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Short communication

Oxidative stress protection of *Trypanosomes* requires selenophosphate synthase

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ABSTRACT

Selenoproteins are characterized by the incorporation of at least one amino acid selenocysteine (Sec-U) encoded by in-frame UGA stop codons. These proteins, as well as the components of the Sec synthesis pathway, are present in members of the bacteria, archaea and eukaryote domains. Although not a ubiquitous pathway in all organisms, it was also identified in several protozoa, including the Kinetoplastida. Genetic evidence has indicated that the pathway is non-essential to the survival of *Trypanosoma* growing in non-stressed conditions. By analyzing the effects of RNA interference of the *Trypanosoma brucei* selenophosphate synthetase *SPS2*, we found a requirement under sub-optimal growth conditions. The present work shows that *SPS2* is involved in oxidative stress protection of the parasite and its absence severely hampers the parasite survival in the presence of an oxidizing environment that results in an apoptotic-like phenotype and cell death.

The amino acid selenocysteine (Sec – U) is present in organisms from all three domains of life (bacteria, archaea and eukarya), and is the main biological form of incorporation of selenium into selenoproteins at an in-frame UGA stop codon [1,2]. Its biosynthesis and subsequent introduction into a protein sequence requires the function of at least four genes in prokaryotes and more than seven in eukaryotes [3,4] in addition to a specific tRNA expressed from the *SELC* gene [5]. Previous studies [6,7] identified the presence of the Sec biosynthesis pathway in Kinetoplastid parasites. The synthesis of selenocysteine requires the highly reactive reduced selenium donor compound, monoselenophosphate [8,9], generated by the enzyme selenophosphate synthetase (SPS2), a central enzyme in the pathway and also present in *Leishmania major*, *T. cruzi* and *Trypanosoma brucei* [10].

The protection against oxidative stress has been considered a valid drug target as a consequence of genetic validation on trypanothione-related enzymes [11]. Most known selenoproteins act as oxidoreductases regulating the cellular redox state or are involved in redox related functions [12,13]. Here we present evidence by RNA interference experiments to propose that the selenocysteine synthesis pathway is involved in the oxidative stress protection. Previous work [14,15] has suggested that this pathway is dispensable in *T. brucei* procyclic and bloodstream life stage forms. Our results extended this observation, indicating that the selenocysteine synthesis pathway is relevant for long-term protection against the exposure to oxidative stress.

The nucleic acid and amino acid sequences of *T. brucei* selenophosphate synthetase (*TbSPS2* accession no. XP_823164) were identified in *T. brucei* genomic databases. The SPS2 nucleotide sequence showed no significant identity with other *T. brucei* genes as well as with their human and *Escherichia coli* homologues. For RNAi analysis [16], a 1076 bp fragment of *TbSPS2* was amplified by the polymerase chain reaction (PCR) from *T. brucei* 427 procyclic genomic DNA. The PCR product was cloned into the pCR2.1TOPO vector (Invitrogen), sequenced and cloned into the *Eam1105* I sites of a modified version of the p2T7^{TABlue} vector [17,18]. This construction was linearized by *Not* I cleavage and transfected into procyclic *T. brucei* 29-13 (PCF) [19] and *T. brucei* single marker bloodstream (BSF) [20] cell-lines by electroporation. The cells were cloned by the limiting dilution technique and selected under 2.5 μg/mL phleomycin to obtain stable cell lines as previously described [21].

To induce the synthesis of the double strand RNA (dsRNA), cells were incubated in medium containing tetracycline (1 μg/mL) and their growth followed by counting in a hemacytometer. Cell cultures were diluted to 5×10^5 cells/mL (PCF) and 5×10^4 cells/mL (BSF) at intervals of four days until reaching the stationary phase. To determine the *TbSPS2* mRNA depletion, total RNA was isolated before and after RNAi induction at different time points. The *TbSPS2* mRNA was estimated by semi-quantitative RT-PCR (RevertAid^{TM}

Abbreviations: SPS2, selenophosphate synthetase 2; RNAi, RNA interference.

