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Addendum

Dihydroxyacetone Induced Autophagy in African Trypanosomes

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Addendum to:

Antiproliferative Effect of Dihydroxyacetone on* Trypanosoma brucei *Bloodstream Forms: Cell Cycle Progression, Subcellular Alterations and Cell Death

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ABSTRACT

Dihydroxyacetone (DHA) was examined to explore its trypanocidal activity. The compound is easily taken up by trypanosomes via its aquaglyceroporins but is not converted to a glycolytic intermediate due to the lack of a respective kinase. Investigating the DHA-induced cell death it became evident that parasites die by autophagy rather than by necrosis or apoptosis. Since autophagy is not well studied in African trypanosomes our work offers a way to investigate the importance of autophagy for trypanosomes not only for stress coping but also for organelle reconstruction during differentiation.

AFRICAN TRYPANOSOMES AND AQUAGLYCEROPORINS

Trypanosomes are parasitic protozoa that are distributed worldwide. Of the more than 100 species known, only two are human infective: *Trypanosoma cruzi* in South and Latin America causing Chagas disease and the Brucei-group members *T. gambiense* and *T. rhodesiense* in sub-Saharan Africa causing sleeping sickness. Following a bite of an infected tsetse fly the latter parasites enter the blood and lymphatic system of the human host (Fig. 1) and-at a later stage-cross the blood brain barrier (BBB) and populate the cerebrospinal fluid. During the blood-lymphatic stage of the disease, symptoms are unspecific and variable, while the cerebral stage leads to severe behaviorial changes with a dysregulation of sleep-wake cycles and somnolence during daytime. Untreated, sleeping sickness is a fatal disease leading to death within months (*T. rhodesiense*) or years (*T. gambiense*). Existing drugs are limited to the blood-lymphatic stage (suramin) or to the chronic *T. gambiense* form (difluoromethylornithin), or they are toxic; for example, melarsoprol B crosses the BBB but this leads to severe side effects due to its arsenic component. In addition, drug resistance is an increasing problem, rendering successful medical treatment into often fatal relapses. New and effective strategies are therefore needed to fight the epidemic plague which threatens about 60 million people and leads to over 10 thousand casualties each year.[1]

We cloned and characterized the aquaporins (AQP) of *T. brucei* and showed that all 3 isoforms belong to the aquaglyceroporin sub-family, which transports (besides water) a variety of small solutes, including glycerol.[2,3] Interestingly, it turns out that TbAQPs have also a remarkably high capacity to transport dihydroxyacetone (DHA, Fig. 2). Since blood stage trypanosomes depend exclusively on glycolysis for ATP formation and belong to the small group of organisms where most of this metabolic pathway is located within a specific organelle, the so called glycosome,[4] we became interested in determining if secretion of DHA is a necessity for the parasite to cope with anaerobic conditions. To our surprise, however, DHA is readily taken up by the parasite and kills the cells in a dose-dependent manner (Fig. 3). On the other hand, DHA is used by many people as a food supplement for sunless tanning, and to increase endurance capacity, and is well tolerated even at high concentrations.[5,6] We were thus interested about the cell death mechanism in trypanosomes and the possibility that DHA may serve as a lead compound for drug development.

DHA INDUCED CYTOTOXICITY IN AFRICAN TRYPANOSOMES

Effects of DHA in trypanosomes have been investigated by several means: (1) metabolic conversion, (2) growth inhibition, (3) morphological changes and (4) determination of the induced cell death mechanism. Metabolic studies clearly revealed

that DHA is not converted to DHA phosphate due to the absence of a trypanosomal DHA kinase. Hence, while human cells will convert DHA to DHA phosphate, which is further converted via glycolysis, DHA accumulates in trypanosomes leading to cell death, as evidenced by growth inhibition using in vitro cultivation experiments (Fig. 3). Trypanosomes belong to the order of kinetoplastida, which are characterized by a kinetoplast, i.e., a highly catenated system of small DNA rings representing the mitochondrial genome. Since the parasite divides by binary fission, cell cycle progression can simply be analyzed by counting the number of cells containing one nucleus (N) and one kinetoplast (K), 2 N 1 K, 2 N 2 K and 1N 2K. The results revealed that cell cycle arrest increased in a dose dependent manner. In addition, fluorescence activated cell sorting analysis counting cells in G_1, S and G_2 phase showed a 70% increase of cells in G_2/M in the presence of DHA.[7] The morphology of treated cells has been analyzed by light and electron microscopy, which also supports the conclusion of a cell cycle arrest. Investigating the cell death mechanism revealed that an increase of necrosis or apoptosis markers was negligible, while induction of autophagy was clearly visible from transmission electron micrographs (Fig. 4).

