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Effect of oltipraz on the metabolism of glutathione in *Schistosoma mansoni*

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Oltipraz (RP 85 972) is a slow acting drug active against *Schistosoma* infections [1]. Bueding *et al.* [2] have recently shown that the antischistosomial action of oltipraz is concomitant with the lowering of glutathione (GSH) level in the worms and have suggested that this depletion would be damaging to the parasite. This lowering effect of oltipraz is limited to the parasite; in contrast, administration of the drug results in increasing GSH level in the host's tissues. These results suggest that a major metabolic difference exists between host and parasite in the regulation of intracellular GSH, a difference which may contribute to elucidate the mechanism of action of oltipraz.

In this present study, we have examined the GSH metabolism in *S. mansoni* compared with that of the mammal and the effects of oltipraz on the GSH biosynthesis in the parasite.

Methods

L-γ-Glutamyl-L-cysteine disulfide was prepared from oxidized GSH [3]. Prior to use, the disulfide was reduced with dithiothreitol [4]. L-γ-Glutamyl-L-α-aminobutyrate was synthesized as described [5]. L-γ-Glutamyl-L-cystine was prepared by hydrolysis of L-cysteine-glutathione [3], obtained according to [6].

Protein concentrations were measured by the Lowry method [7]. TLC were performed on silica gel chromatoplates Merck 60 F 254. The development solvent was butanol, pyridine, acetic acid, water 4/6/1/4. Detection was performed by spraying with ninhydrin, or using a Berthold betacamera BF 290 HR. To detect oxoproline, the plates were treated by 4 N HCl, before ninhydrin. The radioactive materials were from Amersham (England). For enzymatic studies, a suspension (1 mg/ml) of micronized oltipraz (95% of particles smaller than 5 μm, specific surface 13,100 cm^2/g) either in water or Tween 80 1‰ was used.

Organisms and preparation of extracts. Unseparated pairs of worms were collected from the mesenteric and portal veins of CD_1 female mice (Charles River, France, Saint-Aubin-les-Elbeuf) infected two months previously with 120 cercariae of a brazilian strain of *S. mansoni*. The worms pairs (300) were washed two times in Tyrode's salts solution, suspended in 1 ml of 150 mM KCl, 5 mM dithiothreitol and 1 mM $MgCl_2$ and disrupted at 4° using a Thomas potter homogeniser. The homogenate was centrifuged (10,000 *g*, 15 min) and the supernatant used for enzymatic determinations.

GC synthetase was characterized according to Meister [8, 9]. The reaction mixture (total volume 0.6 ml) contained final concentrations [^{14}C]-L-glutamate (8.3 mM, SA 5 mCi/mmol), L-α-aminobutyrate or L-cysteine (8.3 mM). The reaction initiated by adding 0.2 ml of the worm homogenate, was monitored compared with a blank in which cysteine or aminobutyrate was omitted. After incubation (37°, 15 min), 10% trichloracetic acid (0.1 ml) was added and the mixture centrifuged; the supernatant was neutralized with 1 N NaOH and chromatographed on Dowex-50 cation exchange resin (H^+ form). Radioactive fractions eluted with water were collected and total radioactivity estimated. After lyophilization, radioactive oxoproline was identified by TLC. The silica gel plates were cut into 0.5 cm strips and the percentage of oxoproline calculated. From the total radioactivity eluted with water, the amount of oxoproline formed during the reaction was calculated. Similarly, the radioactive fractions eluted with NH_4OH were lyophilized and chromatographed on Dowex-1 anion exchange resin. After elution with 1 N formic acid, radioactive γ-glutamyl-α-aminobutyrate or γ-glutamyl-cysteine as disulfide and reduced forms were identified and their amounts calculated as above.

GC synthetase was also estimated according to Beutler and Gelbart [10]: the amount of [^{32}Pi] released from γ-[^{32}P]ATP in the presence of glutamic acid and cysteine was measured compared with a blank. Using this method, enzyme activity was linear for up to 30 min and proportional to the amount of extract up to 0.18 mg protein per assay.

