus *et al.* [17]. Hepatocytes were treated with BCNU and allowed to recover their GSH levels essentially as described previously [13, 18]. These techniques yielded *ca* 250×10^6 cells with 70–80% viability post-treatment. The extent of GSH reductase inhibition, assayed according to Racker [19], was between 90 and 95%.

Cell incubations. All incubations were performed in a volume of 10 ml Krebs–Henseleit buffer, pH 7.4, containing HEPES (12.5 mM) at 37° under a carbogen (95% O_2, 5% CO_2) atmosphere. Incubations contained 2×10^6 cells/ml: additions of diquat (final concentration 1 mM) and Ebselen (final concentration 50 μM) were made in water and DMSO respectively. Additions of NAC and GSH were made in aqueous solution to a final concentration of 1 mM. Further additions of 0.5 mM (final concentration) made at 30 min intervals in some experiments. Aliquots (100 μl) of incubation were centrifuged at 50 *g* for 3 min, whereupon the supernatant and the cell pellet (carefully washed once with 1 ml buffer) were collected for analysis of reduced and oxidized thiols. Lipid peroxidation was assessed in aliquots (0.5 ml) of incubation treated with trichloroacetic acid to precipitate cellular protein.

Biochemical assays. Reduced and oxidized thiols in incubations were determined by derivatisation of samples with monobromobimane (mBBr), separation of adducts by reversed phase HPLC and quantitation of adducts by fluorescence, essentially as described by Cotgreave and Moldéus [15, 16]. Lipid peroxidation was assessed as thiobarbituric acid (TBA)-reactive products in incubations as described previously [20]. H_2O_2 in chemical and enzymatic incubations was determined by formation of the ferrithiocyanate complex according to Thurman *et al.* [21].

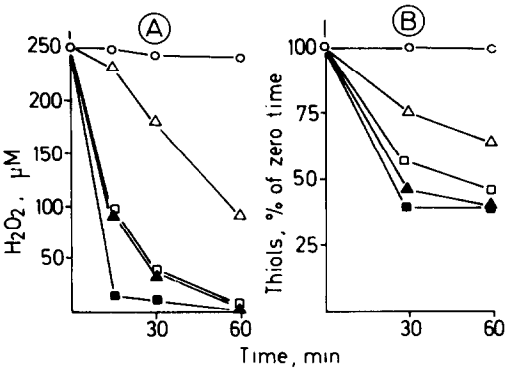

Fig. 1. Interaction of H_2O_2 (250 μM) with NAC (1 mM) and GSH (1 mM) in the presence and absence of Ebselen (50 μM). Figure 1A shows the effect on H_2O_2 concentration and Fig. 1B the effect on the concentration of free thiol. Incubations were performed as described in the Methods section. Values are means from three experiments. ○—○, control incubations, panel A, no thiol added, panel B, no H_2O_2 added; △—△, H_2O_2 plus NAC; □—□, H_2O_2 plus GSH; ▲—▲, H_2O_2 + NAC + Ebselen; ■—■, H_2O_2 + GSH + Ebselen.

RESULTS

In agreement with previous observations [7], Ebselen was shown to increase the GSH-dependent reduction of H_2O_2 (Fig. 1A). This was accompanied by a depletion of GSH in the incubation (Fig. 1B), presumably by oxidation to GSSG since 100% of the GSH was recovered following reduction of the sample with dithiothreitol (DTT). The addition of Ebselen in combination with NAC to the incubations also resulted in a reduction of H_2O_2 and a depletion of free thiols, however, the catalysis was slower than that observed in incubations containing GSH (Figs 1A and B). In the absence of GSH or NAC, Ebselen had no effect on the H_2O_2 level. In a second series of experiments in which H_2O_2 was continuously generated by the action of glucose oxidase on glucose, the GSH- and NAC-dependent reduction of H_2O_2 was also enhanced by the presence of Ebselen (Fig. 2A) with depletion of the thiol (Fig. 2B) due to oxidation as above. Furthermore, there was an apparent threshold for the reaction between both thiols and H_2O_2 in these experiments (Fig. 2). This threshold decreased significantly in the presence of Ebselen and indeed, H_2O_2 levels were almost undetectable for the first 60 min when GSH and Ebselen were provided concomitantly. An increase in H_2O_2 levels was observed subsequent to the complete depletion of thiols from the incubation (Fig. 2).