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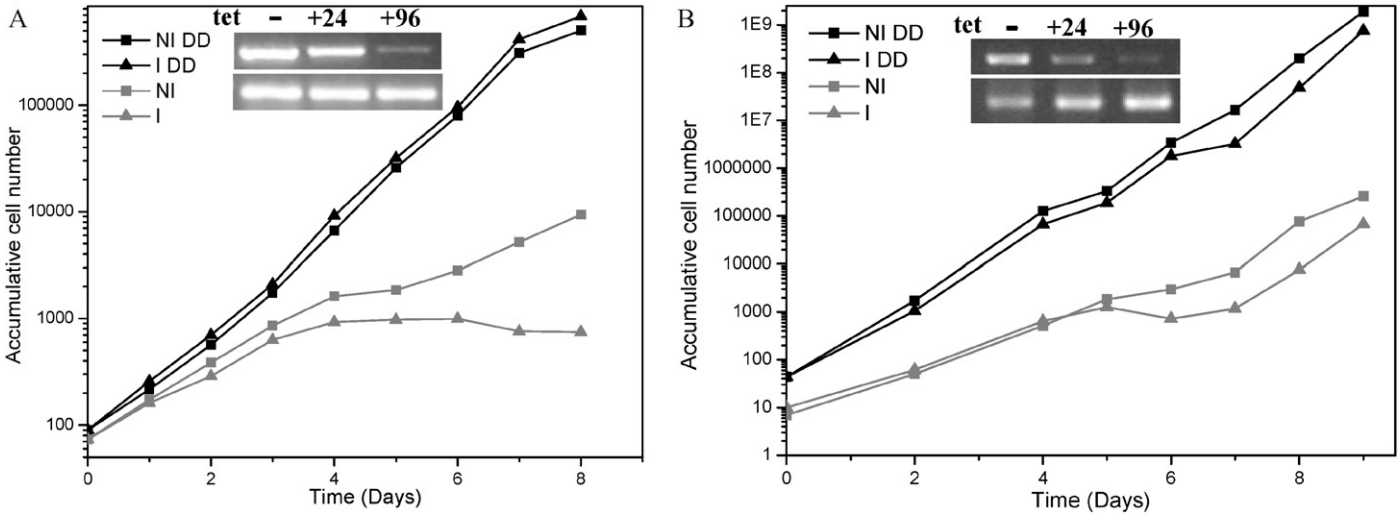

Fig. 1. Effect of SPS2 knockdown only affects *T. brucei* growing in sub-optimal conditions. Knockdown of *TbSPS2* by RNAi was achieved with a specific fragment PCR-amplified from genomic DNA using gene-specific primers (*TbSPS2a* 5′-GCG*CTCGAG*GACTACACACGGCTTAAGGGTTGCAG-3′, *TbSPS2b* 5′-CGC*AAGCTT*TTAATCATTTACCTTCGCTCCCACCT-3′) and cloned into a modified p2T7^{TAblue} with phleomycin resistance. The recombinant plasmids were linearized to accomplish their integration in the ribosomal repeat spacer of the *T. brucei* genome. PCF 29–13 were cultured at 26 °C in Cunningham's medium supplemented with 10% fetal bovine serum (panel A). BSF, SM strain, were cultivated in HMI-9 medium containing 10% of heat-inactivated fetal bovine serum at 37 °C with 5% CO_2 (Panel B). Cultures were diluted daily (DD, black symbols) to 1×10^6 cells/mL (PCF) and 1×10^5 cells/mL (BSF) or every four-day (D4, gray symbols) to 5×10^5 cells/mL (PCF) and 5×10^4 cells/mL (BSF) in the presence (I) and in the absence of tetracycline (NI). A representative of three experiments is shown. Insets shown the semi-quantitative RT-PCR (RevertAid^{TM} M-MuLV Reverse Transcriptase) performed using the sequence specific primers (*TbSPS2c* 5′-ATTGTGAAACTGCAGCACAAGGATGAGACC-3′, *TbSPS2d* 5′-CAGCGTTATATAGTTCTTCAATCTCCTCCGAG-3′) from total RNA isolated using Trizol (Invitrogen) before and after tetracycline addition at the indicated points. It produces a PCR fragments of 531 bp. *T. brucei* glycosomal glyceraldehydes 3-phosphate dehydrogenase gene (*gGAPDH*, X59955.1) was used as control with the primer pair *TbGAPDHa* 5′-CCTCGGGAATGAGATTGATGTCGTTGCTGT-3′ and *TbGAPDHb* 5′-CACATACTCCACACCAAGCTTTCCC-3′ in a standard RT-PCR, resulting in PCR fragments of 244 bp.