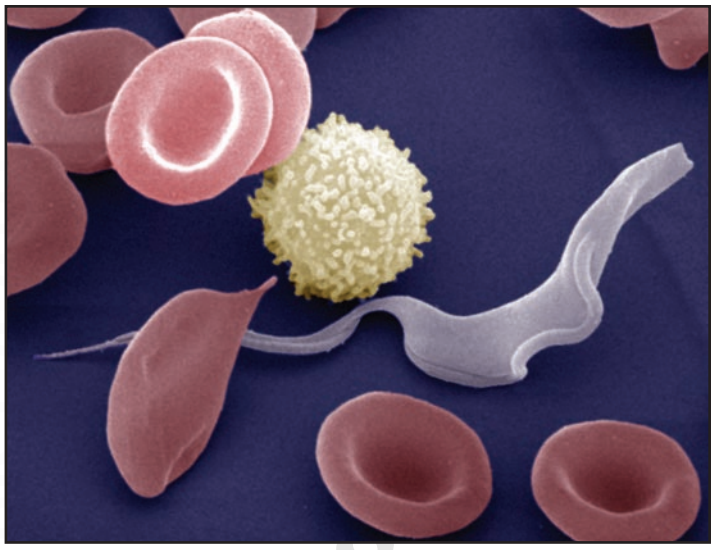

Figure 1. Scanning electron micrograph of *T. brucei* in blood between red blood cells and a single lymphocyte.

AUTOPHAGY IN TRYPANOSOMES

Autophagy plays a key role during the life span of every eukaryotic cell. On the one hand it is a self defense mechanism to cope with nutrient deprivation by degradation and reuse of dispensable cell components,[8] and on the other hand it allows the degradation of unwanted cell organelles during adjustment of the cell to different conditions.[9] In any case, a plethora of proteins and lipids are involved to provide the machinery for the controlled and organized self destruction.[10] Interestingly, trypanosomes lack about half of the proteins known to be engaged in autophagy in yeast and higher eukaryotes.[11-13] The question thus is whether trypanosomes need just a reduced form of autophagy because the nutrient supply is never limited in blood, or if their autophagy proteins comprise a rather simple machinery due to their evolutionary stage. Indeed, as judged from phylogenetic studies, kinetoplastida branched off the evolutionary tree very early and represent in many respects less advanced, although by no means primitive, organisms, well suited to study complex cellular responses and behavior in a more comprehensive way.[13] However, autophagy is not well studied in this parasite and nonambiguous hallmark features have yet to be clearly defined. At least a TOR homolog is easily detected in the trypanosome gene bank[11] and thus autophagy should be inducible using rapamycin. We have investigated necrosis and apoptosis in blood stage trypanosomes following prostaglandin treatment[14,15] and compared DHA induced cell death with the data obtained earlier. Using reasonable DHA concentrations in the range of the IC_{50}, i.e., below 5 mM, autophagy was the only cell death mechanism detected, as shown by electron microscopy in comparison with rapamycin-induced apoptosis (Fig. 4).

