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Auranofin-induced oxidative stress causes redistribution of the glutathione pool in *Taenia crassiceps* cysticerci

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ABSTRACT

Previously, we have studied the effect of the gold-compound auranofin (AF) on both thioredoxin-glutathione reductasa (TGR) activity and viability of *Taenia crassiceps* cysticerci. It was demonstrated that micromolar concentrations of AF were high enough to fully inhibit TGR and kill the parasites. In this work, the dynamics of changes in the glutathione pool of *T. crassiceps* cysticerci following the addition of AF, was analyzed. A dose-dependent decrease in the internal glutathione concentration, concomitant with an increase in ROS production was observed. These changes were simultaneous with the formation of glutathione-protein complexes and the export of glutathione disulfide (GSSG) to the culture medium. Incubation of cysticerci in the presence of both AF and N-acetyl cysteine (NAC) prevents all the above changes, maintaining cysticerci viability. By contrast, the presence of both AF and buthionine sulfoximine (BSO) resulted in a potentiation of the effects of the gold compound, jeopardizing cysticerci viability. These results suggest the lethal effect of AF on *T. crassiceps* cysticerci, observed at micromolar concentrations, can be explained as a consequence of major changes in the glutathione status, which results in a significant increase in the oxidative stress of the parasites.

1. Introduction

The tripeptide glutathione (γ-glutamylcysteinylglycine) is the major low-molecular-mass thiol compound in most cells [1] and participates in such diverse biological processes: detoxification of both reactive oxygen species (ROS) and reactive electrophilic compounds [2,3], maintenance of the cellular redox status [4], redox-dependent signaling [5], cell proliferation and differentiation [6,7] and apoptosis [8].

Cell glutathione may be present in three forms: reduced (GSH), oxidized (GSSG) and in a complexed form, mainly with proteins (PSSG) [9]. Under physiological conditions, GSH is significantly favored over GSSG due to the activity of the NADPH-dependent flavoenzyme glutathione reductase (GR, E.C. 1.6.4.2. NAD[P]H:GSSG oxidoreductase) [10]. However, some pathological conditions [11], as well as ROS-dependent signaling-processes [12] have been found to result in a decreased [GSH]/[GSSG] ratio due to oxidative stress, which increase the formation of glutathione-protein mixed disulfides [13] and the GSSG excretion [14,15]. Nevertheless, few works have studied the dynamics of glutathione under physiological or oxidative-stress conditions [16–19].

In 2001, the multifunctional properties of TGR were characterized in *Mus musculus* [24]. The first report of this atypical disulfide reductase in flatworms, was in the cestode *Moniezia expansa* in 1995 [25], although it was erroneously considered as a GR. To date, TGR has been reported in the trematodes *Schistosoma mansoni* [26], *Fasciola hepatica* [27] and *S. japonicum* [28], as well as in the cestodes *Echinococcus granulosus* [29] and *Taenia crassiceps* [30]. Subsequent studies showed that in flatworms cytosolic and mitochondrial isoforms are encoded by a single gen [31] although with different kinetic properties [32].

In spite of the significant number of reports on TGR in parasitic flatworms, no information concerning the glutathione or the thioredoxin pools in these organisms is available. Due to the different nature of the detoxification systems between flatworms and their vertebrate hosts, TGR has been proposed as a drug target [33]. We have previously demonstrated both *in vitro* [30] and *in vivo* the inhibition of TGR by auranofin (AF), as well as its effect on the viability of *T. crassiceps* cysticerci [34]. AF is an antirheumatic

Abbreviations: AF, auranofin; BSO, buthionine sulfoximine; GR, glutathione reductase; GSH, reduced-form glutathione; GSSG, glutathione disulfid; NAC, *N*-acetyl cysteine; PSSG, protein-complexed glutathione; ROS, reactive oxygen species; TGR, thioredoxin-glutathione reductase; Trx, thioredoxin;; TrxR, thioredoxin reductase.

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gold-derivatized salt [35], which specifically inhibits selenoproteins, such as mammalian TrxR and TGR [36].

The correct equilibrium in the glutathione pool between its oxidized and reduced states is critical for cell survival. In this work, the importance of glutathione in redox homeostasis and its global distribution in *T. crassiceps* cysticerci in response to the oxidative stress caused by AF was evaluated. Our results show that in this parasite, AF cause significant changes in the [GSH]/[GSSG] ratio, *S*-glutathionylation and the GSSG excretion rate, suggesting a key role of glutathione in the redox homeostasis of these organisms.

2. Material and methods

2.1. Chemicals and reagents

Auranofin (AF) was obtained from Alexis Biochemicals (Lausen, Switzerland). Dimetilsulfoxide (DMSO) was obtained from MERCK (Hohenbrunn, Germany) while the RPMI-1640 medium was obtained from Life Technologies-Gibco (Grand Island, NY, USA). All others chemicals were purchased from SIGMA–ALDRICH Chemical Co. (St. Louis, MO, USA). Water purified by reverse osmosis was used in the solutions preparation.

2.2. Growth of T. crassiceps cysticerci

Female Balb/c mice were inoculated with about 20 cysticerci of the *T. crassiceps* HYG strain by injection into the peritoneal cavity, as previously described [37]. Two to four months later, cysticerci were recovered from the peritoneal cavity under sterile conditions. Before use, larvae were washed thoroughly with 10 mM phosphate buffered saline solution (PBS) pH 7.4. In order to obtain a homogeneous population, cysticerci of about 3.0 mm diameter were selected by filtration through a sieve.

