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SYNTHETIC SELENO-ORGANIC COMPOUND WITH GLUTATHIONE PEROXIDASE-LIKE ACTIVITY IN THE CHICK

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Abstract—The glutathione peroxidase activity catalyzed by the seleno-organic anti-inflammatory drug Ebselen (registered under the trademark of the Natterman Corp. Cologne, FRG) [PZ51, 2-phenyl-1,2-benzisoselenazol-3(2*H*)on], as measured by NADPH oxidation, was inhibited *in vitro* by the selenium-dependent glutathione peroxidase (SeGSHpx) inhibitors aurothioglucose and D-(−)penicillamine HCl. Vitamin E- and selenium-deficient chicks were given 0, 80 or 320 ppm PZ51 in diets devoid of vitamin E and supplemented with low levels of sodium selenite (0.04 ppm selenium added to the basal diet containing *ca.* 0.015 ppm selenium) when a small number of chicks (*ca.* 13%) had exudative diathesis (ED). By 24 hr, the high PZ51 dose (320 ppm) delayed the onset of ED compared to untreated controls. Similarly, vitamin E-deficient chicks fed diets containing 0, 80, 160, 320, 640 or 1280 ppm PZ51 and supplemented with 0.04 ppm selenium showed ED in inverse proportion to log PZ51 dose. Plasma and liver post-mitochondrial supernatant samples from these chicks also exhibited log-linear relationships between dietary PZ51 level and selenium content or SeGSHpx-like activity. The amount of SeGSHpx-like activity for chicks given PZ51 above that determined for untreated chicks was extractable into ethanol, indicating that those PZ51-associated increases were not due to protein-bound selenium or SeGSHpx. This suggests that selenium from PZ51 was not available to support synthesis of SeGSHpx. Dietary PZ51 (1280 ppm) or selenium (0.1 ppm) alone or in combination decreased the acute lethalities of nitrofurantoin or paraquat in vitamin E-adequate chicks. The results indicate that SeGSHpx-like activity in selenium-deficient chicks is increased by oral administration of PZ51, which appears to mimic the true enzyme by affording protection against clinical signs of selenium deficiency (i.e. ED) and pro-oxidant drug lethality.

The synthetic seleno-organic compound 2-phenyl-1,2-benzisoselenazol-3(2*H*)on (PZ51) is a novel anti-inflammatory agent with inherent catalytic activity, which resembles that of the selenium-dependent glutathione peroxidase (SeGSHpx, EC 1.11.1.9) [1, 2]. It has extremely low toxicity in animals probably due to the apparent lack of availability of the selenium contained in this compound for further metabolism [2]. Although the SeGSHpx-like activity of PZ51 has been demonstrated *in vitro*, the relevance of this activity to its anti-inflammatory action *in vivo* has been largely hypothetical [1–3]. Recently, evidence has been presented which suggests that the anti-inflammatory activity of PZ51 may be due to its inhibition of lipoxygenase rather than to its SeGSHpx-like property [3].

Dietary selenium has been shown to protect humans and animals [4] from a variety of pathological conditions associated with selenium deficiency. In the chick, combined deficiencies of vitamin E and selenium produce an edematous condition known as exudative diathesis (ED) [5–7]. Dietary selenium can prevent this disorder to the extent that it increases tissue SeGSHpx activities [8, 9]. Additionally, uncomplicated selenium deficiency increases the acute lethalities of the pro-oxidant compounds paraquat (1,1′-dimethyl-4,4′-bibyridinium hydrochloride, methyl viologen) and nitrofurantoin (*N*-[5-nitro-2-furfurylidine]-1-amino-hydantoin) in the chick [10, 11]. In recent studies, we have shown that two anti-arthritic drugs, aurothioglucose and D(−)penicillamine, inhibit chick SeGSHpx in crude tissue fractions *in vitro* and increase ED in vitamin E-deficient, selenium-supplemented chicks while decreasing the activities of SeGSHpx in various tissues [12]. These inhibitors were shown to increase the acute toxicities of both paraquat and nitrofurantoin in selenium-supplemented chicks [13].

The present study was conducted to determine whether PZ51 could be used to increase SeGSHpx-like activity in marginally selenium-deficient chicks without affecting the native enzyme activity. Indications of PZ51 action in the chicks were assessed in selenium-deficient chicks for properties attributed to dietary selenium, including increases in SeGSHpx-like activity and protection against ED and pro-oxidant compound toxicities.

