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A NOVEL BIOLOGICALLY ACTIVE SELENO-ORGANIC COMPOUND—III

EFFECTS OF PZ 51 (EBSELEN) ON GLUTATHIONE PEROXIDASE AND SECRETORY ACTIVITIES OF MOUSE MACROPHAGES

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Abstract—PZ 51 (2-phenyl-1,2-benzisoselenazol-3(2H)-on), a selenium-containing compound with glutathione peroxidase (GSH-Px)-like activity, was administered to selenium-deficient mice for 5 days. A significant increase in peritoneal macrophage GSH-Px activity after treatment was only observed when basal GSH-Px activity was almost zero (i.e. in 19 weeks selenium-deficient animals), possibly due to binding of PZ 51 to the macrophages. This indicates that PZ 51 releases very little, if any, free selenium for incorporation into endogenous GSH-Px. The compound *in vitro* exerted a concentration-dependent inhibition of the generation of chemiluminescence by resident mouse peritoneal macrophages and a partial inhibition of the production of prostaglandin E_2 by resident peritoneal macrophages. β-Glucuronidase production by *C. parvum*-activated peritoneal macrophages was unaffected by PZ 51. These *in vitro* data can be explained on the basis of a selective peroxide scavenging and/or GSH-Px-like activity of PZ 51, offering a novel approach to anti-inflammatory therapy.

Glutathione peroxidase (GSH-Px) is a selenium-containing enzyme present in various mammalian tissues which scavenges damaging peroxides generated by oxidative metabolism, such as that which occurs in the liver or following stimulation of leucocytes [1, 2]. The active site of GSH-Px is a selenocysteine molecule and administration of selenocysteine, its metabolic precursor selenomethionine or selenite to Se-deficient animals restores the activity of endogenous GSH-Px [2]. However, dietary supplementation with selenite does not enhance GSH-Px activity in animals with Se-adequate diets [3].

The effectiveness of oral Se in enhancing depressed GSH-Px activity raises the possibility of the therapeutic usefulness of Se-containing compounds in treating conditions associated with overproduction of H_2O_2 or lipid peroxides. Increased levels of peroxides have been detected in rheumatoid synovial fluid and experimental inflammatory exudates [4–7] and serum Se levels are reduced in patients with rheumatoid arthritis [8]. Recently, we have shown that in Se-deficient rats adjuvant arthritis is more severe, while in Se-deficient mice impaired lymphocyte responses to mitogens are associated with decreased macrophage GSH-Px activity and increased macrophage H_2O_2 release during phagocytosis [9]. These data suggest that immuno-inflammatory responses may be sensitive to therapy with Se-containing compounds and indeed, anti-inflammatory effects of selenite have been reported [10]. We now present data on the effects of PZ 51, a Se-containing compound with GSH-Px-like activity [11, 12] (Fig. 1), on mouse macrophage function.

MATERIALS AND METHODS

Animals. Male inbred CBA mice (weighing approx. 20 g) were obtained either from Bomholtgard, Ry, Denmark or from Dr Ivanovas, Kisslegg in Allgäu, F.R.G. For studies on GSH-Px activity the mice were fed a Se-deficient diet† specially prepared by Altromin GmbH, Lage, F.R.G. All other animals received a normal diet (Altromin Standard Diet—1324). The mice were kept in sawdust-lined Makrolon-cages, with regular 12 hr periods of light and darkness and constant temperature and had access to food and water *ad libitum*.

Macrophages. Resident macrophages were obtained by peritoneal lavage as described previously [13]. These cells were used for determination of GSH-Px activity, chemiluminescence and prostaglandin E_2 generation, since resident macrophages generate far more prostaglandins than do elicited or activated cells [14]. On the other hand, release of β-glucuronidase by macrophages activated by *Corynebacterium parvum* (*C. parvum*) is much greater than that by resident macrophages (unpublished

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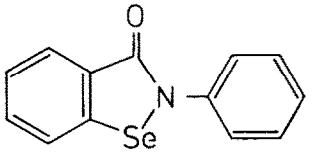
† Altromin-Sonder-Diät C1045: Torula yeast 34%, starch 38%, saccharose 10%, cellulose powder 4%, olive oil 6%, vitamin C1000 2%, minerals and trace elements C1045 6%. Control animals in this experiment received the same diet supplemented with 0.25 ppm Se as sodium selenite.