As indicated in these chemical and enzymatic test systems, NAC itself reacts rather poorly with H_2O_2. Thus, there were only very marginal protective effects of NAC (1 mM) against diquat-induced GSH depletion (Fig. 3A) and cytotoxicity (Fig. 4A) in BCNU-treated hepatocytes, even with repeated additions of NAC at 30 min intervals. Effects on

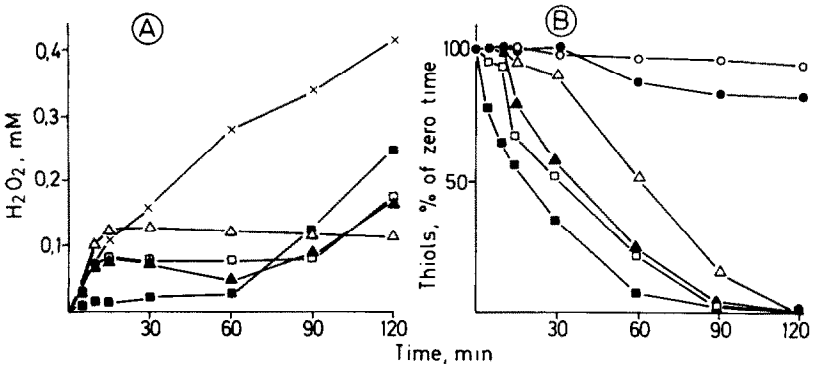

Fig. 2. Interaction of H_2O_2 derived from glucose–glucose oxidase with NAC (1 mM) and GSH (1 mM) in the presence and absence of Ebselen (50 μM). Figure 2A shows H_2O_2 concentration and Fig. 2B the effect on the concentration of free thiol. Incubations were performed as described in the Methods section. Values are means from three experiments. ×—×, H_2O_2 generating system but not thiol addition; ○—○, GSH but no H_2O_2 generating system; ●—●, NAC but no H_2O_2 geneating system; △—△, H_2O_2 plus NAC; □—□, H_2O_2 plus GSH; ▲—▲, H_2O_2 + NAC + Ebselen; ■—■, H_2O_2 + GSH + Ebselen.

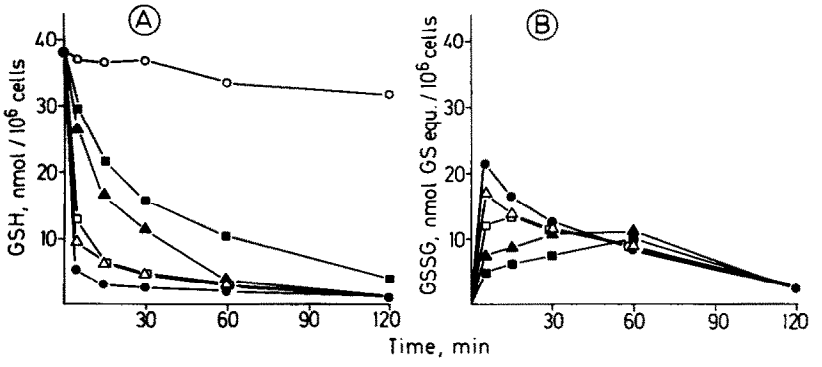

Fig. 3. The intracellular content of GSH (A) and GSSG (B) of BCNU-treated hepatocytes incubated with diquat (1 mM) in the presence and absence of NAC and Ebselen (50 μM). Incubations were performed as described in the Methods sections. Results shown are from one experiment typical of three. ○—○, cells with no additions; ●—●, cells plus diquat; △—△, cells plus diquat and NAC (1 mM); ▲—▲, cells plus diquat, NAC (1 mM) and Ebselen; □—□, cells plus diquat and NAC (1 mM and further 0.5 mM additions at 30, 60 and 90 min); ■—■, cells plus diquat, NAC (as □—□) and Ebselen. In Fig. 3B GSSG in control cells was below the level of detection.

