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Prostaglandin D_2 induces programmed cell death in *Trypanosoma brucei* bloodstream form

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Abstract

African trypanosomes produce some prostanoids, especially PGD_2, PGE_2 and $PGF_{2\alpha}$ (Kubata *et al.* 2000, *J. Exp. Med.* 192: 1327–1338), probably to interfere with the host's physiological response. However, addition of prostaglandin D_2 (but not PGE_2 or $PGF_{2\alpha}$) to cultured bloodstream form trypanosomes led also to a significant inhibition of cell growth. Based on morphological alterations and specific staining methods using vital dyes, necrosis and autophagy were excluded. Here, we report that in bloodstream form trypanosomes PGD_2 induces an apoptosis-like programmed cell death, which includes maintenance of plasma membrane integrity, phosphatidylserine exposure, loss of mitochondrial membrane potential, nuclear chromatin condensation and DNA degradation. The use of caspase inhibitors cannot prevent the cell death, indicating that the process is caspase-independent. Based on these results, we suggest that PGD_2-induced programmed cell death is part of the population density regulation as observed in infected animals.
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Abbreviations: dUTP, desoxyuridinetriphosphate; $\Delta\Psi_m$, mitochondrial membrane potential; FACS, fluorescence-activated cell sorter; PCD, programmed cell death; PG, prostaglandin; SEM, scanning electron microscopy; TEM, transmission electron microscopy; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling

Introduction

African trypanosomes cause sleeping sickness in humans and severe epidemics in livestock. Owing to antigenic variation, the course of infection is characterized by parasitic waves, which peak every 8 to 10 days; untreated, the infection is lethal. Although the decline of parasite density is attributed to the appearance of specific antibodies,[1,2] immunosuppressed animals also show a regular increase and decrease in parasite numbers,[3–5] and this behavior is even observed *in vitro*, if culture media are replaced at regular intervals.[6] Thus, beside the control of parasitemia by the immune system of the host, cell density is also regulated by the parasite itself.[7–9]

Following up reports about an increase of prostaglandin (PG) levels in the serum and cerebrospinal fluid of sleeping sickness patients,[10] we have shown that trypanosomes produce PGD_2, PGE_2 and $PGF_{2\alpha}$ from arachidonic acid.[11,12] These PGs led to a broad variety of different physiological effects in higher eukaryotes[13] and their accumulation in serum coincides remarkably with symptoms observed during trypanosomiasis, such as fever, pain, immunosuppression, dysregulation of sleep/wake cycles and others. So far, it is not clear why protozoa produce PGs, but it is tempting to speculate that these parasites may have adopted the formation of PGs to modify host reactions for their own benefit. In addition, we also found that $PGF_{2\alpha}$ was mainly produced in fast dividing forms of the parasite such as the slender bloodstream form and the procyclic insect form and was scarcely secreted into the media, while PGD_2 was mainly produced by the nondividing stumpy bloodstream form and was primarily secreted.[11]

Here we report the effect of PGD_2 on cellular growth of *Trypanosoma brucei* bloodstream form under *in vitro* culture conditions. As judged from our results, PGD_2 (but not PGE_2 and $PGF_{2\alpha}$) induces a programmed cell death (PCD) with characteristic features of apoptosis. Interestingly, apoptosis was also induced by PGs in higher eukaryotes.[14–17]

Since protozoa do not contain caspases,[18] the classical apoptosis mechanism cannot work in single-cell organisms. However, published data indicate that apoptosis can occur in the complete absence of caspases.[19–22] Additionally, a considerable number of investigations have been reported, showing that at least some of the typical PCD features like DNA fragmentation, autophagy, phosphatidylserine exposure, decrease of mitochondrial membrane potential, etc can be observed in protozoa like *Leishmania*,[23,24] *T. cruzi*,[25] *T. brucei*,[26,27] *Tetrahymena*,[28,29] *Blastocystis*,[30] *Dictyostelium*,[19,31,32] yeast[33] and even in bacteria.[34] We propose that the induction of cell death by PGD_2 in bloodstream form trypanosomes could be involved in cell density regulation.

Results

Growth inhibition induced by PGD_2

Trypanosomes produce PGs from arachidonic acid, which are released into the culture media.[11] In addition to effects on the host, like immune suppression or sleep induction, we questioned if PGs may also have an influence on the growth characteristics of trypanosomes. Indeed, addition of PGD_2 to trypanosomes in culture led to a dose-dependent decrease of

cell growth (Figure 1a). The IC_{50}, that is, the concentration necessary to inhibit cell growth by 50% was 3.7 μM for PGD_2. Interestingly, the IC_{50} for PGE_2 was 159 μM and for $PGF_{2\alpha}$ 199 μM. The PGD_2 inhibition was confirmed by a proliferation assay measuring the incorporation of [^{3}H]-thymidine into newly formed DNA (Figure 1b). PGD_2 and PGE_2 are very closely related isomers, which differ only by the inverse position of the keto- and the hydroxyl-group on carbon atoms 9 and 11, respectively (Figure 1c). Since the IC_{50} of PGE_2 was 43-fold higher as compared with PGD_2, our results clearly indicate that the inhibitory effect of PGD_2 on cell growth of *T. brucei* bloodstream forms is specific and not due to the mere hydrophobic structure of the compound.

