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A NOVEL BIOLOGICALLY ACTIVE SELENOORGANIC COMPOUND—IV

PROTECTIVE GLUTATHIONE-DEPENDENT EFFECT OF PZ 51 (EBSELEN) AGAINST ADP–Fe INDUCED LIPID PEROXIDATION IN ISOLATED HEPATOCYTES

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Abstract—PZ 51 (Ebselen) is capable of inhibiting ADP–Fe-induced lipid peroxidation in isolated hepatocytes, assessed by generation of low-level chemiluminescence, and the formation of alkanes (ethane, *n*-pentane) and malondialdehyde. The sulphur analog PZ 25 is much less active.

PZ 51 is ineffective in glutathione-depleted hepatocytes that were obtained after phorone pretreatment of the animals. However, other antioxidants, like (+)-cyanidanol-3 or diethyldithiocarbamate are effective in inhibiting lipid peroxidation also in glutathione-depleted cells. The results are attributed to a GSH peroxidase-like activity of PZ 51.

Addition of the chemically synthetized compound, PZ 51-SG, a glutathione adduct of PZ 51, does not provide extra protection in GSH-depleted cells in comparison to the parent compound, PZ 51. Dithioerythritol exhibits a protective effect, and PZ 51 is more efficient than in the absence of this thiol reductant. Dithioerythritol also exerts an effect in glutathione-depleted cells. A peroxidase-like activity using DTE as reductant is demonstrated *in vitro*.

Recently, a novel biologically active selenoorganic compound exhibiting glutathione peroxidase (GSH Px)-like* activity *in vitro* and antioxidant activity against microsomal lipid peroxidation was described [1, 2]. The latter activity, the antioxidant capacity, was independent of exogenous GSH and was similar to that observed with diethyldithiocarbamate (DDC) [3]. The selenium moiety in PZ 51 is not bioavailable for incorporation into the selenoprotein GSH Px [2]. Further, it was found that incubation of resident mouse macrophages with PZ 51 revealed a partial inhibition of prostaglandin E_2 production, implying a peroxide scavenging GSH Px-like activity of this compound [4].

In the present work we show that PZ 51 protects against oxidative attack by ADP–Fe in intact cells. The effects of the compound on ADP–Fe induced lipid peroxidation with isolated hepatocytes was assessed by detection of low-level chemiluminescence, ethane and *n*-pentane production, and the formation of thiobarbituric acid-reactive material. It was compared to PZ 25, the sulphur analog, and experiments were also carried out in the state of glutathione depletion, in order to test the requirement for GSH.

* Abbreviations used: GSH, glutathione; GSH Px, glutathione peroxidase; PZ 51, 2-phenyl-1,2-benzoisoselenazol-3(2*H*)on; PZ 51-SG, adduct of GSH with PZ 51; PZ 25, 2-phenyl-1,2-benzoisosulfazol-3(2*H*)on; DDC, diethyldithiocarbamate; ADP–Fe, complex of adenosinediphosphate with $FeSO_4$; DTE, dithioerythritol.

MATERIALS AND METHODS

Biological materials and chemicals. Hepatocytes were prepared from male Wistar rats fed on stock diet (Altromin, Lage, F.R.G.). PZ 51 (Ebselen), PZ 51-SG, and PZ 25 were a gift from Dr. E. Graf (Nattermann & Cie. GmbH, Cologne, F.R.G.), and (+)-cyanidanol-3 was a gift from Dr. G. Hennings (Zyma GmbH, Munich, F.R.G.). Other chemicals and biochemicals were purchased from Fluka (Buchs, Switzerland), Merck (Darmstadt, F.R.G.), and Boehringer (Mannheim, F.R.G.).

