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Short Communication

Nitric Oxide Production within *Leishmania tarentolae* Axenic Promastigotes and Amastigotes is Induced by Carbaporphyrin Ketals

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Abstract

Previous studies indicated that carbaporphyrin ketals (CK) offer great promise in the treatment of Leishmania diseases, especially since they induce the in vitro formation of reactive oxygen species (ROS) in Leishmania tarentolae promastigotes. We have utilized confocal fluorescence microscopy to probe the response of Leishmania tarentolae axenic promastigotes and amastigotes in vitro for formation of nitric oxide (NO, a reactive nitrogen species) upon addition of CK to the cultures. Human promonocytic cells were also tested. The detection of NO following CK addition in all three cell types is described in this communication.

INTRODUCTION

Leishmaniasis is a parasitic disease with some 310 million people at risk of infection. Most of the seriously affected people are in poor and developing countries [1]. The CDC considers leishmaniasis to be an emerging infectious disease because globalization and environmental changes may threaten populations in regions in which the disease is not endemic [2]. Treatment of the disease has mostly relied on the utilization of pentavalent antimonial compounds, which require the intervention of health professionals and large doses of the medicine. Some species of the parasite have already developed resistance to the antimonial compounds, thus stimulating the search for new therapies. Other compounds, such as amphotericin B, miltefosine, and pentamidine, have emerged and are currently prescribed in many countries around the world [2]. Carbaporphyrin ketals Figure (1) show promise for the treatment of leishmaniasis. Morgenthaler et al., while investigating the mechanism of action of CK, demonstrated that they induce them *in vitro* production of reactive oxygen species (ROS) in *L. tarentolae* axenic promastigotes [3]. Intracellular ROS such as singlet oxygen and superoxide may lead to the death of the parasite. Continuing the investigation into the mechanism of action of carbaporphyrin ketals, we hypothesized that they may also induce the formation of reactive nitrogen species (RNS), specifically nitric oxide (NO) within axenic promastigotes and amastigotes of *L. tarentolae*. It is well documented that *Leishmania* parasites are able to counter oxidative burst from ROS produced by macrophages [4-6].There is strong evidence indicating that macrophages rely on the endogenous production of NO via oxidation of L-arginine mediated by nitric oxide synthase (NOS) and stimulated by cytokines and lymphokines such as tumor necrosis factor α (TNFα) and interferon-γ (INFγ) to kill the parasites [7-9]. Interestingly, Genestra et al., have reported that *Leishmania* parasites are able to produce NO which may play a regulatory role on the interactions between parasite

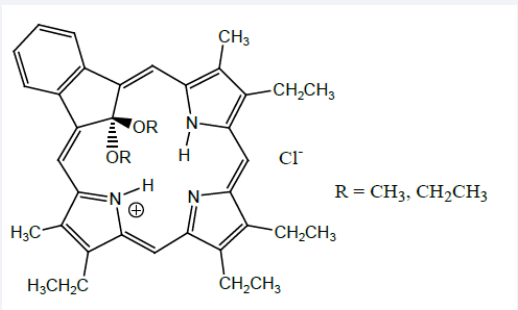

Figure 1 Structure of dimethyl or diethyl carbaporphyrin ketals (CK) [3].

and macrophages which modulates the parasites' response to exogenous NO [10-12]. Also soluble NOS has been purified and characterized from axenic *L. amazonensis* [10]. This current study focused on detection of intracellular nitric oxide following incubation of *L. tarentolae* axenicpromastigotes, amastigotes or human promonocytic cells with various concentrations of CK. Nitric oxide detection was carried out by confocal fluorescence microscopy employing a nitric oxide specific fluorescent probe [13,14].