PZ 51

Fig. 1. Structure of PZ 51 (2-phenyl-1,2-benzisoselenazol-3(2H)-on).

observations). Consequently, for measurement of β-glucuronidase release, each mouse received an i.p. injection of *C. parvum* (1.4 mg in 0.2 ml; Deutsche-Wellcome, Burgwedel, F.R.G.) for 5 or 8 days before harvesting of macrophages.

Treatment with PZ 51. In order to determine whether PZ 51 provides Se for incorporation into macrophage GSH-Px, the drug was dissolved in 1% Tylose and given orally at 5 mg/kg per day for 5 days to groups of mice which had been fed a Se-deficient diet for 8, 11, 14 and 18 weeks. (The dose of 5 mg/kg per day had previously been found to exert anti-inflammatory effects in rats.) Control animals, also Se-deficient, received an equal volume of vehicle. Peritoneal macrophages were harvested within 2 hr after the last dose of PZ 51.

In all *in vitro* experiments, PZ 51 was initially dissolved in ethanol and diluted to give a final concentration of 5% ethanol, irrespective of the drug concentration. All control incubations were carried out in 5% ethanol.

Glutathione peroxidase. Resident macrophages were centrifuged at 300 *g* for 10 min and resuspended in potassium phosphate buffer, pH 7 to a final concentration of 3×10^6 cells/ml. Aliquots (0.1 ml) were taken for duplicate determination of GSH-Px activity by the method of Wendel [15] using *t*-butyl-hydroperoxide as substrate.

Chemiluminescence. Resident peritoneal macrophages were centrifuged at 300 *g* for 10 min, resuspended to 5×10^6 cells/ml Hank's solution and pre-incubated at a final concentration of 1×10^6 cells/ml with PZ 51 for 10 min at 37°. Chemiluminescence was generated in the presence of lumanol by serum opsonized zymosan (0.17 mg/ml, final conc.) and measured after 5 min in a Lumac Biocounter 2000, as described previously [13]. In order to rule out interference of PZ 51 with the oxidation of lumanol by active oxygen species produced by the macrophages, an identical incubation procedure was used as described above, except that the opsonized zymosan and macrophage suspension were replaced by H_2O_2 (9.2 mM final conc.). Inhibition of chemiluminescence 5 min after H_2O_2 addition thus reflected interference with the oxidation of lumanol by H_2O_2.

Prostaglandin E_2 generation. Resident peritoneal macrophages were resuspended in Medium 199 to a final concentration of 2×10^6 cells/ml and 1 ml aliquots of this suspension were added to individual wells of 24-well cell culture plates (Nunc, Wiesbaden, F.R.G.). The cells were allowed to adhere for 2.5 hr at 37° under 5% CO_2, after which the medium was removed, the cells washed twice and 0.5 ml Medium 199 added (with or without test compound). After 10 min pre-incubation (37°, 5% CO_2), 0.5 ml opsonized zymosan (0.5 mg/ml final conc.) was added and the plates were incubated for a further 3 hr. Supernatants were then removed, acidified to pH 2.5–3 with 50% citric acid and shaken for 5 min with 6 ml cyclohexane:ethyl acetate (1:1). After centrifugation (2000 *g*, 5 min), the organic phase was removed and evaporated to dryness in a Büchi Rotavapor. Dried extracts were resuspended in 1 ml benzol:ethyl acetate:methanol (60:40:2, Eluent 2) and added to Bio-Rad Econo columns prepared by suspending silica gel (Malfinckrodt CC-4, 0.5 g/3 ml) in benzol:ethyl acetate (60:40, Eluent 1).

Neutral lipids were first eluted with 5.5 ml Eluent 1 and prostaglandin E_2, then eluted with 12 ml Eluent 2. This fraction was evaporated to dryness for assay. [Recovery of ^{3}H-PGE_2 (2000 cpm Amersham-Buchler) through extraction and chromatography procedures was always >95%.] PGE_2 concentrations were determined using a radioimmunoassay kit with a ^{125}I-labelled tracer (New England Nuclear), antiserum-bound PGE_2 being precipitated with 16% polyethylene glycol and radioactivity being counted in an LKB Gamma counter.