Morphological changes of trypanosomes induced by PGD_2

In order to detect morphological changes due to PGD_2 treatment, trypanosomes were grown for 24 h in the presence or absence of 5 μM PGD_2, monitored by light microscopy and prepared for scanning (SEM) and transmission (TEM) electron microscopy. Using light microscopy, we detected that the amount of cells containing only one nucleus and expressing more than one flagellum was drastically elevated (Figure 2a and b). This observation of unusual dividing forms was clearly confirmed by SEM images, which also revealed a high number (up to 80%) of trypanosomes with at least two flagella (Figure 2d–f), and by TEM images (Figure 3h). Interestingly, here we also observed the appearance of two flagella emerging from the same flagella pocket, indicating an abnormal cell division (Figure 3f and g). In addition to these observations, condensation of chromatin in distinct areas of the nucleus, a characteristic feature of apoptosis in higher eukaryotes, was often detected (Figure 3b–d). Finally, we observed dilated mitochondria (readily recognized by their two membranes; Figure 3k, m and n) and an increase of vacuole-like structures (Figure 3b, d, f and k).

Search for necrosis

Necrosis was excluded using propidium iodide, which stains only necrotic cells due to distortion of the plasma membrane integrity. Accordingly, 95% of digitonin-treated cells showed a clear staining after an incubation time of 5 min as judged by flow cytometric analysis. In contrast, only 2.7% of trypanosomes treated for 24 h with PGD_2 and 2.4% of control cells were stained. Even after an incubation time of 32 h, staining of treated cells remained below 5% (see Figure 6a and b, necrosis).

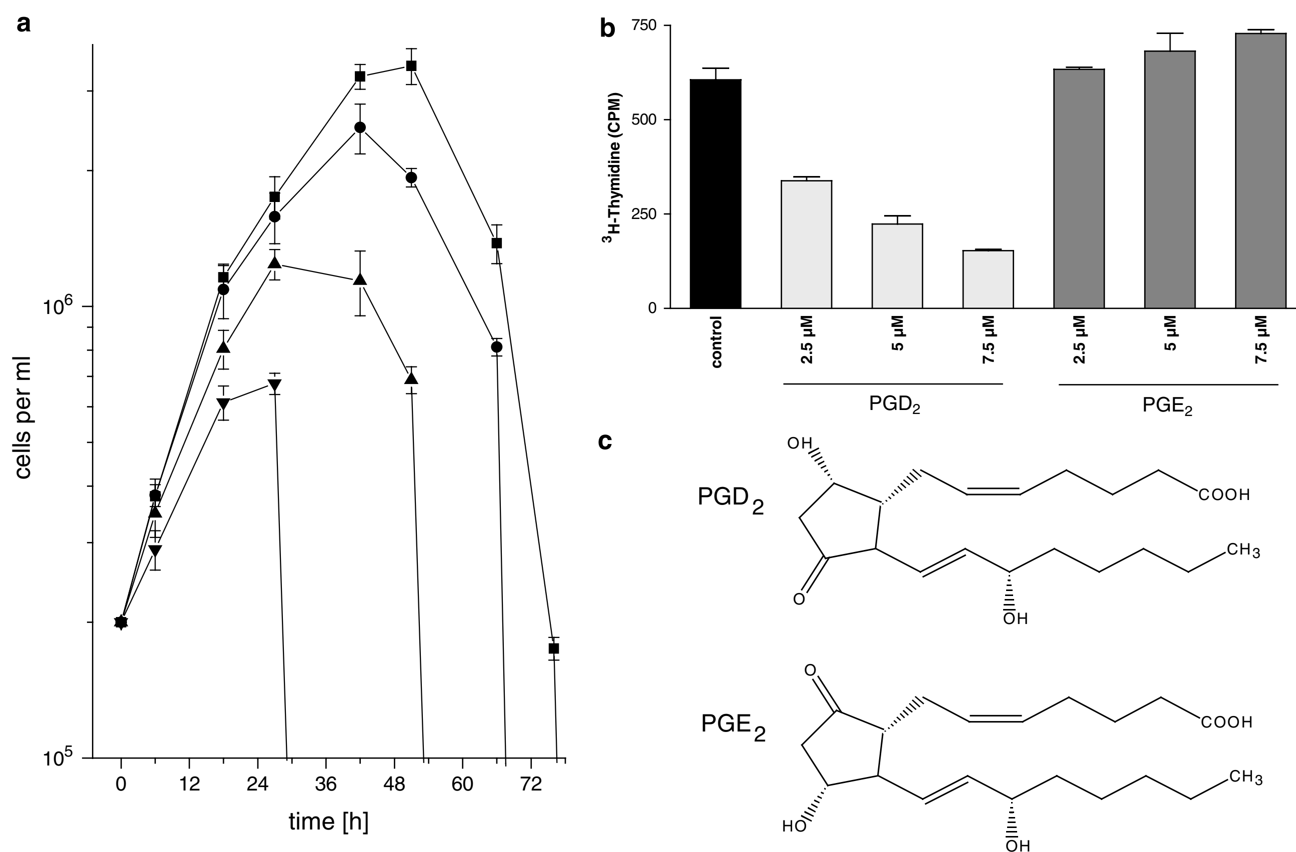


Figure 1 (**a**) Growth curve of *T. brucei* bloodstream forms in axenic culture in the presence or absence of different PGD_2 concentrations. Control cells (■); 2.5 μM PGD_2 (●); 5 μM PGD_2 (▲); 7.5 μM PGD_2 (▼). (**b**) [^{3}H]-thymidine incorporation after 24 h of control cells and treated cells with different concentrations of PGD_2 and PGE_2. (**c**) PGD_2 and PGE_2 structures