MATERIALS AND METHODS

Animals and diets. Selenium- and vitamin E-depleted, day-old Single Comb White Leghorn chicks produced from laying hens fed a low-selenium low-vitamin E practical diet [12] were used in all experiments. Chicks were housed in thermostatically controlled, wire-floored battery brooders with a 15-

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hr day. Ten chicks were housed per pen; they were fed a low-selenium (0.015 to 0.020 ppm), tocopherol-free purified diet [12] used routinely in this laboratory to produce ED in chickens. Dietary supplementation with vitamin E was performed using all-*rac*-alpha-tocopheryl acetate; selenium was added as Na_2SeO_3.

Drug administration and exudative diathesis determination. PZ51 was provided by Dr. E. Graf of the Natterman Corp. (Cologne, FRG). It was mixed into the diet at the levels indicated; in the acute feeding experiment, these diets were employed starting at 7 days of age when a small but statistically insignificant number of chicks (*ca.* 13%) showed ED (day 7). The diet used to produce ED contained no vitamin E, and 0.04 ppm selenium was added to produce marginally selenium-deficient chicks. In the chronic PZ51 feeding experiment, chicks were fed the drug from 1 day of age until the termination of the experiment at 12 days. Exudative diathesis was assessed daily in all feeding experiments by checking for the following signs: abdominal edema accompanied by a purple to purple–green discoloration, and edema and discoloration of the wings, feet or upper neck caudal to the beak.

Paraquat (Sigma Chemical Co., St. Louis, MO) was dissolved in distilled water and given in a volume of 1.0 ml/kg body wt and at a dose of 175 mg/kg body wt by oral intubation to the crop. Nitrofurantoin (Sigma Chemical Co.) was given in solid form via No. 3 size gelatin capsules (Eli Lilly & Co., Indianapolis, IN) delivered *per os* to the crop. Nitrofurantoin doses were adjusted for chick body weight by treating chicks within 10 g weight classes with quantities of the drug based on the mean body weight of each weight class. Both drugs were given to chicks fed selenium-deficient diets or vitamin E-supplemented (100 I.U./kg) diets, containing the indicated amounts of selenium and/or PZ51, for 8 days. Previous studies indicated that, by this time, 60–80% mortality should result from either drug in chicks receiving no selenium supplements [10, 11, 13]

Analytical methods. SeGSHpx activity was assayed by the coupled assay of Paglia and Valentine [14], as modified by Lawrence and Burk [15], using 0.25 mM H_2O_2 as substrate and including 1.0 mM sodium azide to inhibit catalase. PZ51 was dissolved in 50% ethanol and added in 50 μl in the incubation mixture. An equivalent volume of 50% ethanol had no effect on the blank (no enzyme) determination or on SeGSHpx activity in chick liver post-mitochondrial supernatant fraction (the enzyme source for inhibition experiments). Aurothioglucose and D(−)penicillamine HCl (both obtained from the Sigma Chemical Co.) were dissolved in distilled water and were added in 50 μl to the incubation mixtures to give the final concentrations indicated (50 μl H_2O added for no inhibitor). The highest concentrations used for both SeGSHpx inhibitors were not sufficient to significantly reduce glutathione reductase activity and, therefore, the coupled assay could still be used [12]. Determination of ethanol-extractable SeGSHpx-like activity (due to PZ51) in tissue fractions was performed first using PZ51-spike tissue samples, where 100% of SeGSHpx-like activity due to PZ51 was recovered in the ethanol extract, with no significant amount of true SeGSHpx found in the ethanol fraction. This procedure was also performed using liver post-mitochondrial supernatant fractions from chicks chronically fed PZ51. In these experiments, 1 vol. of distilled water (control) or absolute ethanol (ethanol-extract) was added to 0.5-ml samples. The mixtures were vortexed for 1 min and then centrifuged at 2000 *g* for 5 min at room temperature. The resulting supernatant fraction (100 μl) or an equivalent volume of H_2O or 50% ethanol was assayed for SeGSHpx-like activity as described above, or for selenium by the method of Olson *et al.* [16]. In all cases, protein was determined by the dye-binding method of Bradford [17].

Chicks were randomly selected for biochemical analyses from the chronic PZ51 treatment group at the termination of the experiment (i.e. 12 days). Blood was obtained by cardiac puncture with a heparinized syringe; 1.0 ml blood was transferred to tubes containing 0.1 ml heparin (1000 units/ml), 0.9% NaCl, to prevent hemolysis. Plasma was prepared by centrifugation and frozen at −20° until assays were preformed. Organs were dissected out and frozen at −20° until homogenates could be prepared (within 7 days). Tissues were thawed, blotted dry on filter paper, and were homogenized for 10 sec at intermediate intensity/speed using a Polytron tissue homogenizer (Brinkmann Instruments, Westbury, NY) in 3 vol. of 0.25 M sucrose, 11 mM GSH and 1.0 mM EDTA at 4°. Post-mitochondrial supernatant fractions were prepared by centrifugation of homogenates at 12,000 *g* for 10 min at 4° and were held at −20° until assayed.