M-MuLV Reverse Transcriptase) using sequence-specific primers. The *T. brucei* glycosomal glyceraldehyde 3-phosphate dehydrogenase gene (*gGAPDH*, accession no. X59955.1) was used as control. As shown in the insets of Fig. 1A and B, the expression of *TbSPS2* dsRNA led to degradation of the specific mRNA. The unrelated transcript, *TbGAPDH*, remained unaffected in the samples analyzed. The non-induced *T. brucei* cells transfected with *TbSPS2* RNAi construct showed a small reduction of the corresponding mRNA as compared to the non-transfected cells (data not shown). This phenomenon is commonly seen in this experiments due to the expression leakage in the T7 dual-promoter system, which is compatible with the instability observed in the transfected but non-induced cells compared to wild type [22].

When replacing the medium every day, no effect on cell growth was observed following RNAi induction for both PCF and BSF (Fig. 1A and B, black symbols). These results are in agreement with previously published data describing that selenium methionine pathway is dispensable in *T. brucei* [14,15,23]. However, when replacing the medium every 4th day, expression of *TbSPS2* dsRNA resulted in severe growth inhibition of induced PCF and BSF cultures (Fig. 1A and 1B, gray symbols). These results suggest that *TbSPS2* could be required for cells growing in suboptimal conditions).

Since SPS2 is credited with the central role of providing the selenium donor for the whole pathway, we examined in more detail the RNAi effect of this gene. The cell morphology of *T. brucei* cells shows that the non-induced cells appeared normal (Fig. 2B) while the *TbSPS2* knockdown cells presented no, or fragmented nuclei (Fig. 2A). To examine the DNA content of the non-induced (NI) and induced (I) *TbSPS2* RNAi PCF (1×10^6 cells), were subjected to tetracycline induction under stress conditions (DT4) and in the daily-diluted condition (DD). Flow cytometer analysis of cells stained with propidium iodide revealed that *TbSPS2* cells growing in stressed conditions had a 37% decrease in the G2 (C2, being C the DNA content) in induced cultures when compared to control cultures (Fig. 2C). More importantly, an almost 4-fold increase in the degraded DNA peak (C < C1) corresponding to dead parasites, from 7% (NI) to 29% (I), was visible. This same peak remains constant in the non-induced (7.5%) and induced (9.6%) cells growth on the daily dilutions condition, as shown in the histogram of Fig. 2B. These results are consistent with an apoptotic-like phenotype generated by the *TbSPS2* knockdown under stressed condition [24].

The known role of selenoproteins in redox pathways resulting in a protection against oxidative stress [25] in mammalian cells and the RNAi experiments results, under stress conditions, led us to test the sensitivity of the *TbSPS2* dsRNA cell lines to the hydrogen peroxide exposure, in both procyclic and bloodstream forms. As described earlier, a short-term exposure to H_2O_2 in standard growth medium that contains β-mercaptoethanol, resulted in no significant culture viability effect [14]. To investigate the long-term oxidative stress effect in *TbSPS2* depleted *T. brucei* cells, daily-diluted growth curves were performed with addition of H_2O_2. After the 4th day of continuous exposure to H_2O_2, only RNAi induced cells (PCF and BSF) presented impaired growth (Fig. 3A and B).

Therefore, the increased sensitivity to oxidative stress induced by H_2O_2 of *TbSPS2* knockdown *T. brucei* cells, demonstrates that selenoproteins are involved in long-term oxidative protection of *Trypanosoma* cells. The exact role of selenium containing proteins in this protective effect requires further investigation. Recently, the involvement of a *Trypanosoma* homologue selenoprotein, SELK, in the stress-regulation and protection of mammalian cells to endoplasmic reticulum stress agent-induced apoptosis has been described [26]. At any rate, the data presented here clearly demonstrate that the selenocysteine pathway in *Trypanosoma* plays a relevant role for the parasite survival.