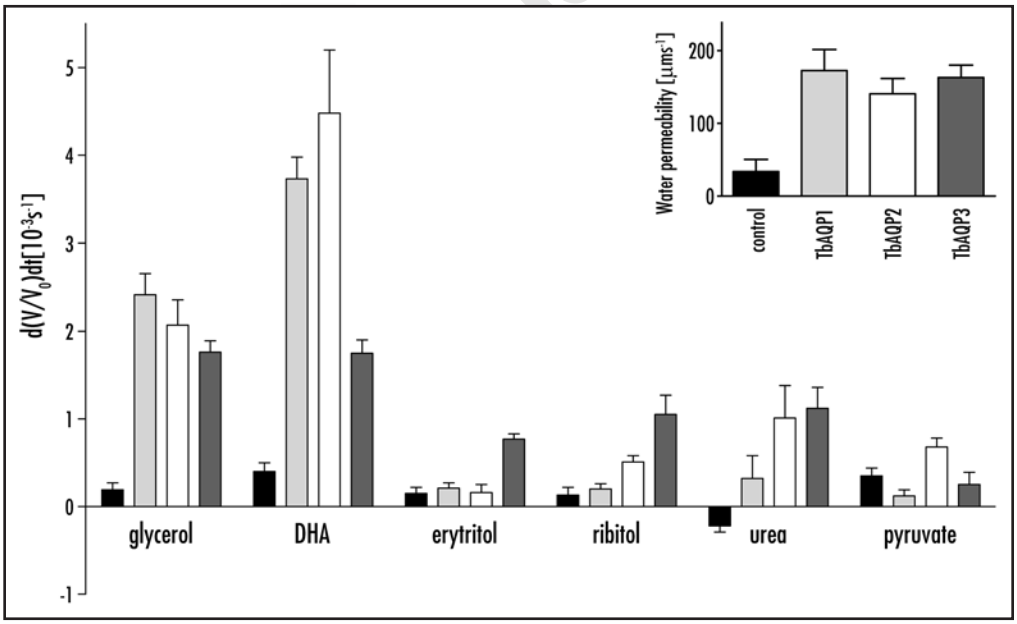

Figure 2. Transport rates for water and different solutes (glycerol, DHA, erythritol, ribitol, urea and pyruvate) by TbAQP1-3 heterologously expressed in *Xenopus* oocytes. Swelling assays were carried out at room temperature and were video-monitored. The relative oocyte volume was calculated from the covered area. Osmotic water permeability (P_f, µM/s) was calculated from the oocyte surface area (S = 0.045 cm^2), the initial slope of the relative volume increase ($d(V/V_0)/dt$ in s^{-1}), the molecular water volume (V_w = 18 cm^3/mol), and the osmotic gradient (osm_{in}-osm_{out}) by the following equation: $P_f = V_0 \times d(V/V0)/dt[S \times V_w \times (osm_{in}\text{-}osm_{out})]$. The initial swelling rates (d(V/V0)/dt in s^{-1}) were used to compare solute permeabilities. See reference 2 for more details. Note the high permeabilities of TbAQP1 and 2 for DHA as compared with glycerol.

During a blood meal, trypanosomes are taken up by the tsetse fly and develop into a procyclic insect form, which multiplies within the midgut of the insect. During differentiation, trypanosomes change their mitochondrion from a degenerated "pro" form with very few cristae and a fairly reduced enzyme content to a fully

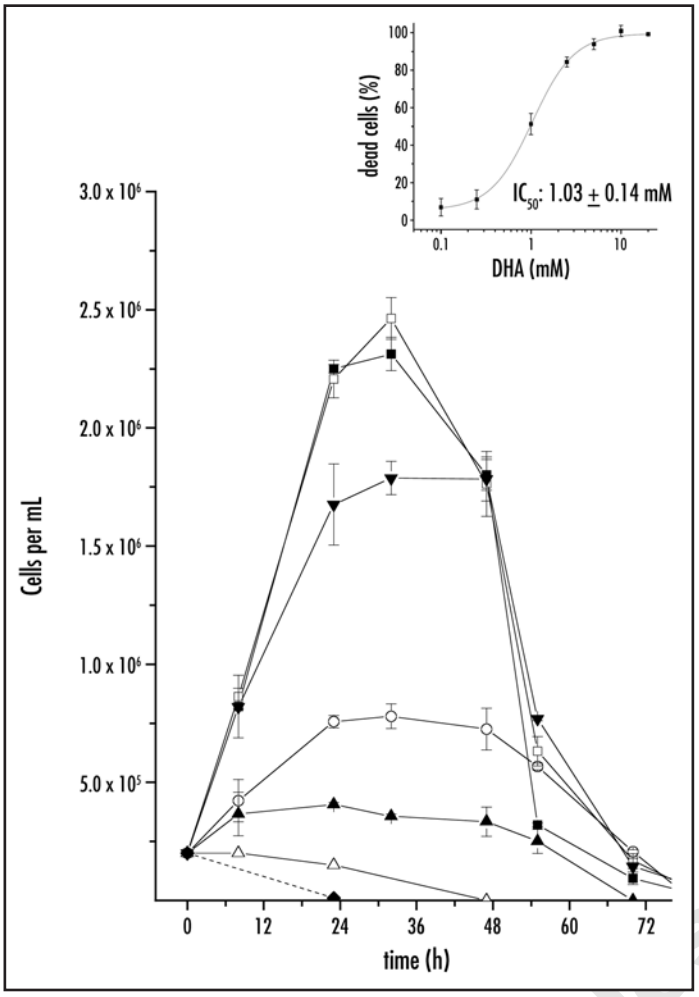

Figure 3. Growth inhibition of *T. brucei* by DHA and determination of the IC_{50} (inset). Blood stage trypanosomes were seeded at a cell density of 2 x 10^5 cells ml^{-1} and grown in the absence or presence of different DHA concentrations in axenic culture. (■) = control; (□) = 0.5 mM DHA; (▼) = 1 mM DHA; (●) = 2 mM DHA; (▲) = 3 mM DHA; (△) = 4 mM DHA; (◆) = 5 mM DHA.