Incubation of hepatocytes. Hepatocytes were isolated from rat liver as described previously [5]. Depletion of hepatic glutathione was obtained by pretreatment of rats with phorone at a dose of 250 mg/kg body weight injected i.p. as a 25% solution in olive oil [6] 2 hr before preparation of the hepatocytes. About 90–95% of the cells were intact as determined by Trypan Blue exclusion and by leakage of lactate dehydrogenase from the cells. The hepatocytes were stored at 4° before use. Incubations of hepatocytes (2.1×10^6 cells/ml) were carried out under constant oxygenation at 37° in 7.5 ml Krebs–Henseleit medium additionally supplemented with glucose (10 mM), lactate (2.1 mM), and pyruvate (0.3 mM) as described previously [7]. Lipid peroxidation was started upon addition of ADP (5.1 mM) and $FeSO_4$ (0.3 mM).

Lipid peroxidation assays. Lipid peroxidation in the cell mixture was monitored by three different methods, i.e. detection of low-level chemiluminescence [8], formation of alkanes [9], and thio-

barbituric acid-reactive material (malondialdehyde accumulation) [10]. Low-level chemiluminescence was monitored with an EMI-9658 AM photomultiplier (EMI-Gencom, Plainview, NY) sensitive in the 350–800 nm range. Assay conditions for chemiluminescence are the same as in [7]. Alkane evolution experiments were performed in 43 ml sealed flasks as described previously [3, 7]. The system was calibrated with a standard gas mixture (Messer-Griesheim, Duisburg, F.R.G.), and the amounts of alkanes were calculated using a correction formula for alkane dilution [9] and expressed in nmoles alkanes formed per 10^8 cells. Malondialdehyde accumulation was determined at 535–570 nm (ε = 156 mM^{-1} cm^{-1}) with a Sigma ZWS II Spectrophotometer (Biochem. Co., Munich, F.R.G.).

Glutathione determination. Total glutathione (GSH + 2GSSG) was determined by the kinetic recycling assay following the reduction of DTNB (5,5'-dithiobis(2-nitrobenzoic acid)) in an Eppendorf photometer at 405 nm [11].

Peroxidase-like activity of PZ 51 with DTE. Peroxidase-like activity was determined with DTE (0.2 mM), *t*-butyl hydroperoxide (1 mM) and PZ 51 (16 μM) in potassium phosphate buffer (50 mM) pH 7.0 at 25°. Similar to the assay with GSH as reductant [1], DTE oxidation was measured at different time points; samples (0.02 ml) were taken from the reaction mixture and mixed rapidly with 1 ml of DTNB (0.5 mM) in phosphate buffer (50 mM, pH 7.0). The reduction of DTNB with residual DTE was determined as described above using an $\varepsilon = 12.7\ mM^{-1}\ cm^{-1}$. Corresponding blank in the absence of PZ 51 was subtracted.