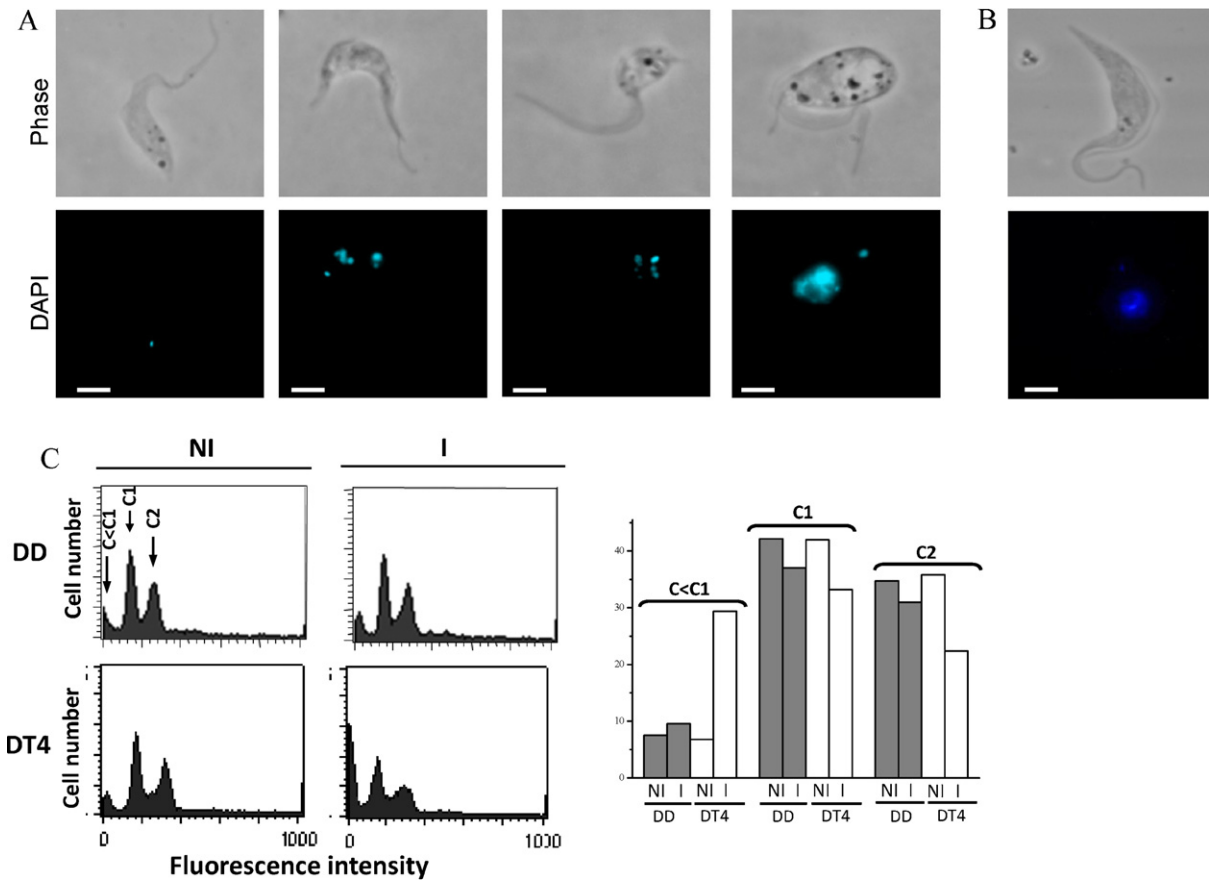

Fig. 2. SPS2 knockdown affects the nucleus morphology in PCF of *T. brucei.* (A) The panel shows examples of PCF 8 days after RNAi induction for SPS2 as observed after fixation with 4% paraformaldehyde in PBS by phase contrast microscope (Phase) and fluorescence after staining with 2 μg/mL 4′,6-diamidino-2-phenylindole (DAPI). Cells exhibited a morphological apoptotic phenotype with abnormal cellular and nuclear morphology. Bars = 2 μm. (B) Non induced cells. Panel (C) show flow cytometer analysis of PCF stained with propidium iodide. Cells (1×10^6) were washed with PBS, resuspended in 500 μL of ice-cold 70% methanol, at 4 °C. The fixed parasites were washed once with PBS and incubated for 20 min at 37 °C with 10 μg/mL of DNAse-free RNase A (Roche-Diagnostics). Samples were washed in PBS, resuspended in 500 μL of PBS containing 20 μg/mL propidium iodide and analyzed using a Becton–Dickinson Excalibur flow cytometer. The data corresponding to 10,000 events was analyzed using the WinMid 2.8 software. At right are shown four FACS distribution graphs of DNA content in control (NI) or after