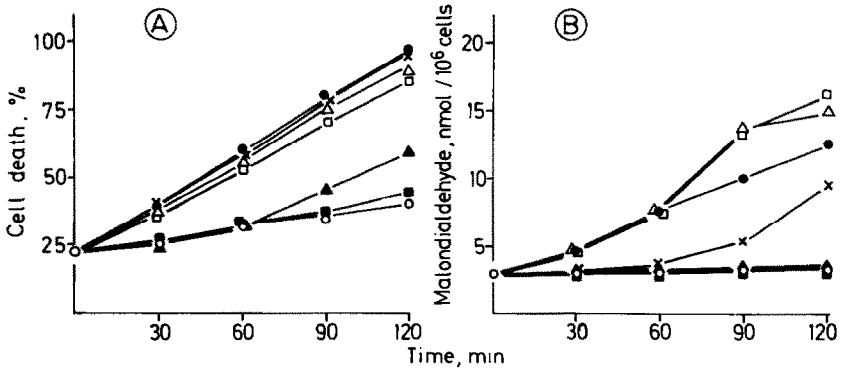

Fig. 4. Cell viability (A) and lipid peroxidation (B) in BCNU-treated hepatocytes incubated with diquat (1 mM) in the presence and absence of NAC and Ebselen (50 μM). Incubations were performed as described in the Methods section. Results shown are from one experiment typical of three. ○—○, cells with no additions; ●—●, cells plus diquat; ×—×, cells plus diquat and Ebselen; △—△, cells plus diquat and NAC (1 mM); ▲—▲, cells plus diquat, NAC (1 mM) and Ebselen; □—□, cells plus diquat and NAC (1 mM and further 0.5 mM additions at 30, 60 and 90 min); ■—■, cells plus diquat, NAC (as □—□) and Ebselen.

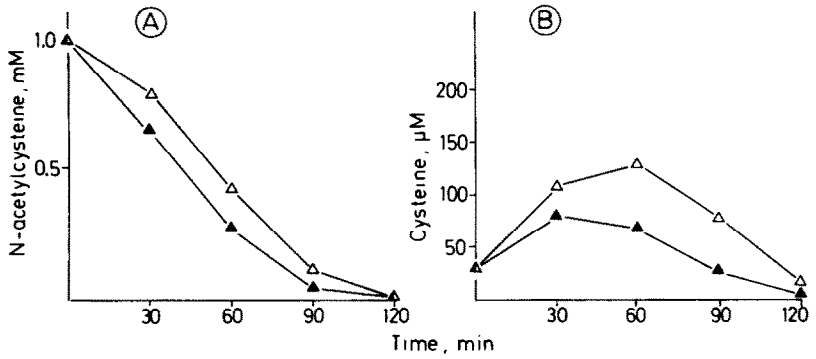

Fig. 5. Concentrations of NAC (A) and cysteine (B) in incubations of BCNU-treated hepatocytes exposed to diquat (1 mM) and NAC (1 mM) with (▲—▲) and without (△—△) Ebselen (50 μM). Experimental details as for Fig. 3.