Data from each experiment were analyzed using a catalogued statistical system (Systat Version 2.1 for the IBM PC, Systat Inc., Evanston, IL) for analysis of variance (two-tailed test employing one-pass provisional algorithm and double precision arithmetic [18, 19]) using a 5% multiple range test of variance (Duncan's procedure employing exact probabilities of the Studentized Range [20]) and then reanalyzed after eliminating extreme observations using Dixon's criteria [21] for each treatment group where appropriate. Dose–response relationships were determined using linear regression analysis, and the results of dose versus log dose analysis were compared (highest r^2 term selected as best analysis).

RESULTS

The effects of the SeGSHpx inhibitors aurothioglucose and D(−)penicillamine HCl on the SeGSHpx-like activity of PZ51, as measured by the oxidation of NADPH in the presence of reduced glutathione and glutathione reductase, are shown in Table 1. Aurothioglucose was approximately 46 times more potent for inhibition of this SeGSHpx-like activity than was penicillamine.

Because PZ51 had good SeGSHpx-like activity and was inhibitable by SeGSHpx inhibitors, it was tested in an acute feeding study with vitamin E- and selenium-deficient chicks. When dietary exposure to PZ51 began on day 7, a small, but statistically insignificant, number of chicks showed ED (Table 2). PZ51 had no significant effects on daily or total feed intake or feed efficiency (feed intake/gain). The incidence of ED increased with age in the untreated

Table 1. Inhibition of selenium-dependent glutathione peroxidase-like activity of 10 μM PZ51 by aurothioglucose and D(−)penicillamine HCl

Inhibitor	Inhibitor conc (mM)	SeGSHpx-like activity (nmoles/NADPH/min)	(%)
None		14.8	100.0
Aurothioglucose	0.0125	14.0	94.6
	0.0250	9.0	60.8
	0.0500	7.2	48.8
	0.100	4.0	27.0
	0.200	2.3	15.5
	50% inhibition = 0.048 mM		
D(−)Penicillamine HCl	0.25	12.5	84.5
	1.00	11.4	77.0
	2.00	9.5	64.1
	4.00	5.2	35.1
	8.00	2.2	14.9
	50% inhibition = 2.2 mM		

Table 2. Effects of short-term dietary response to PZ51 on the manifestation of exudative diathesis in vitamin E- and selenium-deficient chicks*

Dietary PZ51† (ppm)	Exudative diethesis (%) by hr after start of PZ51 feeding			
	0	24	48	72
0	12.7 ± 12.7^{e}‡	50.0 ± 11.1^{c}	66.0 ± 8.3^{bc}	93.0 ± 3.5^{a}
80	21.0 ± 10.5^{de}	45.3 ± 7.9^{cd}	62.3 ± 2.3^{bc}	86.0 ± 4.0^{ab}
320	6.7 ± 6.7^{e}	27.7 ± 15.0^{de}	58.7 ± 5.9^{c}	69.0 ± 1.0^{abc}

* Chicks were fed the basal vitamin E-free diet supplemented with 0.04 ppm selenium (as Na_2SeO_3) to 10 days of age.

† PZ51 was included in the diet for days 7–10 only.

‡ $\bar{X} \pm$ SEM, N = three lots of nine to ten chicks each; means not sharing a common letter superscript are significantly different ($P < 0.05$).

Table 3. Effects of chronic dietary exposure to PZ51 on the manifestation of exudative diathesis (ED) and on plasma and liver postmitochondrial supernatant SeGSHpx-like activity in 12-day-old vitamin E- and selenium-deficient chicks*

Dietary PZ51 (ppm)	ED (%)	SeGSHpx-like activity†			
		Plasma	Liver total	Liver EtOH-extract	Liver residual‡
		(nmoles NADPH · min^{-1} · mg $protein^{-1}$)			
0	78.1 ± 7.3§	6.3 ± 2.4‖	12.4 ± 2.5¶ (4)	0.8 ± 0.5** (4)	11.6 (94%)††
80	60.0 ± 9.5	4.5 ± 1.3	24.9 ± 4.2	8.3 ± 1.8	16.6 (67%)
160	58.0 ± 5.8	11.8 ± 3.6	31.0 ± 6.7 (4)	15.5 ± 3.6 (4)	15.5 (50%)
320	36.0 ± 5.1	8.2 ± 1.8	40.2 ± 15.4 (3)	23.3 ± 8.2 (3)	16.9 (42%)
640	28.0 ± 8.0	12.2 ± 2.3	53.9 ± 10.0	37.2 ± 6.5	16.7 (31%)
1280	12.0 ± 4.9	24.8 ± 4.8	66.1 ± 2.6 (4)	52.2 ± 0.4	13.9 (21%)

* Chicks were fed the basal vitamin E-free diet supplemented with 0.4 ppm selenium (as Na_2SeO_3) until 12 days of age; diets were supplemented with PZ51 as indicated throughout the entire period.