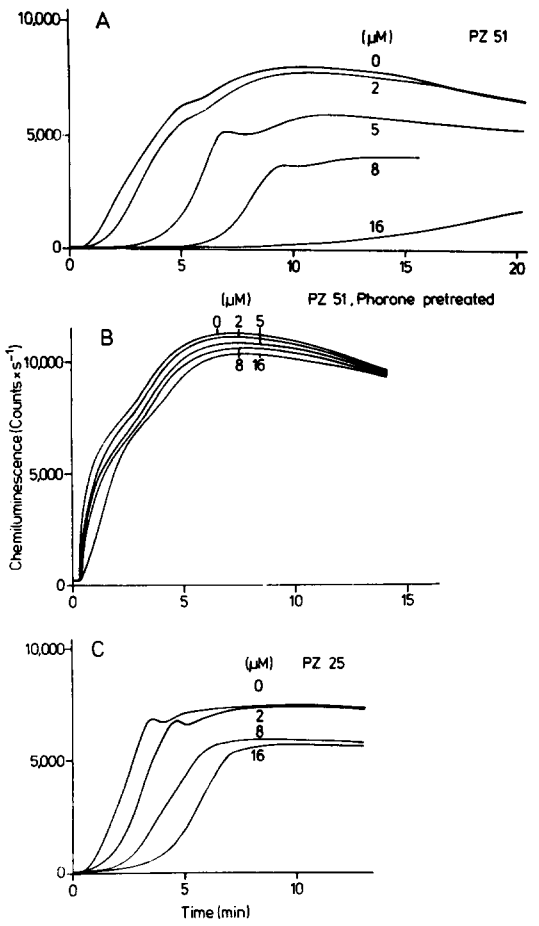

Fig. 1. Time course of ADP–Fe induced generation of low-level chemiluminescence, and the effect of different concentrations of PZ 51 and PZ 25 in isolated hepatocytes. (A) and (C), hepatocytes from untreated rats; (B), hepatocytes from phorone-pretreated rats. The curves are representative of 3 independent experiments.

RESULTS AND DISCUSSION

Low-level chemiluminescence

Incubation of isolated hepatocytes under constant oxygenation produces low-level chemiluminescence reflecting lipid peroxidation as shown previously [12]. Addition of ADP–Fe to a hepatocyte suspension accelerates the generation of light emission, reaching a maximum after 10 min (Fig. 1A) instead of 120 min in the controls [12]. In the presence of PZ 51 at low concentrations in the μM range, the generation of low-level chemiluminescence induced by ADP–Fe is markedly delayed and inhibited (Fig. 1A). The effective concentrations of PZ 51 with cells are 5–10 times higher than those needed for a temporary protection against lipid peroxidation on isolated microsomes [1].

Pretreatment of rats with phorone led to a depletion of total glutathione in hepatocytes to 0.27 ± 0.04 μmole per 10^8 cells (means ± S.E.M., N = 9), compared to control values from untreated animals of 3.40 ± 0.10 μmole per 10^8 cells (means ± S.E.M., N = 9). Chemiluminescence intensity is about 50% higher in GSH-depleted hepatocytes compared with control hepatocytes under similar assay conditions. ADP–Fe induced low-level chemiluminescence is practically unaffected by PZ 51 in GSH-depleted hepatocytes (Fig. 1B). PZ 25, the sulphur analog, exerts a distinct antioxidant activity on cells from control rats (Fig. 1C), but the concentrations needed for PZ 25 are about doubled as compared to PZ 51 (Fig. 1A).

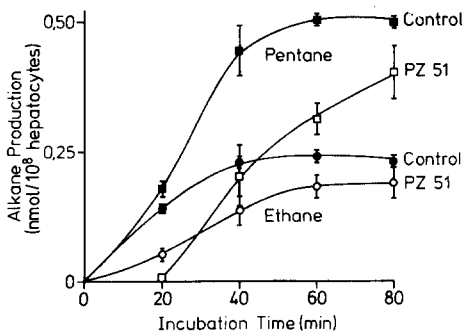

Fig. 2. Time course of ADP–Fe induced ethane and *n*-pentane production in isolated hepatocytes from control rats. PZ 51 was present at 8 μM concentration. Assay conditions as in Fig. 1. Values are means ± S.E.M. from 3–5 independent experiments.

Alkane production and malondialdehyde formation

The effect of PZ 51 in lengthening the lag phase is also demonstrated by ethane and *n*-pentane production as illustrated in Fig. 2. The dependence on the concentration of PZ 51 and PZ 25 is shown for ethane (Fig. 3A), *n*-pentane (Fig. 3B), and malondialdehyde formation (Fig. 3C) at 40 min of incubation. The effects observed with other antioxidants, such as DDC and (+)-cyanidanol-3 are also shown in Fig. 3. The effects of PZ 51 and PZ 25 agree with the results obtained with low-level chemiluminescence shown in Fig. 1. (+)-Cyanidanol-3, a free radical scavenger [13], is effective at higher concentrations than PZ 51, half maximal effect being obtained at 20 μM (data not shown). This compound is known as an inhibitor of lipid peroxidation [14, 15]. In contrast, DDC is effective in much lower concentrations (Fig. 3), similar to results obtained with rat liver microsomes [3].