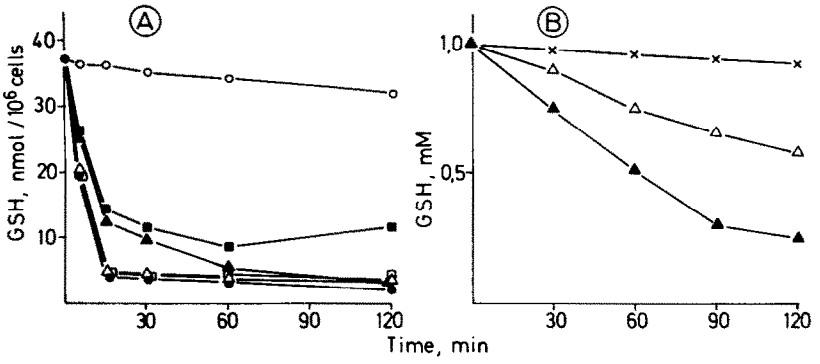

Fig. 6. Intracellular (A) and extracellular (B) GSH content of incubations containing BCNU-treated hepatocytes incubated with diquat (1 mM) in the presence or absence of GSH and Ebselen (50 μM). Incubations were performed as described in the Methods section. Results shown are from one experiment typical of three. ○—○, cells with no additions; ●—●, cells plus diquat; ×—×, cells plus GSH (1 mM); △—△, cells plus diquat and GSH (1 mM); ▲—▲, cells plus diquat, GSH (1 mM) and Ebselen; □—□, cells plus diquat and GSH (1 mM with further 0.5 mM GSH additions at 30, 60 and 90 min); ■—■, cells plus diquat, GSH (as □—□) and Ebselen.

diquat-induced lipid peroxidation of NAC *per se* were complex. NAC was shown to stimulate lipid peroxidation in these cells after 60 min of incubation (Fig. 4B). This may be due to the decline in NAC concentration (Fig. 5A) and the simultaneous accumulation of free cysteine (Fig. 5B) observed in the incubations, which is probably the result of metabolic deacetylation of NAC by the cells. The accumulation is maximal at 60 min of incubation, declining thereafter. In solution, cysteine is well known to undergo auto-oxidation producing superoxide ($O_2^{\cdot}$), H_2O_2 and hydroxyl radicals ($^{\cdot}OH$) which, in a cellular environment, may induce lipid peroxidation [22].

In the presence of Ebselen (50 μM), however, NAC significantly delayed the oxidation of intracellular GSH (Fig. 3A) in diquat-treated hepatocytes. Lipid peroxidation was also almost completely eliminated (Fig. 4B). Despite this, there was still considerable cytotoxicity, but it was markedly delayed (Fig. 4A). These protective effects were more pronounced if additional NAC was added at 30 min intervals (Fig. 4A). Moreover, when Ebselen and NAC were present together the decline in the concentration of NAC was accelerated (Fig. 5A), with a commensurate decrease in the accumulation of cysteine (Fig. 5B).

Glutathione (1 mM) added extracellularly was also ineffective in preventing the depletion of intracellular GSH caused by diquat redox cycling in BCNU-treated hepatocytes (Fig. 6A). Only minimal protection from the resultant cytotoxicity and lipid peroxidation was noted (Figs 7A and B, respectively).

In the presence of Ebselen (50 μM) there were significant reductions in the diquat-induced depletion of intracellular GSH and cytotoxicity in these cells (Figs 6A and 7A, respectively). Additionally, accumulation of TBA-reactive products was totally eliminated (Fig. 7B). As with NAC, these protective effects were more pronounced if extra GSH was added to the incubations at 30 min intervals.

Analogous to Ebselen's acceleration of the depletion of NAC (Fig. 5A), depletion of extracellular GSH was also enhanced in the presence of Ebselen (Fig. 6B). Unlike NAC, however, this GSH depletion was solely due to extracellular oxidation, since uptake of GSH into hepatocytes is minimal due to the negligible γ-glutamyltranspeptidase activity of these cells.