GSH synthetase was determined according to Meister [8]. The reaction mixture contained, in a final volume of 0.5 ml: [^{14}C]glycine (12.5 mM, SA 2 mCi/mmol or 5 mM, SA 4 mCi/mmol), γ-L-glutamyl-L-cysteine or γ-L-glutamyl-L-cystine (2.5 mM) and 0.1 ml of the worm homogenate. The reaction was monitored compared with a blank in which the corresponding dipeptide was omitted. After incubation (30 min, 37°) the mixture was treated and chromatographed on Dowex-1 as previously described for the GC synthetase. The radioactive products eluted with 1 N formic acid were collected, counted and after lyophilization analysed by TLC compared with reduced and oxidized GSH. Enzyme activity was linear for up to 30 min and is proportional to the amount of extract up to 1.2 mg protein per assay.

γ-GTP was determined according to Meister [11]. Worm pairs (300) were disrupted in 100 mM Tris, HCl (pH 8), 10 mM $MgCl_2$ (1.8 ml). The reaction was monitored, comparatively with a blank, using 0.2 ml of crude worm homogenate (corresponding to 7.3 mg/ml protein). Enzyme activity was linear for up to 10 min.

GSH determination. Worm pairs (5) were homogenized in a final volume of 0.2 ml: EDTA (0.005 M), Na_2HPO_4 (0.1 M), metaphosphoric acid (5%). After centrifugation (10,000 *g*, 10 min), GSH was analyzed according to Hissin and Hilf at pH 8 [12].

Results and discussion

From the data summarized in Table 1, it appears that *S. mansoni* is capable of synthesizing GSH according to the classical pathway, i.e. catalysed successively by (a) the L-γ-glutamyl-cysteine (GC) synthetase (EC 6.3.2.2) and (b) the GSH synthetase (EC 6.3.2.3):

$$\text{L-glutamic acid} + \text{cysteine} + \text{ATP} \xrightarrow{(a)} \text{L-}\gamma\text{-glutamyl-L-cysteine} + \text{ADP} + \text{Pi}$$

$$\text{L-}\gamma\text{-glutamyl-L-cysteine} + \text{glycine} + \text{ATP} \xrightarrow{(b)} \text{L-}\gamma\text{-glutamyl-L-cysteine-glycine} + \text{ADP} + \text{Pi}$$

In contrast to the mammal's tissues where the two synthetases have been often found in similar concentrations, the level of activities of the GC and GSH synthetases have been found markedly different; this suggests that the biosynthesis of GSH in *S. mansoni* could be regulated by another way. The formation of 5-oxoproline from [^{14}C]-glutamate observed during the GC synthetase determination is due to the presence in *S. mansoni* of L-γ-glutamyl cyclotransferase (EC 2.3.2.4), which catalyses the conversion: L-γ-glutamyl aminoacid ⟶ aminoacid + 5-oxoproline [13].

On the other hand, we have shown the possibility for *S. mansoni* to use L-γ-glutamyl-L-cystine as precursor of GSH. Such a transformation has been described by Anderson and Meister [14] in the kidney cell GSH metabolism. These authors propose a pathway in which L-γ-glutamyl-cystine is formed by transpeptidation between GSH and cystine. γ-Glutamyl-transpeptidase (γ-GTP- EC 2.3.2.2) activity

Table 1. Enzymatic activities in *S. mansoni*

Enzyme	Substrate	Product identified	Activity
GC synthetase[a]	L-Ab[e]	L-γ-glu-Ab	3.75 ± 0.65
		5-oxoproline	7.45 ± 1.85
	L-cys	L-γ-glu-cys	6.20 ± 1.70
		5-oxoproline	4.0 ± 0.40
GC synthetase[c]	L-cys[d]		10.5 ± 1.0
GSH synthetase[b]	γ-glu-cys[d]	GSH	96 ± 27
	γ-glu-cys ‖ cys	GSH	57 ± 5

Activities are expressed [a]as nmol of product, [b]as pmol of GSH, formed per min per mg of protein contained in the 10,000 *g* supernatant worm homogenate, [c]estimated according to [10], activity expressed as nmol of Pi released per min per mg of protein. Values represent the mean of 3 experiments ± SD except [d]8 experiments, [e]L-Ab: L-α-aminobutyrate.