On the right-hand side of Fig. 3, experiments carried out with GSH-depleted hepatocytes are shown for comparison. The increases in the control values for ethane (1.5-fold) and pentane (2-fold) are shown on the ordinates (100% values). Interestingly, the protective effect of PZ 51 is essentially abolished, whereas the antioxidants, (+)-cyanidanol-3 and DDC, exhibit effects similar to those seen in the control cells (left-hand side of Fig. 3). Thus, the dependence of the protective effect of PZ 51 on the

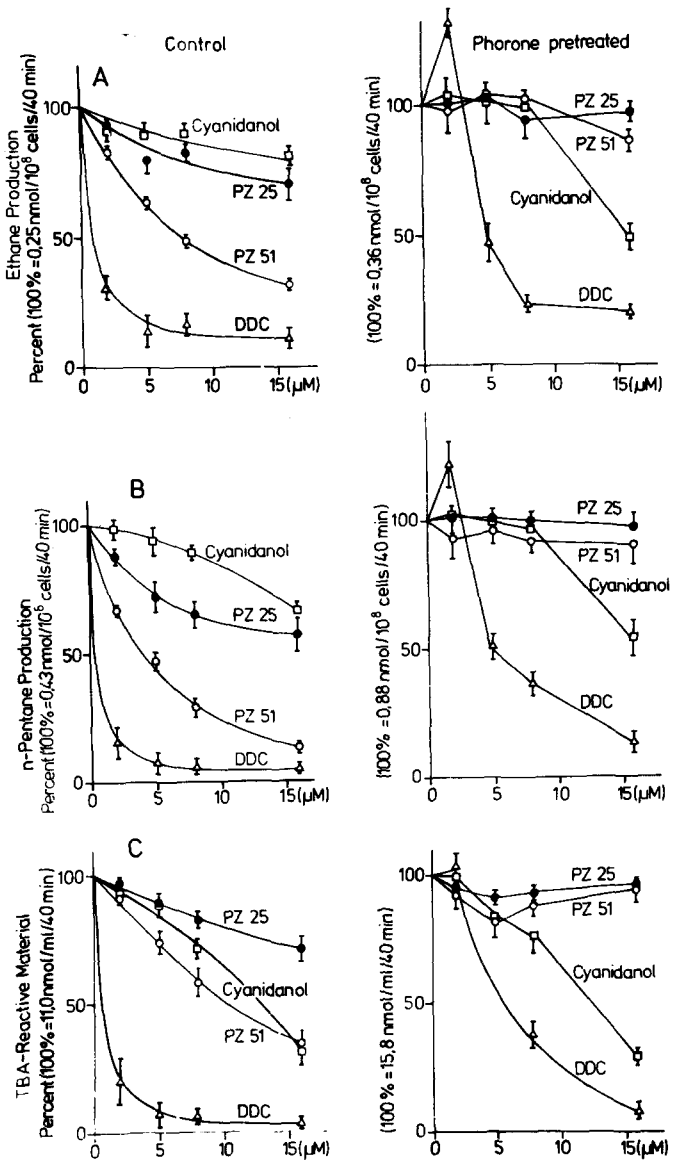

Fig. 3. Dependence of ADP–Fe induced ethane (A), *n*-pentane (B), and malondialdehyde formation (C) on concentration of various antioxidants in hepatocytes from control and phorone-pretreated rats. Assay conditions as in Fig. 1. Values are means ± S.E.M. from 5 independent experiments for the PZ compounds and 3 independent experiments for cyanidanol and DDC.