developed organelle with an extensive increase of the inner membrane surface and containing, e.g., ATP synthases and all respiratory chain complexes.[16] In addition, glycosomes are rebuilt as well with a largely increased enzyme content.[17] It is most likely that in both cases autophagic processes are used to adjust the cells for survival in a completely changed environment. Our experiments showed that autophagy is induced in trypanosomes as a way to cope with stress conditions. More investigations are needed to define if autophagy is also involved in cell differentiation.

CONCLUSIONS

Considering the relatively low number of proteins involved in autophagy in trypanosomes it seems likely that this parasite could be used as a model organism to unravel the complex machinery involved in cell differentiation and stress response. Investigating these processes in more detail, the respective interactome in kinetoplastida might become accessible leading to a comprehensive and concise picture of autophagy at the beginning of evolutionary refinement. This knowledge may also offer a handle for medical control of the disease by interfering with vital and essential cellular processes.

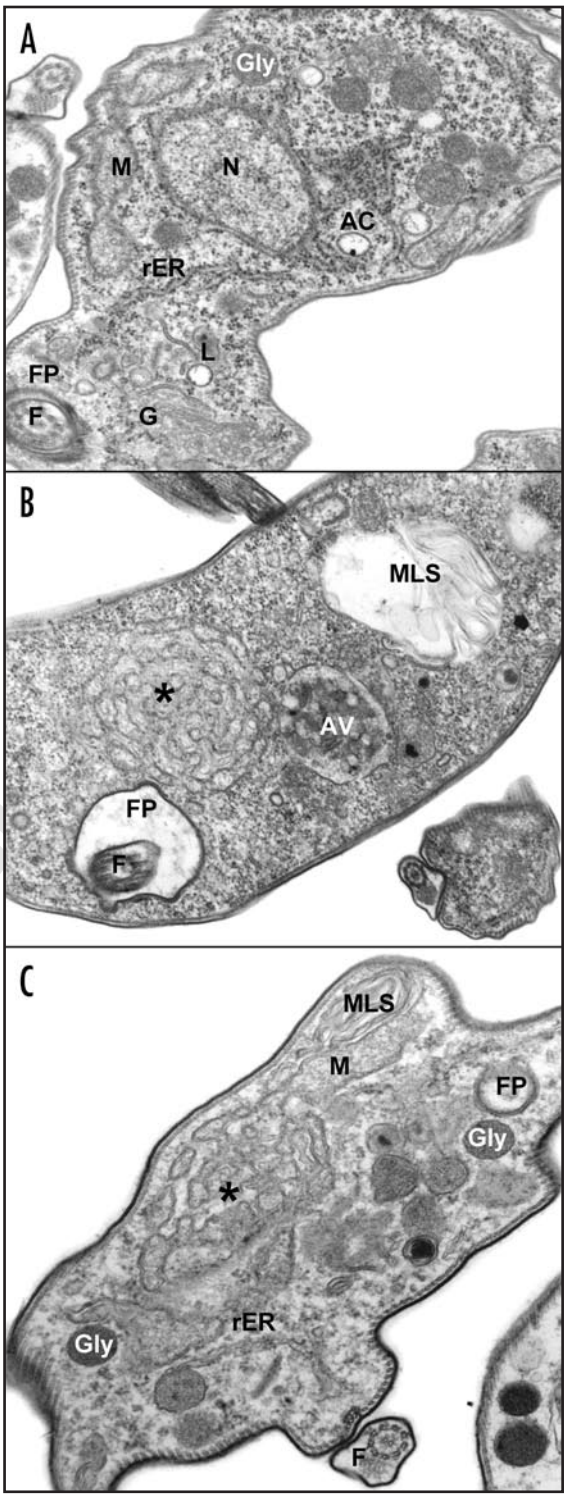

Figure 4. Electron micrographs of *Trypanosoma brucei*. (A) Untreated control cell. (B) Cells treated with 5 µM rapamycin. (C) Cells treated with 3 mM DHA. AC, acidocalcisome ; AV, autophagic vacuole; F, flagellum; FP, flagellar pocket ; G, Golgi apparatus; Gly, glycosome; L, lysosome; M, mitochondrion; MLS, myelin-like structure; N, nucleus; rER, rough endoplasmic reticulum; ◊, characteristic autophagic structure probably of mitochondrial origin.

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