EXPERIMENT

Leishmania tarentolae promastigotes were obtained from American Tissue Culture Collection (ATCC # 30143, Manassas, VA). Hemin and penicillin-streptomycin (10,000 U/mL penicillin and 10 mg/mL streptomycin; pen-strep) were obtained from Sigma Chemical Company (St. Louis, MO). *Leishmania tarentolae* amastigotes were obtained following the method of Taylor et al., [15] from the LEM-125 strain donated by Professor Larry Simpson, University of California at Los Angeles. Schneider insect medium and RPMI-1640 medium were purchased from Sigma Chemical Company (St. Louis, MO). U937 promonocyticcells were obtained from ATCC (#CRL-1593.2). The nitric oxide probe for confocal fluorescence microscopy, [4-amino-5-methlyamino-2',7'-difluorofluorescein diacetate (DAF-FM diacetate)] was obtained from Invitrogen (#D23844 Eugene, OR). Brain heart infusion (BHI) was obtained from Beckton, Dickinson and Company (Sparks, MD). Dialkyl carbaporphyrin ketals (CK) were synthesized as previously described [16,17] and dissolved in absolute ethanol. Confocal fluorescence microscopy was carried out in a Leica DMIRE2 from Leica Microsystems (Exton, PA). *L. tarentolae* axenic promastigotes were cultured at 25 °C, for four days, in 25 cm^2 canted neck flasks from Corning (Corning, NY) containing 10 mL of brain heart infusion (BHI) medium supplemented with 20 μM hemin and 100U/mL penicillin and 0.1 mg/mL of streptomycin, before submitting the cultures to testing by adding either ethanol only or CK dissolved in ethanol. All work with *Leishmania* and promonocytes was done under sterile conditions until the confocal fluorescence microscopy analysis. *L. tarentolae* amastigote cells were cultured at 32°C for three days, in 25 cm^2 canted neck flasks from Corning (Corning, NY) containing 9 mL Schneider's Insect Medium adjusted to pH 5.2 and supplemented with 1mL fetal bovine serum (FBS), 2 mM L-Glutamine and 100U/mL penicillin and 0.1 mg/mL of streptomycin, before submitting the cultures to testing by adding either ethanol only or CK dissolved in ethanol. U937 promonocyte cells were incubated under 5% CO_2 at 37°C for three days, in 25 cm^2 canted neck flasks from Corning (Corning, NY) containing 9 mL RPMI-1640 medium supplemented with 2 mM L-Glutamine, 1 mL FBS, and 100U/mL penicillin and 0.1 mg/mL of streptomycin, before submitting the cultures to testing by adding either ethanol only or CK dissolved in ethanol. For confocal fluorescence microscopy, axenic promastigotes, amastigotes or promonocyte cells with and without various concentrations of the CK were evaluated for NO production using the DAF-FM fluorescent probe. An argon ion laser set to 488 nm excited the probe while emission was collected in the 500-550 nm range and detected with a photomultiplier tube (PMT). One mL aliquots of four day old cultures were placed in sterile 1.5 mL polypropylene centrifuge tubes and ινχυβατεδ with ethanol or CK in ethanol for 3 hours. After incubation, 100 μL aliquots of the inoculated cultures were placed in non-sterile centrifuge tubes and incubated with 1 μM NO probe (DAF-FM diacetate) for 30 minutes. Then slides were prepared and evaluated by confocal fluorescence microscopy.

RESULTS AND DISCUSSION

Control cultures of axenic promastigotes in the presence of ethanol (0.5%) and the NO probe (Figure 2) showed a small number of fluorescent cells (about 5%) compared to cell cultures containing CK (Figure 3). For control samples, the gain and offset of the PMT were adjusted until there was no fluorescence seen. With the microscope at these standard settings (blanked), slides of parasites exposed to CK were prepared and observed under the microscope in both the fluorescence and transmission modes. Parasites and promonocytes incubated in the presence of the NO probe but without the CK or ethanol did not show any fluorescence (data not shown). Parasites and promonocytes with CK but without the NO probe did not show any fluorescence (data not shown), verifying that the fluorescence was not directly due to CK. Samples with cells incubated with the NO probe and CK concentrations ranging from 0.5 μM to 5 μM showed fluorescence indicating nitric oxide production. The number of fluorescent parasites and intensity of fluorescence was dependent on the concentration of CK Table (1) Figure (3) for promastigotes.

Our data clearly indicate that CK induces the generation of nitric oxide inside *L. tarentolae* axenic promastigotes and amastigotes (Figure 4) under the incubation conditions tested. This is interesting because NO produced by macrophages has been recognized for its effectiveness against *Leishmania* [7]. Mice lacking inducible nitric oxide synthase (iNOS) are unable to fight *Leishmania* infection while the macrophages from these mice are unable to eliminate promastigotes in culture [18]. It has also been shown that contact between macrophages and *Leishmania*

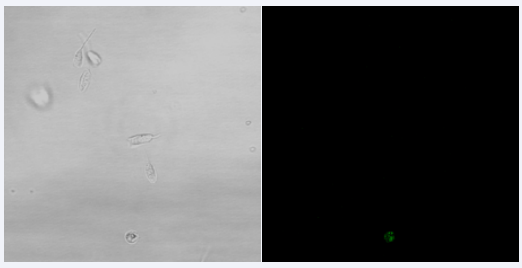

Figure 2 Promastigotes incubated with 0.5% ethanol and nitric oxide specific probe observed by confocal fluorescence microscopy. Right: fluorescence image, left: simultaneous transmission image.

Table 1: Percentage of fluorescent cells as a function of CK concentration.