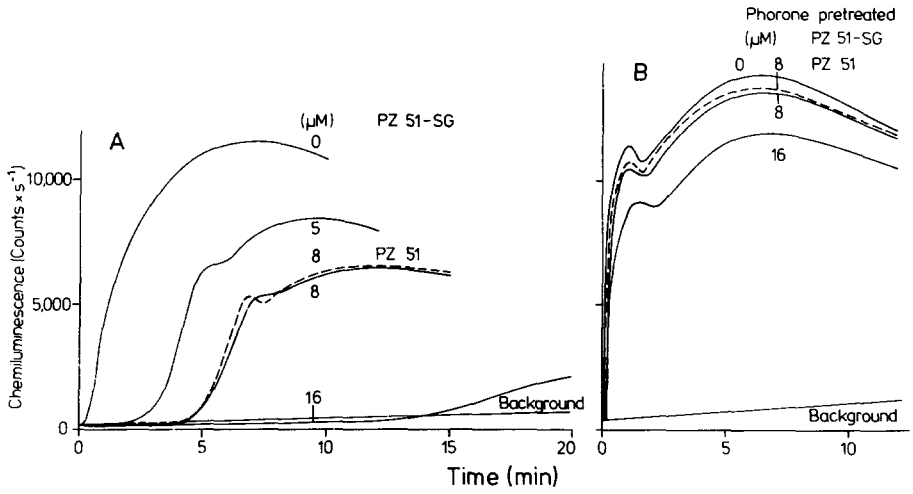

Fig. 4. Effect of PZ 51-SG and PZ 51 on ADP–Fe induced low-level chemiluminescence in isolated hepatocytes from control (A) and phorone-pretreated rats (B). Dotted line: effect of PZ 51. Assay conditions as in Fig. 1.

presence of GSH appears to indicate that the GSH Px-like activity [1] is responsible for the biological effect; it may be recalled that the antioxidant effect of PZ 51 in isolated microsomes does not require GSH [1].

It is noted that DDC has a somewhat higher efficiency in control cells as compared to GSH-depleted cells. This may be explainable by the fact that oxidized DDC (disulfiram) can be reduced by GSH [16].

Effect of the glutathione adduct, PZ 51-SG

The above results indicate that GSH is required for the protective effect of PZ 51. In order to evaluate whether an adduct between GSH and PZ 51, PZ 51-SG, can exert activity, experiments with the chemically synthetized adduct were carried out. As shown in Fig. 4 and Table 1, both compounds gave similar responses in chemiluminescence and, importantly, there was no difference in GSH-depleted hepatocytes (Fig. 4B). This indicates that the adduct, PZ 51-SG (in the concentration range of 0.23–0.76 μmol/10^8 cells), provides no extra protective effect with the presently employed model in comparison to the parent compound, PZ 51.

The lack of additional effects between PZ 51 and PZ 51-SG adduct, observed in Figs. 4A and B, could be explained in terms of (a) an insufficient amount of GSH supplied as PZ 51-SG (lower than residual GSH in phorone-pretreated rats), and (b) an inability of the PZ 51 adduct with GSH to enter the cell. This would be supported if PZ 51-SG were subject to a thiol exchange on the membrane, and therefore only PZ 51 would enter the cell. However, at the present time we have no experimental evidence for the occurrence of such thiol exchange; it cannot be excluded that the adduct penetrates the cell, as known for other GSH conjugates [17].

Effect of thiol reductant, DTE

In order to assess the effect of the thiol redox

Table 1. ADP–Fe induced low-level chemiluminescence and the effect of different PZ compounds in isolated hepatocytes

	Low-level chemiluminescence (% of controls)				
	Control			Phorone-pretreated	
PZ concentration (μM)	PZ 51	PZ 51-SG	PZ 25	PZ 51	PZ 51-SG
0	100	100	100	100	100
2	97 ± 1	94 ± 1	99 ± 1	99 ± 1	94 ± 1
5	70 ± 4	78 ± 3	87 ± 2	97 ± 2	97 ± 2
8	46 ± 3	58 ± 3	80 ± 2	95 ± 2	96 ± 2
16	3 ± 1	3 ± 1	76 ± 2	93 ± 1	86 ± 2

Assay conditions as in Fig. 1. Values are means ± S.E.M. from 3 independent experiments obtained after 10 min incubation. 100% = 9600 ± 400 counts/sec, N = 9 (control cells), 100% = 13,000 ± 480 counts/sec, N = 6 (phorone-pretreated cells). Data from Figs. 1 and 4.