† Hydrogen peroxidase activity was assayed as described in the text.

‡ Values were obtained by subtracting ethanol-extractable activity from the total hepatic activity.

§ $\bar{X} \pm$ SEM, N = five lots of ten chicks each unless indicated in parentheses; significant linear regression ($P < 0.05$) with $r^2 = 0.608$ for log(dietary PZ51) vs percent of chicks with ED.

‖ $\bar{X} \pm$ SEM, N = five chicks for each assay if not indicated in parentheses; significant linear regression ($P < 0.05$) with $r^2 = 0.400$ for log (dietary PZ51) vs plasma SeGSHpx activity.

¶ Significant linear regression ($P < 0.05$) with $r^2 = 0.523$ for log (dietary PZ51) vs liver SeGSHpx activity.

** Significant linear regression ($P < 0.05$) with $r^2 = 0.767$ for log (dietary PZ51) vs EtOH-extractable liver SeGSHpx activity.

†† Percentage of total hepatic activity shown parenthetically.

group, and was clearly reduced in chicks receiving 320 ppm PZ51 after 24 hr. After 3 days, almost all of the untreated chicks had ED, whereas the high dietary level of PZ51 delayed the onset of these deficiency signs ($P < 0.05$ within time period). Tissue SeGSHpx activity did not conclusively indicate the presence of PZ51, so clearer evidence for a PZ51 effect was sought through a chronic feeding study using graded levels of the drug. Table 3 shows the results of feeding PZ51 on the manifestation of ED in 12-day-old vitamin E- and marginally selenium-deficient chicks. The results show a clear inverse log (PZ51 dose)-linear(ED incidence) relationship ($P < 0.05$). Chicks fed 0.2 ppm selenium-supplemented diets had no signs of ED by this time. As in the previous experiment, PZ51 did not affect feed consumption or feed utilization efficiency, nor did it produce any overt signs of toxicity. The plasma and liver post-mitochondrial supernatant SeGSHpx-like activities (Table 3) and liver selenium (Table 4) of those chicks indicated direct relations with log (PZ51 level). Extraction of these samples with ethanol showed no significant SeGSHpx activity or selenium in untreated chicks, indicating that low levels of native SeGSHpx enzyme and other protein-bound forms of selenium were responsible for the basal activity resulting from low dietary selenium intake. Similarly, untreated selenium-adequate chicks (0.2 ppm selenium in diet) had no extractable liver selenium or SeGSHpx activity. Within the error of calculation, apparently all the observed increases in SeGSHpx-like activity and selenium were extractable in ethanol, indicating that these increases were due to the presence of PZ51 in these preparations, rather than to selenium made available to the chick by metabolism of the drug. The strong correlation between PZ51 level, SeGSHpx-like activity and ED incidence is suggestive of PZ51 functioning as SeGSHpx in the chick.

The effects of PZ51 and selenium on the acute lethalities of paraquat and nitrofurantoin (Table 5) show similar results. PZ51 and selenium each increased chick survival from a high dose of paraquat ($P < 0.05$). In fact, the administration of PZ51 to selenium-fed chicks was more effective in protecting against paraquat lethality than was either treatment alone. Similarly, the overall analysis of variance indicated that selenium ($P < 0.05$) and PZ51 ($P < 0.05$) were each capable of decreasing nitrofurantoin lethality. This is not seen in the post-hoc analysis as clearly as for paraquat; nevertheless, dietary selenium and PZ51 each protected against the acute toxicity of this pro-oxidant compound.

Table 5. Effects of dietary selenium and PZ51 on survival of vitamin E-adequate chicks treated with 175 mg paraquat/kg or 100 mg nitrofurantoin/kg (p.o.) 8 days after hatching

Dietary treatment* Selenium† (ppm)	PZ51 (ppm)	Paraquat 24-hr survival (%)	Nitrofurantoin 72-hr survival (%)
0	0	30.0 ± 4.1^{a}‡ (4)	57.0 ± 6.6^{a} (3)
0	1280	52.5 ± 4.8^{b} (4)	72.6 ± 6.3^{ab} (3)
0.1	0	70.0 ± 0.0^{c} (4)	80.0 ± 0.0^{bc} (3)
0.1	1280	86.7 ± 3.3^{d} (3)	93.3 ± 3.3^{c} (3)

* Selenium-deficient basal diet was supplemented with 100 I.U. vitamin E/kg.