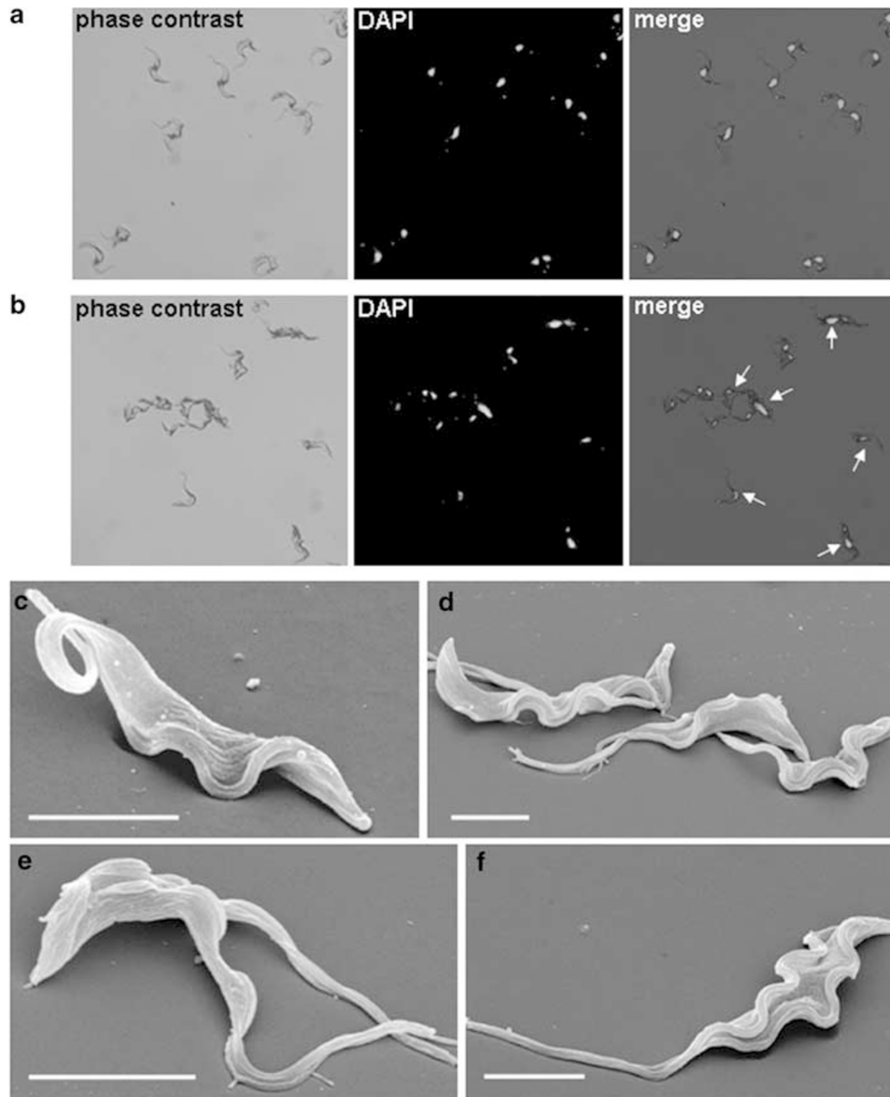


Figure 2 DAPI staining images of control cells (**a**) and PGD_2-treated cells (**b**); arrows show cells containing two or more flagella and only one nucleus. SEM of control (**c**) and PGD_2-treated (**d**, **e**, **f**) trypanosomes. An unusual amount of dividing stages of trypanosomes after 24 h was observed. Bars in S.E.M. represent 5 μM each

Search for autophagy

Morphological alterations following PGD_2 treatment of trypanosomes, especially the increase of vacuole-like structures, prompted us to consider autophagic mechanisms for the observed cell death. Monodansylcadaverine is especially taken up by autophagic cells and stains autophagosome structures.[35] However, a staining difference between control cells and PGD_2-treated cells could not be observed within 36 h. Adenosine, 3-methyladenine and wortmannin have been described as autophagy inhibitors.[36–39] However, as shown in Figure 4a, 3-methyladenine and wortmannin were not able to revert the PGD_2 effects at concentrations up to 100 μM or 50 nM, respectively. Adenosine (50 μM) had a minor effect on PGD_2-treated trypanosomes, as it prolonged the survival time for about 6 h (Figure 4b). Higher adenosine concentrations had themselves an inhibitory effect on cell growth *per se*, while lower concentrations did not change the growth characteristics of PGD_2-treated or nontreated parasites. To further check the observed effects of adenosine, deoxycoformycin (Nipent), a drug well known to enhance the adenosine effect on autophagic cells,[38] was administered together with adenosine, but did not revert the observed cell death (Figure 4b).

Search for apoptosis

In order to analyze PGD_2-induced cell death in *T. brucei*, we used terminal deoxynucleotidyl transferase-mediated desoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) test and fluorescence-activated cell sorter (FACS) analysis, which are well-established methods to investigate apoptosis in higher eukaryotes.

