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Research brief

Leishmania spp.: Delta-aminolevulinate-inducible neogenesis of porphyria by genetic complementation of incomplete heme biosynthesis pathway

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Abstract

To further develop the *Leishmania* model for porphyria based on their deficiencies in heme biosynthesis, three Old World species were doubly transfected as before for *Leishmania amazonensis* with cDNAs, encoding the 2nd and 3rd enzymes in the pathway. Expression of the transgenes was verified immunologically at the protein level and functionally by uroporphyrin neogenesis that occurs only after exposure of the double-transfectants to delta-aminolevulinate. All species examined were equally deficient in heme biosynthesis, as indicated by the accumulation of uroporphyrin as the sole porphyrin and the production of coproporphyrin upon further transfection of one representative species with the downstream gene. The results obtained thus demonstrate that at least the first five enzymes for heme biosynthesis are absent in all species examined, rendering their transfectants inducible with aminolevulinate to accumulate porphyrins and thus useful as cellular models for human porphyrias.

Index Descriptors and Abbreviations: Uroporphyrin; Coproporphyrin; Delta-aminolevulinate dehydratase; Porphobilinogen deaminase; Uroporphyrinogen decarboxylase; Heme biosynthesis; Transgenes; Trypanosomatid protozoa

1. Introduction

The inability of trypanosomatid protozoa to synthesize heme results in their nutritional requirement for hemin, hematin or hemoglobin as a growth factor (Trager, 1974; Chang et al., 1975; Chang and Chang, 1985; Krishnamurthy et al., 2005). Earlier, this deficiency has been delineated genetically by transfecting *Leishmania amazonensis* with mammalian cDNAs, encoding the 2nd and 3rd enzymes of the biosynthetic pathway for tetrapyrroles (Sah et al., 2002) (cf. Fig. 1). Development of uroporphyria in these transfectants in the presence of exogenous delta-aminolevulinate (ALA) led us to suggest that this New World *Leishmania* is functionally defective in at least the first five and possibly the first seven of the eight enzymes known in heme biosynthetic pathway (Sah et al., 2002) (Fig. 1). Of these eight enzymes, only the last one, i.e. ferrochelatase (Fe-C) has been shown to exist functionally by nutritional studies (Chang et al., 1975) and enzyme assays (Salzman et al., 1982) in different trypanosomatid protozoa. Most recently, we have found the putative Fe-C sequences of high homology in all *Leishmania* isolates grouped phylogenetically into some 20 genotypes, including most Old World species, i.e. *L. infantum/donovani*, *L. major*, *L. tropica*, *L. turanica* and *L. gerbilli* (Waki et al., 2007). Sequence conservation of Fe-C suggests that it may function beyond heme biosynthesis, since protoporphyrin (PROTO) is cytotoxic and thus would not be widely available to serve as a substrate of this enzyme to produce the heme needed by *Leishmania*. Fe-C and its two preceding enzymes for heme biosynthesis

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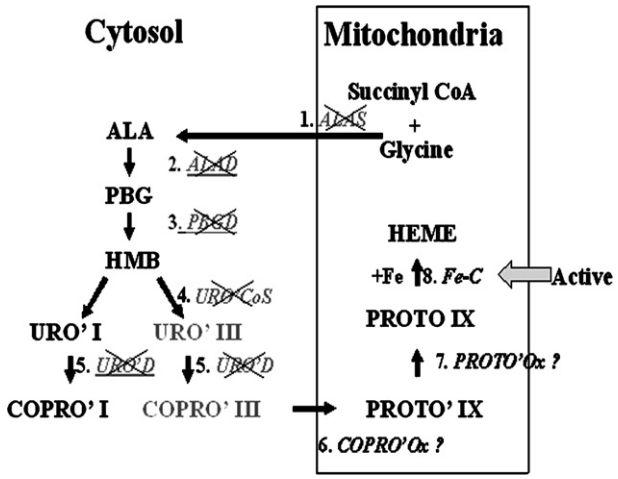

Fig. 1. Diagrammatic depiction of the classic heme biosynthetic pathway and *Leishmania* enzymic deficiencies in this pathway. ×, Enzymes absent in *Leishmania* spp.; ?, putative presence of the last three terminal enzymes based on *in silico* construction of metabolic pathways according to the *L. major* genomic database. Abbreviations: *ALAS*, aminolevulinate synthase; ALA, delta-aminolevulinate; *ALAD*, aminolevulinate dehydratase; PBG, porphobilinogen; *PBGD*, porphobilinogen deaminase; HMB, hydroxymethylbilane; *URO CoS*, uroporphyrinogen cosynthase, URO'I and III, uroporphyrinogen I and III; *URO'D*, uroporphyrinogen decarboxylase; COPRO'I and III, coproporphyrinogen I and III; *COPRO'Ox*, coproporphyrinogen oxidase; PROTO'IX, protoporphyrinogen IX; *PROTO'Ox*, protoporphyrinogen oxidase; PROTO IX', protoporphyrin IX; *Fe-C*, ferrochelatase; Fe, ferrous iron.

were noted and proposed to have a bacterial origin during *in silico* construction of *Leishmania* metabolic pathways from *L. major* genome sequence database (http://www.genedb.org/genedb/leish/) (Opperdoes and Coombs, 2007).