† Added as Na_2SeO_3.

‡ $\bar{X} \pm$ SEM for (N) replicate lots of ten chicks each; means not sharing a common letter superscript are significantly different ($P < 0.05$).

DISCUSSION

The seleno-organic compound PZ51 has some mechanistic similarities with the seleno-enzyme SeGSHpx. These include end product formation, substrate specificity, and the requirements for reduced glutathione and selenium in the active site

Table 4. Effects of chronic dietary exposure to PZ51 on the selenium content of liver postmitochondrial supernatant fractions (PMS) from 12-day-old vitamin E- and selenium-deficient chicks*

Dietary PZ51 (ppm)	Liver PMS selenium		
	Total	EtOH-extractable (μg selenium/g tissue)	Residual†
0	0.05 ± 0.01‡ (4)	0.006 ± 0.003§ (4)	0.04 (89%)‖
80	0.09 ± 0.04	0.03 ± 0.02	0.06 (67%)
160	0.12 ± 0.03 (4)	0.08 ± 0.01	0.04 (33%)
320	0.16 ± 0.05 (3)	0.16 ± 0.02	0.0 (0%)
640	0.33 ± 0.05	0.26 ± 0.06	0.07 (21%)
1280	0.81 ± 0.17 (4)	0.68 ± 0.10	0.13 (16%)

* Same treatment as for chicks described in Table 3.

† Values were obtained by subtracting ethanol-extractable amount from total amount.

‡ $\bar{X} \pm$ SEM, N = five replicates unless indicated in parentheses; significant linear regression ($P < 0.05$), $r^2 = 0.580$ for log (PZ51) vs total liver PMS selenium.

§ Significant linear regression ($P < 0.05$), $r^2 = 0.668$ for log (PZ51) vs EtOH-extractable liver PMS selenium.

‖ Percentage of total amount indicated parenthetically.

for peroxidase activity [1, 2]. The inhibition by aurothioglucose and D(−)penicillamine HCl of the SeGSHpx-like activity of PZ51 is consistent with interactions with a selenium-centered peroxidase. The studies of Chaudiere *et al.* [22, 23] and our subsequent report [12] indicate that aurothioglucose is a far better inhibitor of SeGSHpx than penicillamine. This is also true for the SeGSHpx-like activity of PZ51, for which aurothioglucose was 46 times more inhibitory than penicillamine.

The effect of PZ51 in reducing the incidence of ED clearly indicates some physiological value of PZ51 for the selenium- and vitamin E-deficient chick. The correlation between PZ51 level, SeGSHpx-like activity and ED incidence is similar to (and most easily explained by) the original nutritional studies which demonstrated the correlation between dietary selenium, SeGSHpx activity and the incidence of ED in chicks [8, 9]. These correlative data are only suggestive of that mechanism and do not exclude other pharmacological properties of PZ51 (i.e. lipoxygenase inhibition [3]). Studies in mice by Wendel *et al.* [2] indicate that the selenium in PZ51 is not available for synthesis of SeGSHpx. The present study indicates that this is also true for the chick. This is evidenced by the lack of toxicity of high dietary levels of PZ51; even if as little as 1% of PZ51-selenium was released through metabolic degradation of the drug (it contains 28.8% selenium by weight), then the chicks fed the highest level of PZ51 (i.e. 1280 ppm PZ51 or 369 ppm selenium) would be expected to show signs of selenosis.

The results of the paraquat and nitrofurantoin toxicity experiments are again only suggestive of the SeGSHpx-like function of PZ51 in protection against pro-oxidant compound toxicity. Further experimentation needs to be done to determine how large a part the SeGSHpx-like activity of PZ51 has in this observed protection versus possible effects on drug absorption, disposition, metabolism and excretion.

It appears that increasing dietary selenium or oral treatment with PZ51 results in the same outcomes in selenium-deficient chicks: reduced toxicities of pro-oxidant compounds and decreased incidence of ED. The SeGSHpx inhibitors have opposite effects. It is of interest to determine whether the effects of PZ51 are due in major part to the observed increases in SeGSHpx-like activity *in vivo* or other pharmacological properties of this drug. This determination may offer considerable evidence for the role of the true SeGSHpx in protecting chicks from ED and pro-oxidant compound toxicities.

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