TUNEL staining

This method is applied to detect DNA double-strand ruptures. Cells taken from different growth phases were fixed with

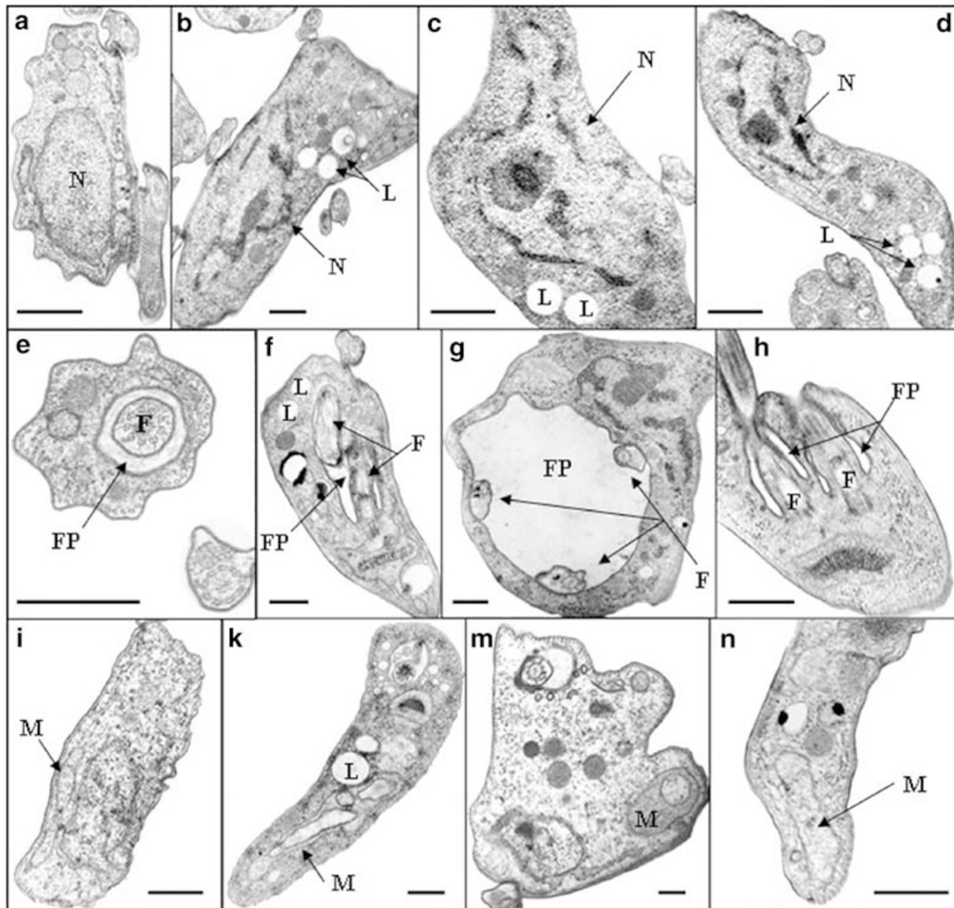


Figure 3 TEM of Epon-embedded cells: the most obvious alterations in PGD_2-treated cells are (1) segmented nuclei (**b**, **c**) and structures of condensed chromatin near the nuclear envelope (**b**–**d**, **g**); (2) appearance of two or more flagella in the same flagella pocket (**f**, **g**) or two flagella pockets in the same cell (**h**); (3) dilated mitochondria (**k**–**n**); and (4) an elevated amount of lysosomes (**b**, **d**, **f**, **k**) as compared with control cells. Note appearance of nuclei, flagella pockets and mitochondria in control cells (**a**, **e**, **i**, respectively). Bars in TEM represent 0.5 μM each. Lysosome (L); nucleus (N); flagellum (F); flagella pocket (FP); and mitochondrion (M)

formaldehyde and placed on immunoslides (Biomerieux, Nürtingen). Free DNA ends were labeled using modified dUTP, and the ratio of labeled and unlabeled nuclei was visually monitored. No staining differences were observed between treated (5 μM PGD_2) and control cells after an incubation time of up to 12 h; only a background staining of about 1% was detected. In contrast, after an incubation time of 24 h in culture about 50% of treated cells showed positive nuclear staining, while only 3% of control cells were labeled (Figure 5a and b). The addition of $PGF_{2\alpha}$ or PGE_2 (10 μM each) gave no positive staining if compared with control cells (data not shown).

FACS analysis

In order to determine the DNA content, treated (5 μM PGD_2) and control cells were lysed hypotonically in 10 mM phosphate buffer containing 6 μM digitonin. DNA within the nuclei was stained using propidium iodide, before the nuclei were sorted according to size and fluorescence labeling using a FACScalibur® cell sorter (BD Biosciences). The respective distribution chart clearly discriminated between nuclei of the G1, S or G2 cell cycle stage and nuclei with a reduced DNA content due to DNA degradation processes. The latter fraction represents an established apoptosis marker in higher eukaryotic cells.[40–42] As shown in Figure 6a, there was virtually no difference between control and treated cells for up to 20 h, while after 24 h most of the nuclei of treated cells showed a shift towards the area of degraded DNA. Quantification of nuclei in the area of DNA degradation, as compared with the number of total nuclei, revealed that the background of cells with a low DNA content was 4.6 ± 0.9%, while in PGD_2-treated cells, the ratio increased to 13.4 ± 2.0 and 57.6 ± 1.5% after 24 and 32 h of cell growth, respectively (Figure 6b, DNA degradation). Nuclei of cells grown in the presence of 10 μM $PGF_{2\alpha}$ or PGE_2 showed the same behavior as control cells with a regular distribution between G1, S and G2 phase. Interestingly, quantification of nuclei in the areas of the different cell cycle stages indicated an increase of about 15% of cells in the G1 phase, which was paralleled by a 10% decrease from the G2 and a 5% decrease from the S phase in PGD_2-treated cells after 24 h (Figure 6c). These differences increased further with time, leading to an increase of about 30% of cells in the G1 phase and a corresponding

