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Endogenous sterol biosynthesis is important for mitochondrial function and cell morphology in procyclic forms of *Trypanosoma brucei*

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ABSTRACT

Sterol biosynthesis inhibitors are promising entities for the treatment of trypanosomal diseases. Insect forms of *Trypanosoma brucei*, the causative agent of sleeping sickness, synthesize ergosterol and other 24-alkylated sterols, yet also incorporate cholesterol from the medium. While sterol function has been investigated by pharmacological manipulation of sterol biosynthesis, molecular mechanisms by which endogenous sterols influence cellular processes remain largely unknown in trypanosomes. Here we analyse by RNA interference, the effects of a perturbation of three specific steps of endogenous sterol biosynthesis in order to dissect the role of specific intermediates in proliferation, mitochondrial function and cellular morphology in procyclic cells. A decrease in the levels of squalene synthase and squalene epoxidase resulted in a depletion of cellular sterol intermediates and end products, impaired cell growth and led to aberrant morphologies, DNA fragmentation and a profound modification of mitochondrial structure and function. In contrast, cells deficient in sterol methyl transferase, the enzyme involved in 24-alkylation, exhibited a normal growth phenotype in spite of a complete abolition of the synthesis and content of 24-alkyl sterols. Thus, the data provided indicates that while the depletion of squalene and post-squalene endogenous sterol metabolites results in profound cellular defects, bulk 24-alkyl sterols are not strictly required to support growth in insect forms of *T. brucei* in vitro.

1. Introduction

Protozoan parasites are responsible for a significant portion of global morbidity, mortality and economic hardship, and in most countries current control and treatment regimes are either failing or under serious threat (Barrett et al., 2003). As the lipid metabolism of trypanosomes differs in many respects from that of its mammalian host, it offers potential and promising targets for the development of urgently needed and new chemotherapeutic drugs to combat these parasites (Lorente et al., 2004; van Hellemond and Tielens, 2006; Lepesheva et al., 2007, 2008; Urbina, 2009). African trypanosomes do not synthesize cholesterol de novo, but instead synthesize ergosterol and other 24-alkylated sterols, similar to plants and fungi (Roberts et al., 2003). However, when cholesterol is present in their environment it is incorporated by the parasite without further metabolism from the host via receptor-mediated endocytosis of low density lipoproteins (LDLs) (Coppens and Courtoy, 2000) followed by lysosomal degradation (Coppens and Courtoy, 1995; Coppens et al., 1995). While bloodstream forms are highly dependent on this exogenous supply of sterols for adequate growth, procyclic trypanosomes are flexible with respect to the source of sterols and adjust their de novo ergosterol biosynthesis to the external supply of cholesterol (Coppens and Courtoy, 1995).

The close similarities to fungi in relation to sterol composition and sterol biosynthesis have offered a unique opportunity for the development of chemotherapy by targeting the sterol biosynthetic pathway using the types of drugs already successfully employed against fungal pathogens (Roberts et al., 2003). In fungi, sterols are responsible for membrane stability and modulate morphogenesis, intracellular trafficking, membrane permeability and the activities of membrane-bound enzymes (Heese-Peck et al., 2002; Sharma, 2006). Indeed, sterol biosynthesis inhibitors have been successfully tested as anti-trypanosomals in the case of *Trypanosoma cruzi* and *Leishmania* spp. (Magaraci et al., 2003; Lorente et al., 2004; Urbina et al., 2004; Hucke et al., 2005). Curiously, certain studies have demonstrated that inhibitors of the enzymes, sterol methyl transferase and sterol 14α-demethylase, are active against bloodstream forms of *Trypanosoma brucei* (Cammerer

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et al., 2007; Lepesheva et al., 2007) even though they rely on host endocytosed cholesterol to satisfy their sterol requirements (Coppens et al., 1995). Furthermore, it has been recently shown that endogenous sterol synthesis does occur in this form of the parasite (Nes et al., 2012). However, despite the therapeutic importance of the sterol biosynthesis pathway, the role of endogenously synthesized sterols in cell viability and morphology has not yet been examined using genetic tools that allow dissection of specific sterol requirements. Here we analyse the consequences of the knockdown of three enzymes involved in sterol biosynthesis on cell morphology and function in procyclic parasite forms in order to establish the role of distinct intermediates in the sterol biosynthetic pathway.

The first enzyme, squalene synthase (SQS, EC 2.5.1.21) (Urbina et al., 2002), catalyses the condensation of two molecules of farnesyl diphosphate to produce squalene, the first committed step of the sterol pathway. Loss of its function in *Saccharomyces cerevisiae* leads to cell death (Jennings et al., 1991) due to the defect in ergosterol biosynthesis. SQS from *T. cruzi* has been cloned and characterised, and quinuclidine inhibitors show a very potent (sub-nanomolar) activity and selectivity for the parasite enzyme (Sealey-Cardona et al., 2007). Also of pharmacological interest is squalene epoxidase (SQE, EC 1.14.99.7) (Leber et al., 2003; Akins, 2005), a flavin adenine dinucleotide (FAD) containing monooxygenase that converts squalene into 2,3-oxidosqualene (Favre and Ryder, 1997; Klobucnikova et al., 2003; Leber et al., 2003). The enzyme plays a key role in the synthesis of essential sterols, hence homozygous disruption of *ERG1* (the yeast gene encoding SQE) was found to have deleterious effects in yeast cells (Leber et al., 1998; Tsai et al., 2004).