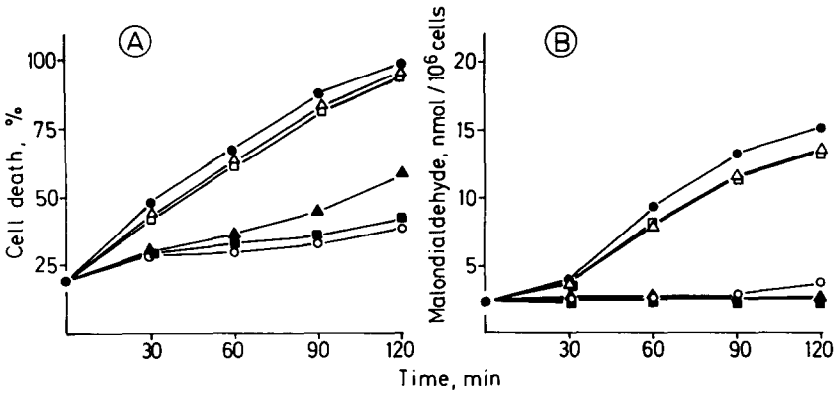

Fig. 7. Cell viability (A) and lipid peroxidation (B) of BCNU-treated hepatocytes incubated with diquat (1 mM) in the presence and absence of GSH and Ebselen (50 μM). Incubations were performed as described in the Methods section. Results shown are from one experiment typical of three. ○—○, cells with no additions; ●—●, cells plus diquat; △—△, cells plus diquat and GSH (1 mM); ▲—▲, cells plus diquat, GSH (1 mM) and Ebselen; □—□, cells plus diquat and GSH (1 mM with further 0.5 mM GSH additions at 30, 60 and 90 min); ■—■, cells plus diquat, GSH (as □—□) and Ebselen.

When Ebselen alone was supplied to incubations, no protective effect was observed upon intracellular GSH levels or cell viability. However, consistent with previous findings [6], some reduction in lipid peroxidation was observed (Fig. 4B).

It is well known that GSH does not penetrate the hepatocyte membrane. Control experiments demonstrated that free NAC was only detected in trace amounts intracellularly when hepatocytes were incubated under the conditions described. These trace levels were not elevated if Ebselen was co-incubated in the presence or absence of diquat. Similarly, oxidized NAC could not be detected intracellularly during any of these control experiments (data not shown).

DISCUSSION

The present study presents data which support previous reports of the GSH-Px-like activity of Ebselen with H_2O_2 [7]. Such activity may have contributed somewhat to the observed anti-lipid peroxidative effects of Ebselen reported previously [6, 7]. Additionally, we have demonstrated that another thiol, NAC, is also effective in supplying reducing equivalents to the peroxidase-like activity of Ebselen. NAC is of particular interest in terms of potential therapeutic applications, since it is a drug utilized in many countries in the treatment of chronic bronchitis and it is the antidote of choice for paracetamol overdosage in humans [24]. In both of these examples, NAC has shown efficacy *per os* and from previous studies it is apparent that NAC itself [16], as well as its metabolites cysteine and GSH [25] are available to the systematic circulation following oral administration. The bipyridyl herbicide diquat is an effective redox cycling compound both *in vitro* and *in vivo* [12, 13]. Previous *in vitro* studies with isolated hepatocytes demonstrated that diquat-induced cytotoxicity was expressed only after cellular defenses against oxidative stress had been compromised in some way [13]. When BCNU pretreatment of hepatocytes was utilized to inhibit GSSG reductase, diquat rapidly decreased intracellular GSH to extremely low levels, and acute cytotoxicity was apparent after only one hour of incubation [13]. This potentiation of toxicity by BCNU inhibition of GSSG reductase strongly suggested redox cycling as the major cause of cytotoxicity in this system. Redox cycling could produce the diquat radical which rapidly reacts with dioxygen to form $O_2^{\cdot-}$ and H_2O_2 by dismutation. The H_2O_2 so formed may then rapidly react in a Fenton-type reaction catalyzed by transition metals to yield $\dot{O}H$, which may induce irreversible damage to the structural integrity of the cells. Several other lines of evidence support the direct involvement of H_2O_2 in diquat toxicity *in vitro*. For example, catalase provided extracellularly protected against diquat toxicity (Moldéus *et al.*, in preparation).