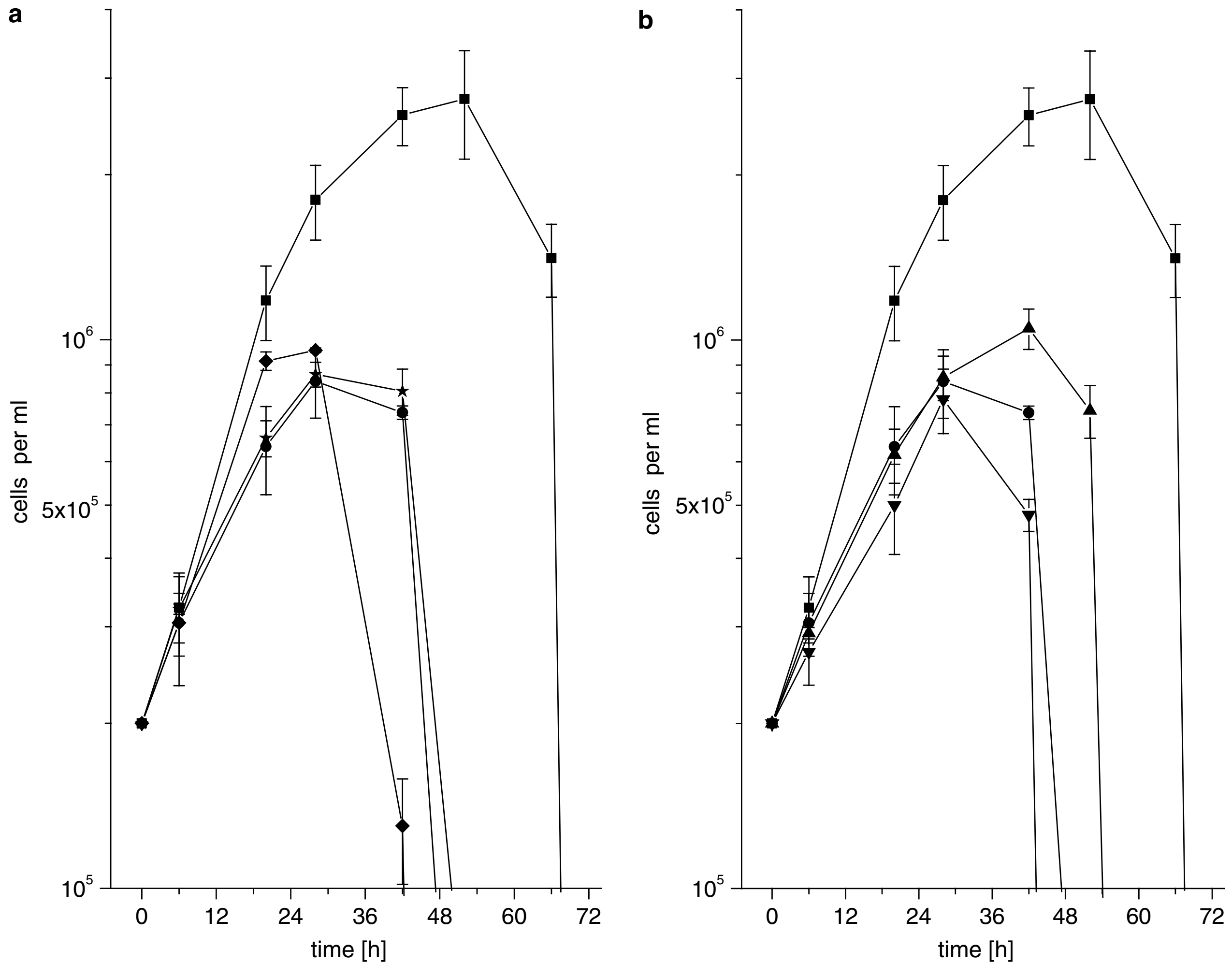


Figure 4 Treatment of cells with PGD_2 and autophagy inhibitors at the same time. (**a**) 3-Methyladenine (100 μM; ◆) and wortmannin (50 nM; ★) did not revert PGD_2 effects. (**b**) Adenosine (50 μM; ▲) prolonged the survival time for up to 6 h which, however, did not increase after the addition of 100 μM deoxycoformycin (▼). Control cells (■); PGD_2-treated cells (●)

decrease by 20% from the G2 and by 10% from the S phase after 32 h (Figure 6c). In addition, cell death induced by PGD_2 was associated with a loss of mitochondrial membrane potential (Ψ_m) as monitored by tetramethylrhodamine uptake (Figure 6a, Ψ_m). After 32 h, 41.8±3.4% of PGD_2-treated cells showed a low Ψ_m compared with only 8.1±2.6% of control cells (Figure 6b, Ψ_m). Control experiments using valinomycin (100 nM) abolished tetramethylrhodamine uptake completely, demonstrating that uptake of this dye is strictly driven by Ψ_m.

Use of caspase inhibitors

In addition to the above-mentioned experiments, we have used the apoptosis inhibitors zVAD and DEVD-CHO each one alone and together with PGD_2, to see if they can stop PCD induction. As shown in Figure 7, both drugs led to a strong growth inhibition of trypanosomes at the maximum concentrations used, but had no visible effects on growth inhibition induced by PGD_2 if administered in sublethal doses. These data suggest that the PCD process induced by PGD_2 treatment is caspase independent.