2.3. Cysticerci maintenance and treatments

The conditions for the cysticerci maintenance in culture medium have been previously described [34]. Briefly, 300 cysticerci (approximately 3 mL) were carried to 20 ml with RPMI-1640 medium (pH 7.0) supplemented with 1% penicillin-streptomycin and incubated during three hours under aerobic conditions in a rotatory shaker at 37 °C in the presence of either 10 mM N-acetyl cysteine (NAC) dissolved in 100 mM HEPES (pH 7) or 100 μM buthionine sulfoximine (BSO) dissolved in PBS. Then, AF (dissolved in 100% DMSO) was added at the corresponding final concentration (0, 2, 4 or 10 μM) and the culture was maintained for an additional 13 h period under the same incubation conditions. Control samples were maintained in the presence of medium plus DMSO at the concentration used in the AF samples. After the treatment, cysticerci were recovered, washed with PBS and separated in aliquots. The maintenance media were also recovered and stored at −70 °C to measure glutathione content.

2.4. Cysticerci viability

Cysticerci viability was evaluated by both vital staining (0.02% trypan blue) and temperature-induced motility, as previously reported [34].

2.5. Determination of gamma-glutamyl-cysteine synthetase activity in crude extract

Immediately after drug exposure, 100 cysticerci were harvested, washed with PBS, and then homogenized with a Teflon pestle in the presence of 86 μM phenylmethylsulfonyl fluoride (PMSF) and centrifuged at 14,600 × *g* for 15 min to discard mitochondria and then at 269,000 × *g* for 45 min. to discard the microsomal fraction. To eliminate low molecular weight metabolites, the high-speed supernatant was dialyzed overnight against 10 volumes of 100 mM Tris/HCl buffer 1 mM EDTA (pH 8.0) at 4 °C. For the determination of gamma–glutamyl–cysteine synthetase activity, an aliquot of 100 μL was incubated at 37 °C with saturating concentrations of 0.8 mM glutamate, 0.8 mM cysteine, 5 mM ATP and 20 mM $MgCl_2$. After 30 min, the reaction was stopped by addition of 6% perchloric acid (PCA). After centrifugation an aliquot of 100 μl was taken off and analyzed by reverse-phase high-performance liquid chromatographic (RP-HPLC) using an analytic column C-18 Spherisorb S5 ODS2 (PSS 831,913), according to Aviles et al. [38]. Commercial gamma-glutamyl cysteine was used as standard.

2.6. Identification of TGR-activity in crude extract

A 100 cysticerci sample homogenized as mentioned above and centrifuged at 38,800 × *g* for 30 min. The supernatant was recovered and dialyzed overnight against 10 volumes of 50 mM Tris plus 1 mM EDTA 1 mM pH 7.8 at 4 °C.

TGR activity was measured using a 100 mM Tris/HCl buffer, 2 mM EDTA (pH 7.8) buffer and 120 μM NADPH. The reaction was started by the addition of 200 μL of dialyzed extract and the endogenous thioredoxin-dependent NADPH consumption was followed at 340 nm.

2.7. Determination of cytosolic pH in T. crassiceps cysticerci

Cysticerci (~500) were punctured with a needle to extract and discard the vesicular liquid. Next, cysticerci were carefully washed three times with bidistilled H_2O (pH 7) and squeezed by centrifugation at 68,000 × g for 45 min. Finally, the pH in the supernatant (cytosolic fraction) was determinated using a pH-meter 320 (CORNING). The measurements were made by triplicate in ten different cysticerci samples.

2.8. Obtention of deproteinized extracts

An aliquot containing 200 cysticerci was harvested immediately after the corresponding treatment, washed with PBS, and then homogenized with a Teflon pestle in the presence of an equal volume of 2 M ice-cold perchloric acid (PCA) containing 4 mM EDTA. The resultant suspension was centrifuged at 68,000 × *g* for 1 h to remove the precipitated protein. The supernatant was neutralized with 2.0 M KOH containing 0.3 M *N*-morpholinopropanesulfonic acid (MOPS), and fractioned in aliquots for determination of both internal glutathione equivalents (GSH + 2 × [GSSG]) and oxidized glutathione (GSSG). An aliquot of the acid supernatant was immediately derivatized with 180 mM of 2-vinylpyridine (2-VP) for GSSG determination. 2-VP does not interfere with glutathione determinations [39]. The sample lacking 2-VP was used for determination of internal glutathione equivalents.

2.9. Reduction of protein-complexed glutathione

To determine the amount of protein-bound glutathione (PSSG) the protein pellet obtained as described above was recovered and washed twice with 2.0 M PCA and 4.0 mM EDTA and centrifuged for 30 min at 25,000 × *g* at room temperature. The pellets were resuspended in 1.0 ml of 100 mM potassium phosphate buffer 1 mM EDTA (pH 7.4), and the pH raised up to 8.2–8.4 with 1 N NaOH; then, dithiothreitol (DTT) at a final concentration of 8 mM was added and the samples were incubated for 20 minutes at room temperature. The reduced sample was again deproteinized and neutralized as

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