SPS2 RNAi induction (I), at the daily diluted condition (DD) and at the stressed condition, culture diluted every four day (DT4). A peak of degraded DNA (C < C1), G1 (C1) and G2 (C2) peaks are indicated, being C the DNA content of the cells. At left is shown the histogram comparison of the % cell population with C < C1, C1 and C2 DNA content. Not included are the cells in S phase and signal due to cell debris.

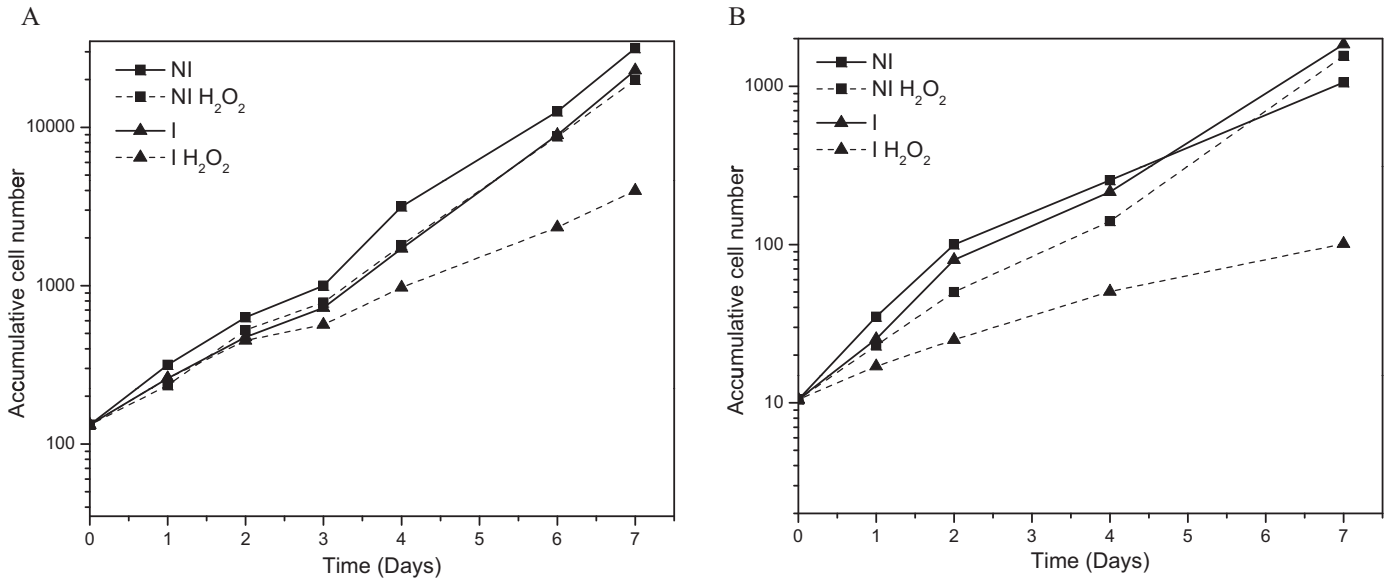

Fig. 3. Sensitivity to after of *TbSPS2* knockdown increases the sensitivity of *T. brucei* to H_2O_2. PCF (panel A) and BSF (panel B) containing the construction for RNAi against *TbSPS2* were daily diluted (DD) to 1×10^6 cells/mL (PCF) and 1×10^5 cells/mL (BSF) in the presence of tetracycline (I, solid), in the absence of tetracycline (NI, solid), in the presence of tetracycline and H_2O_2 (I H_2O_2, dash), in the absence of tetracycline and the presence of H_2O_2 (NI H_2O_2, dash). In PCF tests it was used 5 μM and in BSF tests 50 μM of H_2O_2. Growth curves are representative examples of multiple experiments ($n \geq 3$).

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