Inhibition of PGD_2 effect by cycloheximide

To further explore the mechanism of PGD_2-induced cell death, the translation inhibitor cycloheximide was used. Thus, cells from stationary phase were preincubated for 1 h at 37°C with 10 μg/ml cycloheximide. Thereafter, prostaglandin D_2 was added and cell density was monitored during the next 6 h. As shown in Figure 8a, PGD_2 alone induced cell death, which was interestingly abolished by cycloheximide. Additionally, phosphatidyl serine exposure was assayed. A significant difference ($P<0.05$) was obtained between control cells and PGD_2-treated cells after a 6 h incubation, which was clearly blocked by cycloheximide (Figure 8b). These results suggest that the mode of action of PGD_2 requires *de novo* protein synthesis.

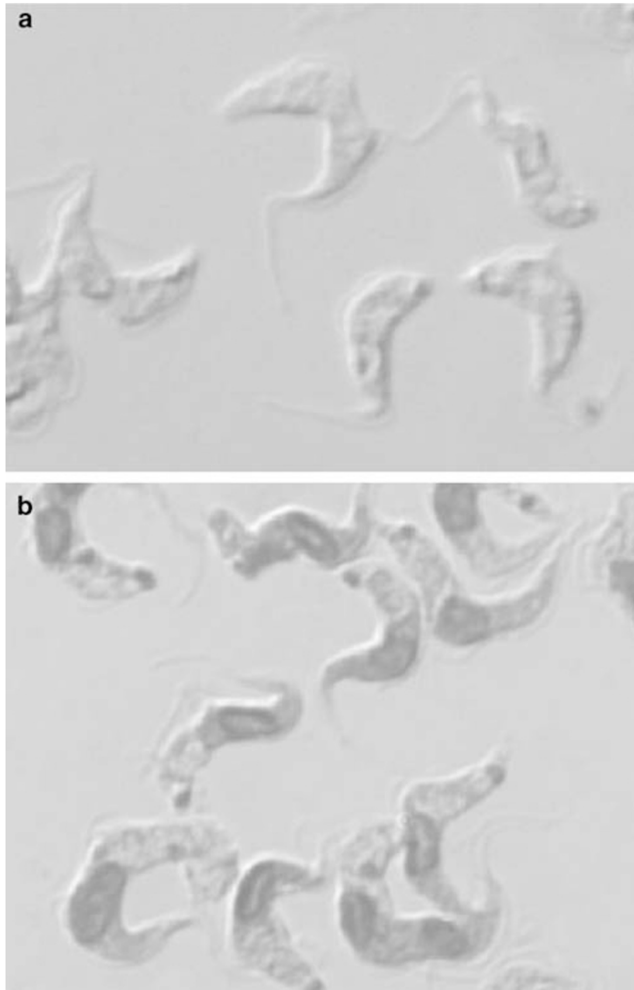


Figure 5 Staining of DNA double-strand nicks using the TUNEL assay. Control cells (**a**), PGD_2-treated cells (**b**). Control cells did not exceed a level of 10%, whereas stained nuclei increased in PGD_2-treated cells to about 50%

Discussion

Trypanosomes produce and release a set of PGs,[11] which certainly influences the host–parasite relationship. Here, we questioned if these cytokines by means of an autocrine or a paracrine regulation have an influence on the parasite's growth. Addition of PGE_2 or $PGF_{2\alpha}$ had no visible effects on trypanosomes, that is, cells grew normally and showed no differences in their growth characteristics or morphology as compared with control cells. In contrast, addition of PGD_2 showed clear inhibitory effects on growing trypanosomes. The IC_{50} values revealed that PGD_2 is about 50-fold more potent than PGE_2 or $PGF_{2\alpha}$. These results clearly confirm that cell growth inhibition induced by PGD_2 is specific.