β-glucuronidase release. C. parvum-activated macrophages were centrifuged at 300 *g* for 10 min, resuspended in Medium 199, containing penicillin/streptomycin to a concentration of 4×10^6 cells/ml and plated in 24-well cell culture dishes as described previously [9]. After adherence for 24 hr and washing, cells were preincubated with PZ 51 for 1 hr, stimulated with opsonized zymosan (0.2 mg/ml) for 2 hr and β-glucuronidase activity in supernatants determined by reaction with phenolphthalein glucuronide (0.56 mg/ml, final conc.) also as described [9].

Lactate dehydrogenase. Lactate dehydrogenase levels were determined in supernatants, under the same conditions as for β-glucuronidase, using a commercially available kit (LDH Monotest, Boehringer Mannheim, F.R.G.).

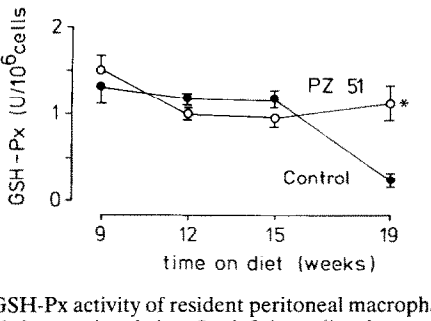


Fig. 2. GSH-Px activity of resident peritoneal macrophages removed from mice fed a Se-deficient diet for various lengths of time and treated for the preceding 5 days with PZ 51 (5 mg/kg p.o.) or vehicle (control). Each point is the mean ± S.E.M. of 3 duplicate observations. *$P \leq 0.05$ (vs control, Mann–Whitney *U*-test). In animals which had received a similar diet supplemented with 0.25 ppm Se for 9 weeks the macrophage GSH-Px activity was 2.14 ± 0.23 U/10⁶ cells.

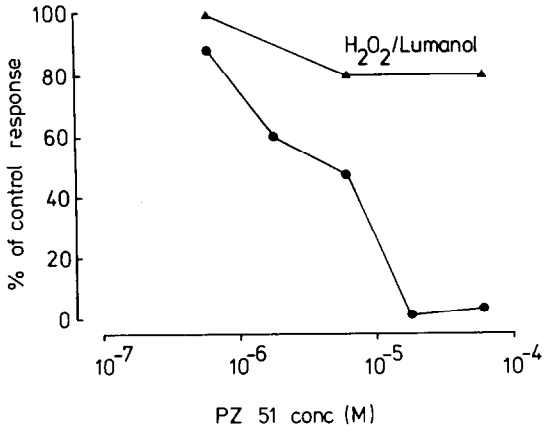


Fig. 3. Inhibition by PZ 51 of the opsonized zymosan-induced generation of chemiluminescence (CL) by mouse resident peritoneal macrophages *in vitro* (100% = 255 relative light units). Each point is the mean of 3 duplicate observations. The upper curve represents the degree of inhibition by the compound of the CL generated by the reaction of 9.2 mM H_2O_2 with lumanol in the absence of cells (100% = 453 relative light units).

RESULTS

Glutathione peroxidase activity

Administration of PZ 51 (5 mg/kg p.o.) to mice for 5 days failed to induce any change in macrophage GSH-Px activity in animals which had been fed a Se-deficient diet for 9–15 weeks (Fig. 2). This confirms the data in the accompanying paper by Wendel *et al.* [12]. Only in mice which had been on a Se-deficient diet for 19 weeks (when macrophage GSH-Px activity was almost zero) could an enhancement of macrophage GSH-Px activity be observed following treatment of the animals with PZ 51.

Secretory activity of macrophages

Chemiluminescence (CL) generated by stimulated phagocytes is a measure of reactive oxygen species, such as the superoxide anion, formed as a result of the hexose monophosphate shunt-dependent oxidative burst [16]. Such CL is enhanced by lumanol, which generates light during oxidation by these reactive oxygen species.

Preincubation of resident macrophages with PZ 51 exerted a concentration-dependent inhibition of the generation of CL from these cells by opsonized zymosan, which could not be attributed to interference with the oxidation of lumanol (Fig. 3). The release of β-glucuronidase by similarly stimulated *C. parvum*-activated macrophages, however, was not significantly affected (Table 1). The production of PGE_2 by opsonized zymosan-stimulated resident macrophages, on the other hand, was partially inhibited following 3 hr incubation with PZ 51 (Table 1). Although a shallow concentration-dependency was observed, PZ 51 only achieved a maximum inhibition of PGE_2 production of 43% at 10^{-5} M. At this dose the compound was non-toxic to the cells, extracellular LDH release being less than 3%.