Concentration of CK (μM)	% fluorescent cells[a]
0.5	25
3	58
5	80

[a]Values were calculated by dividing number of promastigote cells that fluoresce by the total number of promastigote cells observed in transmission mode, times 100.

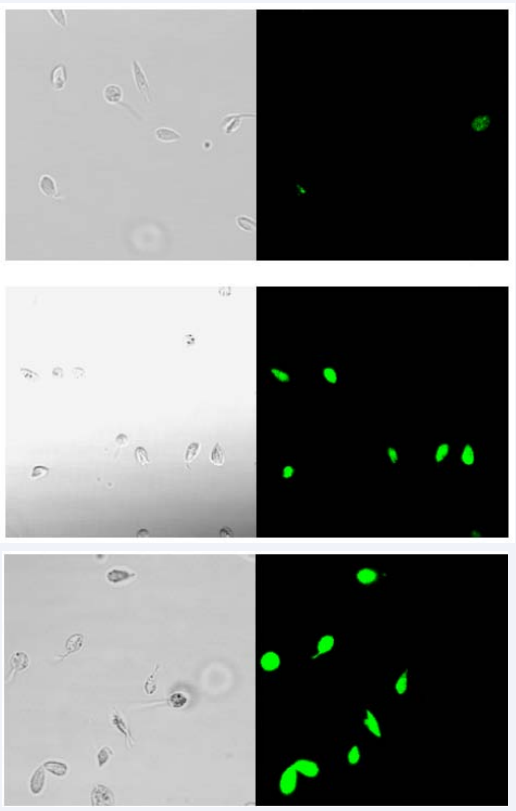

Figure 3 Confocal microscopy images of promastigotes incubated with nitric oxide specific probe and 0.5 μM CK (top), 3 μM CK (middle), and 5 μM CK (bottom). Right: fluorescence image, left: simultaneous transmission image.

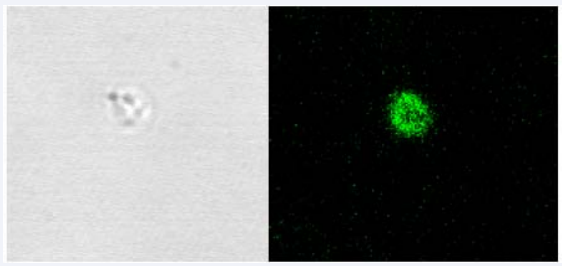

Figure 4 Amastigote incubated with 5 μM CK and nitric oxide specific probe observed by confocal fluorescence microscopy. Right: fluorescence image, left: simultaneous transmission image.

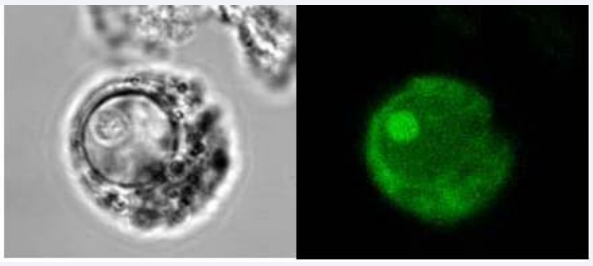

Figure 5 Promonocytes incubated with 5 μM CK and nitric oxide specific probe observed by confocal fluorescence microscopy. Right: fluorescence image, left: simultaneous transmission image.

parasites leads to inhibition of many of the cytokine-inducible responses including NO production by macrophages [19,20] and that such a process may be regulated by production of NO by the parasite. Thus, although *Leishmania* parasites appear to be negatively affected by exogenous NO, the NO they produce may in fact regulate the production of NO by macrophages [21]. We observed that promonocytes were also able to produce NO following addition of CK (Figure 5), which implies that CK may be used to compensate any NO which may be down regulated by the parasite.

The role of NOS in the processes by which *Leishmania* parasites protect themselves against NO remains unknown. It is speculated that NOS pathways could be important during the signaling that involves host cell and parasite interactions [10,22-24]. In a different protozoan, *T. cruzi* epimastigotes, NO has been shown to participate in stimulation of cell motility and down modulation of parasite apoptosis induced by fresh human serum [11].Thus induction of endogenous NO in *L. tarentolae* in response to CK may be an attempt by the parasite to reduce apoptosis and accounts for theobservation by Morgenthaler et al., that 100% decrease in cell viability was not detected at CK concentrations up to 10 μM [3].We have now demonstrated that carbaporphyrin ketals have more than one apparent mode of *in vitro* action since the presence of these compounds induces the production of both reactive nitrogen species and reactive oxygen species in *Leishmania tarentolae* axenic promastigotesin a dose dependent manner.

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