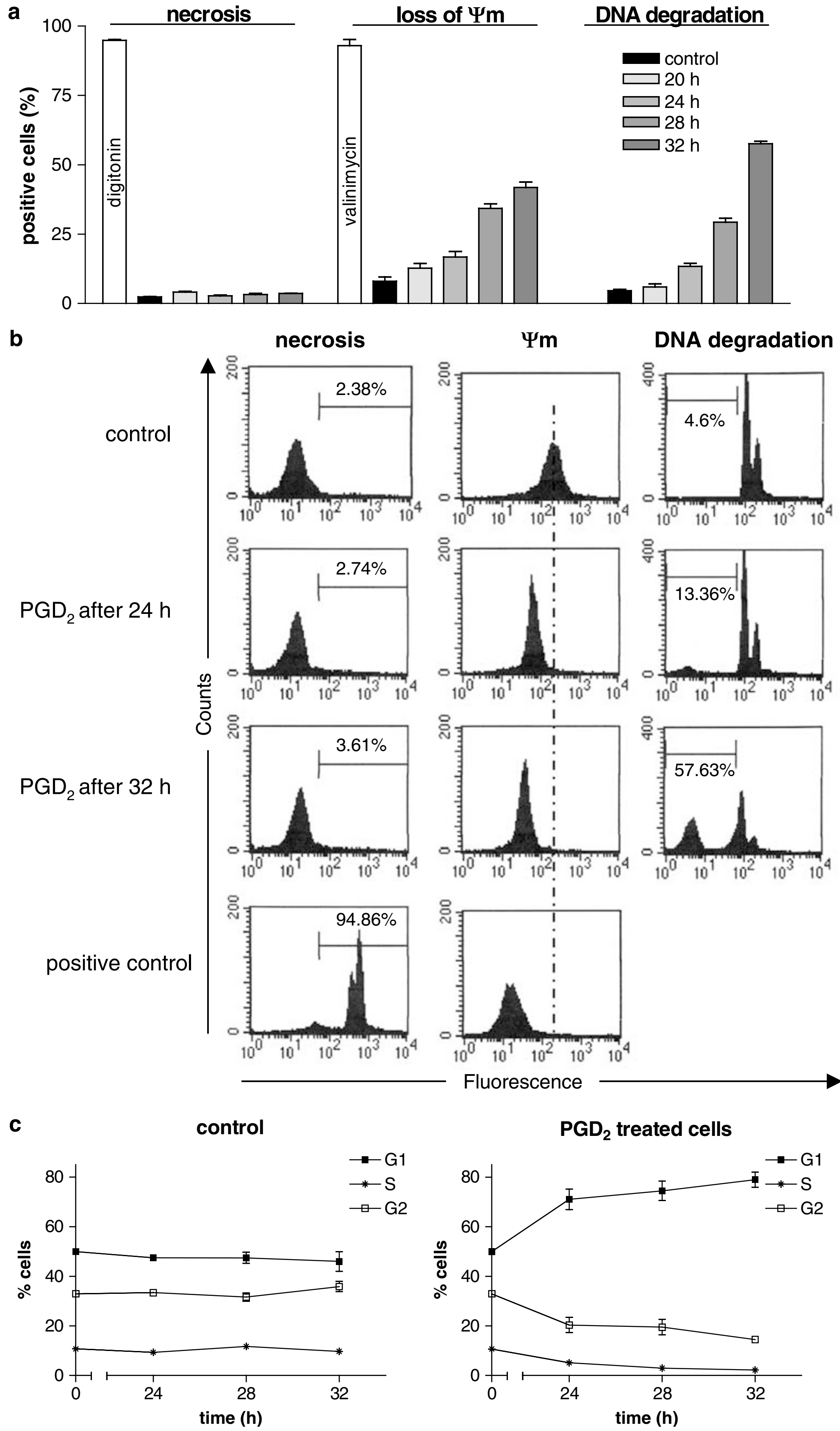
Growth inhibition occurred within 8 to 12 h in logarithmically growing cells and within 2 h in stationary phase cells following PGD_2 treatment. In addition, cells pretreated with 8-(4-chlorophenylthio)-cAMP, a membrane-permeable cAMP analog described to induce differentiation from slender to stumpy form,[43] were more sensitive to PGD_2 than non-pretreated cells (data not shown). These results suggest that PGD_2 works primarily on stumpy forms.

The PGD_2 concentration required to inhibit cell growth of *T. brucei in vitro* was in the micromolar range ($IC_{50} = 3.7\,\mu M$), and thus similar to or lower than concentrations needed in comparable studies using different eukaryotic cells.[15,17,44–46] However, the active concentration of free PGs will be considerably lower, since PGs readily bind to serum albumin.[47–50] Using our conditions, 80% of PGD_2 added to the culture media was bound to serum albumin within 1 h after drug addition. Furthermore, due to the presence of albumin, PGD_2 is readily metabolized to different PGD_2 derivatives.[51] Thus, the effective PGD_2 concentration is in the nanomolar range, which is comparable to physiologically relevant concentrations. We are currently investigating the metabolite production from PGD_2 under our experimental conditions and their possible effects on *T. brucei*.

As judged by FACS analysis, PGD_2 treatment led to a cell cycle arrest in the G1 phase. In addition, observation of cells by light microscopy, SEM and TEM indicated an increased number of cells in a dividing state after 24 h treatment with PGD_2. In this case, most cells contained more than one flagellum but usually only one nucleus, as confirmed by 4,6-diamidino-2-phenylindole (DAPI) staining. Thus, PGD_2 treatment led to an arrest during cell division because formation of new flagella occurred but division of the nucleus could not proceed. These results suggest that PGD_2 is involved in cell cycle regulation of *T. brucei*, as it was also reported for several cell types from other organisms.[52–54]

Since we found no indication for necrosis, we assumed a PCD due to PGD_2 treatment. PCD is accurately defined as an active process that is dependent on signaling events in the dying cell and requires gene expression.[20,55] Treatment with cycloheximide blocked the PGD_2 effect, indicating that cell death induced by PGD_2 is a result of an active cellular process, which can be stopped by protein synthesis inhibition. PCD may appear as caspase-dependent or -independent apoptosis, as autophagy, as PCD type III[56] or as paraptosis.[55] To discriminate between these types, we applied a set of well-known criteria. Although autophagy was suggested by the observation of clearly increased lysosomal structures, we did not observe autophagosomes using monodansylcadaverine and no reversion of autophagy by 3-methyladenine, wortmannin, adenosine or adenosine plus nipent, respectively.