Table 1. Effects of PZ 51 on β-glucuronidase release from mouse *C. parvum*-activated macrophages and on prostaglandin E_2 release from resident macrophages, stimulated in both cases by opsonized zymosan

PZ 51 conc. (M)	PGE_2 (pg/10^6 cells)	β-glucuronidase (units/10^6 cells)
0	129 ± 4 (3)	5.4 ± 0.3 (4)
10^{-7}	94 ± 8 (3)*	5.7 ± 0.2 (4)
10^{-6}	89 ± 3 (3)*	5.2 ± 0.3 (4)
10^{-5}	74 ± 2 (3)*	4.9 ± 0.2 (4)

Values are means ± S.E.M. of the number of observations in brackets.
* $P < 0.05$ (vs control, Mann–Whitney *U* test).

DISCUSSION

Glutathione peroxidase activity is frequently determined in various cells and tissues as a measure of dietary Se status. In Se-deficiency, administration of Se, as sodium selenite or Se-methionine, invariably restores to normal both Se levels and GSH-Px activity [2]. We have now shown that the Se-containing compound PZ 51 does not increase macrophage GSH-Px activity in Se-deficient mice until the basal level approaches zero. Macrophages from mice which have been on a Se-deficient diet for 9–15 weeks already exhibit significantly reduced GSH-Px activity [9], but at this time period administration of PZ 51 was without effect. This indicates that PZ 51 is not a source of free Se for uptake into endogenous GSH-Px as described in the accompanying paper [12]. Indeed, the apparent enhancement of macrophage GSH-Px by PZ 51 in 19-week Se-deficient mice may have been due to limited binding of PZ 51 to the cells which was unmasked by the negligible endogenous enzyme activity. A very slight degree of Se uptake from PZ 51 by the enzyme, though, cannot be ruled out on the basis of the present data.

The concentration-dependent inhibition of macrophage CL by PZ 51 *in vitro* is commensurate with a peroxide scavenging action of the compound. Reactive peroxides are readily formed as secondary products of the superoxide anion initially formed during the macrophage oxidative burst [17] and peroxides rapidly react with luminol to generate light. The slight inhibition by PZ 51 of the oxidation of lumanol by H_2O_2, which we observed, probably reflects competition of the compound and lumanol for the H_2O_2 substrate. PZ 51 may affect the *generation* of reactive oxygen species, as suggested in the preceding paper [11].

The partial inhibition by PZ 51 of the production of PGE_2 by stimulated macrophages can also be explained on the basis of its peroxide scavenging or GSH-Px-like activity. Lands and his colleagues [18, 19] have shown that cyclo-oxygenase (the enzyme responsible for PG synthesis) requires a lipid peroxide activator (PGG_2) for full activity and the biosynthesis of PGs can be inhibited by GSH-Px. The fact that PZ 51, at non-cytotoxic concentrations, only partially inhibited PGE_2 production fully supports the possibility that it was acting by scavenging

the peroxide activator of the macrophage cyclo-oxygenase. Alternatively, inhibition of PGE_2 generation may have been due to diversion of arachidonic acid metabolism toward lipoxygenase products, since GSH-Px has been implicated in the conversion of the lipoxygenase product 12-HETE in platelets [20]. This seems less likely, though. The selectivity of action of PZ 51 is further indicated by the fact that it failed to produce any significant change in macrophage β-glucuronidase release.

The implications of these findings for anti-inflammatory therapy are considerable. Macrophages and neutrophils are both capable of releasing large amounts of highly reactive oxygen species at sites of inflammation and increased levels of peroxides have been detected in rheumatoid synovial fluid and experimental inflammatory exudates [4–7]. We have also shown recently that in Se-deficient rodents decreased macrophage GSH-Px activity is associated with enhanced macrophage H_2O_2 production and more severe adjuvant arthritis [9]. There is thus growing evidence that peroxides may play a role in inflammation. Superoxide has been a candidate for a role as an inflammatory mediator for some time and the use of superoxide dismutase injections for the treatment of inflammatory arthritides has yielded promising results [21]. It is likely that prevention of the formation of secondary products of superoxide, i.e. hydrogen peroxide, contributes to the beneficial action of superoxide dismutase. Scavenging of peroxides at inflamed sites by PZ 51 therefore offers a possible new approach to anti-inflammatory therapy, similar in concept to superoxide dismutase but with the advantage of being orally administrable.

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