The mechanism of diquat-induced hepatotoxicity is, however, still not completely understood. Previous studies in BCNU-treated hepatocytes had demonstrated that lipid peroxidation did not occur prior to cytotoxicity, but was temporally inseparable from it [13]. These studies also demonstrated that lipid peroxidation could be totally prevented, but cytotoxicity only delayed, by the addition of antioxidants such as Trolox C, promethazine and DPPD [13]. The results presented in this manuscript further support the emphasis placed on the involvement of H_2O_2 on diquat toxicity through the demonstration that both NAC and GSH were able to protect the cells from the GSH depletion and toxicity of diquat only in the presence of Ebselen. Furthermore, because toxicity was still observed despite the near total inhibition of lipid peroxidation in these cells in the presence of NAC or GSH and Ebselen, this would again suggest that lipid peroxidation is not the major cause of diquat toxicity in this system.

One may only speculate about the mechanism of the protective action of Ebselen and NAC or GSH against the GSH oxidation and toxicity induced by diquat redox cycling in these cells. Ebselen has pre-

viously been shown to protect against ADP-Fe^{2+}-induced lipid peroxidation in isolated hepatocytes, but only when the full intracellular complement of GSH was present [8]. This dependence upon intracellular GSH content suggested that Ebselen was able to enter the cell due to its lipophilicity and utilize intracellular GSH to detoxify lipid peroxides, much like GSH-Px. In our experiments thiols were added in large quantities extracellularly together with Ebselen, and several protective mechanisms may have prevailed. Firstly, Ebselen may react extracellularly with GSH/NAC to form a Se-thiol adduct [8] which enters the cell and functions intracellularly. It has been shown, however, that the Ebselen–GSH adduct does not protect against ADP-Fe^{2+}-induced lipid peroxidation without the presence of a reducing agent, such as intracellular GSH [8]. GSH itself does not penetrate the hepatocyte plasma membrane and cannot serve as a reducing agent intracellularly in our model where GSH is added extracellularly. Thus, Ebselen or its GSH adduct has to be reduced extracellularly and transported into the cell in the reduced form to serve as an intracellular reductant. Once oxidized, Ebselen or its GSH adduct would then be excreted into the extracellular medium, where it would subsequently be re-reduced and the process could begin again. NAC, on the other hand, is able to penetrate the hepatocyte plasma membrane and may thus reduce Ebselen or the Se-NAC conjugate intracellularly. However, co-incubation of Ebselen with NAC did not increase the normally extremely low intracellular content of free NAC during diquat-induced redox cycling. If the Ebselen–NAC conjugate had entered the cell and functioned intracellularly, this would probably have resulted in the formation of NAC–GSH disulfides as intracellular free NAC levels are so low. Thus transient increases in intracellular oxidized NAC would be noted.

The simplest and most likely explanation for the protective effect of NAC–GSH in combination with Ebselen is that the reactions occur extracellularly and that reduction of H_2O_2 extracellularly results in a drain of H_2O_2 out from the cell into the extracellular medium. This proposed mechanism is also supported by the protective effect of catalase against diquat-induced toxicity, since catalase is unable to penetrate the hepatocyte plasma membrane.

In conclusion, this study has demonstrated the protective effect of Ebselen in combination with either GSH or NAC against cytotoxicity due to redox cycling by a mechanism involving the removal of H_2O_2 formed during this process. It is tempting to speculate about the possible therapeutic efficacy of Ebselen in combination with NAC in instances where rapid H_2O_2 production can be correlated with irreversible tissue damage, for instance during inflammatory processes or during redox cycling of environmental chemicals. *In vivo* studies aimed at testing this hypothesis are presently under way.

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