Finally, the introduction of the methyl group in the 24 position is catalysed by S-adenosyl-L-methionine: $\Delta^{24,(25)}$-sterol methyl transferase (SMT, EC 2.1.1.43) which is found in fungi, yeast, protozoa and plants, but is not present in vertebrate hosts. Thus, SMT imparts one of the key structural differences between ergosterol and cholesterol and is a very attractive target in the search of inhibitors selective for protozoan and fungal sterol biosynthesis. Several studies have shown the anti-proliferative effects of SMT inhibitors in yeast fungi, plants, algae (Nes et al., 1991, 1997; Ator et al., 1992; Acuna-Johnson et al., 1997; Mangla and Nes, 2000) and protozoa such as *Leishmania donovani* (Haughan et al., 1995; Magaraci et al., 2003; Lorente et al., 2004) and *Trypanosoma* spp. (Urbina, 1997; Gros et al., 2006; Zhou et al., 2006, 2007).

Here we show that depletion in *T. brucei* procyclic trypomastigotes of squalene synthase (*Tb*SQS) and squalene epoxidase (*Tb*SQE) results in growth and morphological defects, modifications of the sterol composition, lipid vesicle accumulation and altered mitochondrial function. In contrast, sterol methyl transferase (*Tb*SMT) deficient procyclic cells, while exhibiting defects in C24-alkyl sterol formation, exhibit a normal in vitro growth phenotype. The information obtained will help to establish the role of specific intermediates of this metabolic pathway in cell function and to elucidate the molecular mode of action of sterol biosynthesis inhibitors in trypanosomes.

2. Materials and methods

2.1. Compounds

Foetal bovine lipoprotein depleted serum (LPDS) media supplement was purchased from Kalen Biomedical, USA, and terbinafine, ketoconazole and 22,26-azasterol from Sigma Aldrich, USA. Compounds were dissolved in 100% DMSO. The final concentration of DMSO in cultures did not exceed 0.1% (v/v) and had no effect on cell proliferation.

2.2. Purification of recombinant SQE and SMT from T. brucei and generation of polyclonal antibodies

Escherichia coli BL21 (DE3) cells over-expressing a truncated version of *Tb*SQE (ΔN22/ΔC34) and the full length open reading frame (ORF) for *Tb*SMT, respectively, were used for purification of truncated *Tb*SQE and *Tb*SMT. In the case of *Tb*SMT, expression and purification was performed as previously described (Gros et al., 2006). For *Tb*SQE, a truncated version lacking 22 and 34 amino acids from the amino and carboxy terminal regions, respectively, was obtained by PCR and cloned in the pET28 vector. For purification, a pellet of 2 L of culture was resuspended in PBS supplemented with a cocktail of protease inhibitors. After sonication, the total extract was loaded on a 12% SDS–polyacrylamide gel. The gel bands of interest were excised and the proteins were electrophoretically eluted from the minced gel. After removal of most of the salt and SDS by dialysis, the protein concentration was determined by the method of Bradford and gave a single band in SDS–PAGE. Truncated *T. cruzi* SQS (*Tc*SQS) was expressed and purified as previously described (Sealey-Cardona et al., 2007). Anti-*Tb*SMT, anti-*Tc*SQS and anti-*Tb*SQE serums were generated by immunising rabbits with purified recombinant proteins, which were resuspended in PBS and mixed with FCA before injection into a rabbit. Four inoculations of 250 μg of proteins were carried out before obtaining the antiserums with a titre of 1:131,000,000 for *Tb*SMT, 1:10,000,000 for *Tc*SQS and 1:16,000,000 for *Tb*SQE. To cheque the specificity of the antibodies, the serums were pre-incubated with the corresponding purified protein overnight at 4 °C (Supplementary Fig. S1). The blocked serums were centrifuged at 16,000*g* for 15 min at 4 °C and the supernatants were used in western blots.

2.3. Plasmid constructs for RNA interference (RNAi)

For RNAi studies two different fragments of *TbSQS*, *TbSQE* and *TbSMT* were amplified by PCR. Oligonucleotides used for PCR are listed in Supplementary Table S1. For *TbSQS* the primers used were *TbSQS*1 5′ and *TbSQS*1 3′ (538 bp) and *TbSQS*2 5′ and *TbSQS*2 3′ (553 bp fragment). In the case of *TbSQE* RNAi, *TbSQE*1 5′ and *TbSQE*1 3′ (645 bp) and *TbSQE*2 5′ and *TbSQE*2 3′ (522 bp) were used. For *TbSMT* RNAi, the oligonucleotides were *TbSMT*1 5′ and *TbSMT*1 3′ (515 bp), and *TbSMT*2 5′ and *TbSMT*2 3′ (468 bp).

Firstly, the fragments were cloned into the *Hin*dIII and *Apa*I sites of pGR19 (Clayton et al., 2005). Secondly, the fragments were digested with *Bam*HI for cloning in the opposite orientation (antisense) in the *Bam*HI and *Hpa*I restriction sites of the previous constructs yielding the plasmids pGR19-*TbSQS*1, pGR19-*TbSQS*2, pGR19-*TbSQE*1, pGR19-*TbSQE*2, pGR19-*TbSMT*1 and pGR19-*TbSMT*2.

2.4. Trypanosome culture and transfection

The procyclic *T. brucei* strain, *Tb*449, expressing the tetracycline repressor was grown in SDM-79 medium supplemented with 10% FBS and hemin at 28 °C. Cells were grown in the presence of 0.5 μg mL^{-1} of phleomycin.

For stable transfection of the procyclic form via integration into a rDNA spacer region, the vectors were linearized by *Not*I digestion. Transfection methodology was carried out as previously described (Wirtz et al., 1998, 1999). Procyclic cells were selected in 50 μg mL^{-1} of hygromicin. RNAi was induced by the addition of doxycycline (1 μg mL^{-1}) and screened by northern and western blots.

2.5. Northern blot analysis

For northern blotting, 10 μg of total RNA were separated on formaldehyde gels and blotted onto a membrane (Nitre® Schlei-