Table 2. GSH, protein content, GC and GSH synthetases activities in *S. mansoni*

	Control		After oltipraz treatment[a]	
	per mg protein	per worm pair	per mg protein	per worm pair
GSH	4.72 ± 0.16	0.45 ± 0.16	2.33 ± 0.06	0.185 ± 0.025
protein		93.5 ± 9.0		76.5 ± 3.0
GC synthetase[b]	10.8 ± 1.6	0.98 ± 0.13	13.6 ± 0.5	1.04 ± 0.04
GSH synthetase	81.0 ± 10.0	7.40 ± 0.9	86.0 ± 19.0	6.70 ± 1.5

[a]*S. mansoni* were collected two days after oltipraz treatment (100 mg/kg), [b]GC synthetase was determined using L-α-aminobutyrate as substrate (see Methods); GSH and protein levels are expressed as μg; GC synthetase activities as nmol of glutamic acid transformed and GSH synthetase activities as pmol of GSH formed per min per mg protein contained in the 10,000 *g* supernatant worm homogenate. Values represent the mean of 3 experiments ± SD.

(66.6 ± 12.2 u) [11] was effectively found in the crude homogenate of *S. mansoni*. Furthermore, after centrifugation, no γ-GTP activity could be detected in the supernatant suggesting a membrane localization of the enzyme. These data support the idea that the γ-glutamyl cycle as described by Meister [13] may be effective in *S. mansoni*, and that γ-GTP may participate in amino-acid (especially cysteine) transport.

Effect of oltipraz on GSH biosynthesis in S. mansoni. Oltipraz is a slow acting drug and two months are required until its full schistosomicidal activity [2]. However, if worms were removed from infected mice 6 hr after administration of a single oral dose of oltipraz (500 mg/kg) and then maintained in a survival medium, the death of the parasites was observed 48–72 hr after collecting. In the same conditions, schistosomes from untreated animals lived for ten days.* This result may be explained by a crucial and non-reversing metabolic disturbance occurring in schistosomes after oltipraz treatment.

Therefore, we hypothesized that the decrease in GSH level observed in *S. mansoni* after oltipraz treatment might be due to a non-reversing inhibition of the tripeptide biosynthesis. So, we have studied the effects of oltipraz on the characterized enzymes, according to the hypothesis that the unchanged molecule of oltipraz may interact with the GSH metabolism in *S. mansoni* [15]. Seven weeks after infection, 100 mg/kg of oltipraz (corresponding to the ED_{100} value [1]) was orally administered to some of the mice. The worms were collected according to Beuding *et al.* [2], who have shown that the GSH depletion was significant after two days. Lowering of GSH (50% compared with control) and of protein (20%) levels were observed in *S. mansoni* of treated mice. But GC and GSH synthetases activities estimated from the worms after treatment were quite similar to controls (Table 2).

Oltipraz was also tested *in vitro* as a potential inhibitor of the *S. mansoni* GC and GSH synthetases. Enzymatic activities were determined starting from worm homogenates (obtained from untreated mice) previously incubated (1 hr, 4°) with oltipraz suspension (0.12 mM to 0.6 mM). The synthetases activities were not significantly different from controls (10–20% increase compared to blanks without oltipraz incubation). To check the efficiency of the oltipraz concentration used, worm pairs were incubated 2 hr in Lambert medium [16] supplemented with oltipraz (0.45 mM): GSH content in the worms was effectively depressed to 70% of control (without oltipraz).