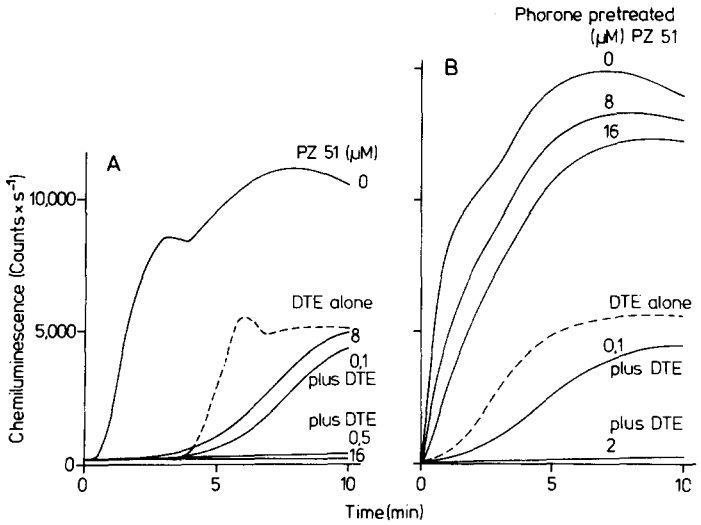

Fig. 5. Effect of DTE (1 mM) and different concentrations of PZ 51 on ADP–Fe induced low-level chemiluminescence in isolated hepatocytes from control (A) and phorone-pretreated rats (B). Dotted line: absence of PZ 51. Assay conditions as in Fig. 1.

state, experiments were performed with the thiol reductant, DTE. As illustrated in Fig. 5A, DTE alone substantially decreases ADP–Fe induced low-level chemiluminescence in control cells. Furthermore, the effect of PZ 51 is intensified in the presence of DTE, indicated by a much lower effective concentration of PZ 51. Importantly, in GSH-depleted cells PZ 51 becomes effective with DTE (Fig. 5B), in contrast to results obtained without DTE (Fig. 1B) or with PZ 51-SG (Fig. 4). The addition of GSH (1 mM) to the hepatocyte incubation mixture was ineffective (not shown), explainable by the fact that GSH cannot penetrate the cell membrane [18].

These observations of DTE with PZ 51 could either be due to the increased level of reduced glutathione from mixed disulfides and GSSG, or to a peroxidase-like activity by PZ 51 utilizing DTE as reductant. Therefore, we assayed peroxidase activity with DTE as a cosubstrate of PZ 51 for the reduction of *t*-butyl hydroperoxide *in vitro*. Activity was observed, even fivefold higher than with GSH under similar conditions, in agreement with recent results from A. Wendel *et al.* (personal communication) who found a threefold higher activity with DTE than with GSH.

Thus, the effect of DTE with PZ 51 in control and phorone-pretreated cells (Fig. 5) is probably attributable to a DTE-dependent peroxidase-like activity. The effect of DTE without PZ 51 on low-level chemiluminescence may be explainable by protective effects of various thiols, including DTE, in the mM concentration range as studied previously with rat liver microsomes [3]. This would not involve the selenoenzyme GSH Px, which is highly specific for GSH [19], but could be due to radical-scavenging activities of thiols.

In summary, the selenoorganic compound, PZ 51, is capable of protecting intact hepatocytes against ADP–Fe induce lipid peroxidation in a manner dependent on GSH.

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