Here, we extend our previous findings of this biosynthetic deficiency from *L. amazonensis* to *L. major*, *L. tropica* and *L. infantum*. As observed previously, only double-transfection of each species with cDNAs encoding ALA dehydratase (ALAD) and porphobilinogen deaminase (PBGD) renders them capable of using exogenous ALA, product of the 1st enzyme, to make uroporphyrin. New experimental conditions were designed to facilitate screening of transfectants for ALA-inducible porphyria and for studying cellular events in this porphyrinogenesis. In addition, genetic complementation of a representative species with an enzyme further downstream resulted in the emergence of coproporphyrin (COPRO) and its intermediates, but not protoporphyrin (PROTO). The results not only provide conclusive evidence for a universal deficiency in heme biosynthesis of all species examined but also make available their mutants suitable for studying porphyric mechanisms at the cellular levels.

2. Materials and methods

2.1. Leishmania cultivation and transfection

Leishmania spp. used for the present study include *L. amazonensis* (LV 78 clone 12-1), *L. major* (RM), *L. tropica* (K27) and *L. infantum* (Adana #7) (see http://66.99.255.20/cms/micro/sample%20list.pdf and Waki et al., 2007 for the origin of the strains used).

Promastigotes of all species were grown to stationary phase at 25 °C in Medium 199 (Sigma) Hepes-buffered to pH 7.4 and supplemented with 10% heat inactivated fetal bovine serum (HIFBS). Single- and double-transfection of these cells by electroporation with pX-*alad* and/or p6.5-*pbgd*, selection for transfectants and their maintenance followed published procedures (Sah et al., 2002). Stable lines of single- and double-transfectants were all maintained in the presence of appropriate selective pressures, e.g. 100 μg/ml of G418 (for pX-*alad*) and/or 20 μg/ml of tunicamycin (for p6.5-*pdgd*) (except those of *L. infantum*, for which the selective pressure was lowered to 5–10 μg/ml of tunicamycin and 50 μg/ml of G418). Double-transfectants of *L. major* with pX-*alad* and p6.5-*pbgd* were further transfected with human cDNA of uroporphyrinogen decarboxylase (~1.1 kb *urod* Accession No. BC001778) placed at the BamHI expression site of a different plasmid, pXG-*hyg*. The triple-transfectants were initially selected with hygromycin alone at up to 20 μg/ml and subsequently passaged in media containing a combination of three antibiotics in different concentrations with the highest level of selective pressures as follows: 20 μg/ml of tunicamycin, 100 μg/ml of G418 and 1000 μg/ml of hygromycin. All transfectants were passaged continuously under above-mentioned selective pressures and grew essentially as well as the wild-type cells in drug-free media.

2.2. Western blot analysis

Transfectants were assessed immunologically for their production of ALAD and/or PBGD (Sassa, 1976) using rabbit antisera specific to these mammalian enzymes. Immunoblots were reacted with HRP-conjugated goat anti-rabbit IgG (1:10,000) and developed by using Pierce SuperWest Pico reagent.

2.3. Induction of double-transfectants for neogenesis of porphyria

Transfectants were grown under selective conditions to late log phase and then harvested for exposure to 1 mM delta-aminolevulinate (ALA) (Sigma/Porphyrin products, UT) at room temperature in dark at 10^8 cells/ml in Hepes-buffered Hank's Balanced Salt Solution (HBSS) (pH 7.4) plus 0.01% bovine serum albumin (BSA) (Sigma). Under these conditions of incubation in the presence of glucose (1 mg/ml), cells did not replicate, but remained fully motile and viable for up to 72 h, independent of the presence or absence of ALA. Experiments were terminated at the end of day 3, beyond which time cells began to show signs of degeneration, e.g. sluggish motility and loss of cell integrity.

2.4. Spectrofluorimetry of porphyrins

Cells (10^8) were harvested from each culture by centrifugation for 5 min at 3500 g at 4 °C for porphyrin extraction,