In summary, the presence in *S. mansoni* of GC and GSH synthetases has been shown. But it appears that reduction of GSH level observed in the worms after oltipraz administration is probably not due to a direct inhibition of the GSH biosynthesis.

An alternative explanation has recently been proposed by Morrison *et al.* [17]. These authors have shown that oltipraz inhibits *in vitro* the cysteine and cystine uptake in *S. mansoni*, and suggested that oltipraz may interact with a membrane receptor which possibly functions as a dipeptidase or transport carrier molecule for amino acid. Using our experimental conditions, we also observed a decrease in cysteine and cystine uptake in *S. mansoni* by 0.45 mM oltipraz (respectively 50% and 30% of control after 2 hr incubation). But, when schistosomes were incubated (1 hr, 37°) with oltipraz (0.45 mM), the γ-GTP activity estimated from the homogenate (33.35 ± 6.15 u) was not significantly different from controls (31.50 ± 2.0 u). This preliminary result supports the idea that γ-GTP, via the γ-glutamyl cycle, is not involved in the inhibition of cysteine transport and suggests that oltipraz may influence a specific transport system for cyst(e)ine leading to the GSH depletion in *S. mansoni*.

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Anagrelide: a potent and selective inhibitor of platelet cyclic AMP phosphodiesterase enzyme activity

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Anagrelide has been characterized previously as a broad spectrum inhibitor of platelet function in a variety of models [1, 2]. It inhibits the *in vitro* aggregation of rabbit and human platelets induced by all the agents that have been tested (ADP, collagen, thrombin, platelet activating factor, antigen/antibody complexes, and arachidonic acid) and also inhibits *ex vivo* aggregation in a variety of species including humans. When *in vivo* models are considered, anagrelide is an effective inhibitor of biolaser-induced intravascular thrombosis in rabbit ears and of electrically-induced carotid artery thrombosis in dogs [2]. The cardiovascular effects of anagrelide in anesthetized dogs include a modest decrease in mean aortic blood pressure probably due to vasodilation [2, 3] and a positive inotropic effect that is particularly prominent in dogs in which cardiac function has been compromised by propanolol [3].

The biochemical mechanism(s) for these effects of anagrelide has not been investigated in any detail. In the only biochemical study published to date, Tang and Frojmovic [4] examined the effects of anagrelide on cyclic AMP metabolism in human platelets. They found that the compound inhibits cyclic AMP phosphodiesterase (PDE) activity by approximately 70% at concentrations of 10 and 50 μM when the substrate concentration is low (0.83 and 0.03 μM), whereas inhibition is less than 25% at higher substrate concentrations. In the same studies, an increase in platelet cyclic AMP levels was not demonstrated although synergism between anagrelide and prostaglandin E_1 with respect to inhibition of platelet aggregation was found. On the basis of these data, Tang and Frojmovic concluded that anagrelide was probably an inhibitor of a "low K_M" cyclic AMP PDE within the human platelet. The present study was undertaken to explore further the effects of anagrelide on cyclic nucleotide PDE enzyme activities in platelets and in other tissues. The compound has been found to preferentially inhibit the cyclic AMP PDE activity in platelets. Several other PDE inhibitors including milrinone, 3-isobutyl-1-methyl-xanthine (IBMX) and theophylline have also been studied for purposes of comparison with anagrelide.

Materials and methods

Preparation of platelet sonicates, tissue supernatant fractions and compounds. Platelets were prepared from freshly drawn blood from human donors who had given informed consent and from rabbits. The citrated blood was centrifuged at 120 *g* for 15 min at 4°, and the platelets were subsequently pelleted from the supernatant fraction by centrifugation at 1000 *g* for 15 min. The platelet pellets were frozen and thawed three times, then resuspended in 0.25 M sucrose, and sonicated (3 × 30 sec, 60 W, Bronwell Biosonic III sonicator). With human donors, platelets from