Figure 6 (**a**) FACS analysis of bloodstream forms stained with propidium iodide or tetramethylrhodamine. Treated (5 μM PGD_2) or control cells were harvested at different time points and prepared for analysis of membrane permeability, DNA content and mitochondrial membrane potential. The values for control cells did not change during the total incubation time of 32 h. The bars in this case represent the mean ± S.D. of three individual cultures measured at each time point. (**b**) Originals histograms of the above results after 24 and 32 h, respectively. The positive controls were performed with digitonin and valinomycin for necrosis and mitochondrial membrane potential, respectively. The vertical dot line represents the mean value from control cells. (**c**) Percentages of cells in G1, S, and G2 phase at 0, 24, 28 and 32 h. The percentages were determined using the CellQuest® software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positive cells (%)
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2.38%
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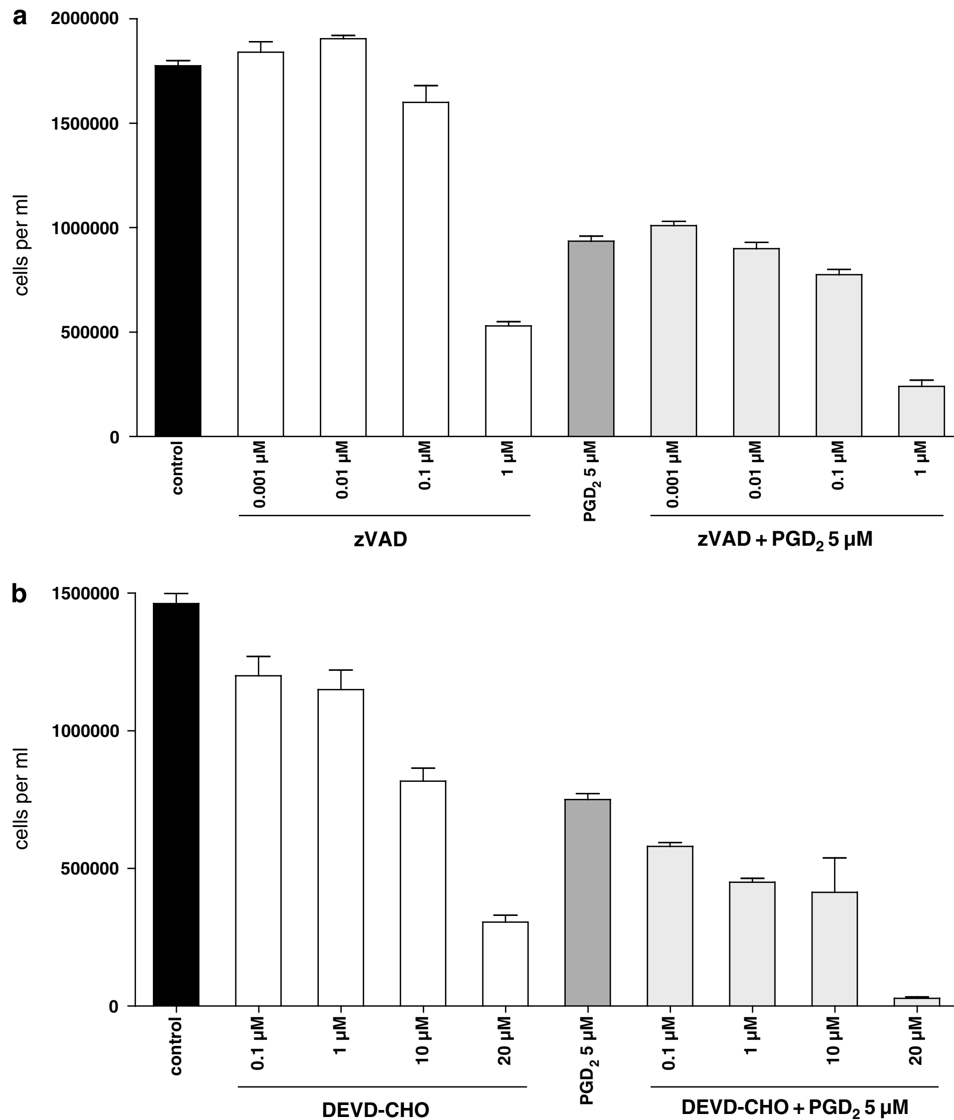

Figure 7 Treatment of cells with caspase inhibitors. Trypanosomes were cultivated in the presence of different concentrations of zVAD (**a**) or DEVD-CHO (**b**) either alone or together with 5 μM PGD_2. After an incubation time of 24 h, cell density was monitored using a hemocytometer. Control cells were cultivated in the presence of solvent alone (0.5% both DMSO and ethanol, final concentration)

Based on these results, there is no evidence for induction of autophagy by PGD_2 treatment. PCD type III is characterized by disintegration of cells into fragments without involvement of lysosomes, which was not observed in our case either. Owing to the lack of caspases in *T. brucei*, a caspase-dependent apoptosis or paraptosis were excluded. Interestingly, we discovered five metacaspase genes in trypanosomes, which have all been cloned and heterologously expressed in yeast. One of the gene products led to a petite phenotype and induced clonal cell death in *Saccharomyces cerevisiae.*[57] However, as judged from our recent results, metacaspases in trypanosomes may be involved in modulation of the dynamics of membrane-associated proteins, but obviously do not participate in PCD.[58]

Our findings indicate that PGD_2-treated cells can undergo cell death that shares essential characteristics with apoptosis in higher eukaryotes and other protozoa.[23,28,30,59] PGD_2 treatment induced nuclear segmentation, chromatin condensation and DNA degradation, as observed by TEM, FACS and TUNEL test. In addition, we observed a dilation of the mitochondrion in TEM, and a decrease of the mitochondrial membrane potential using FACS analysis as well as phosphatidylserine exposure. However, due to the lack of caspases and the failure of the cysteine protease inhibitors DEVD-CHO and zVAD to inhibit PCD, we consider the PCD induced by PGD_2 in *T. brucei* to be caspase independent. This cell death type has also been reported in *Dictyostelium*[19] T-lymphocytes[20] and others (as reviewed in Lorenzo and Susin[21]). It is interesting to note that caspase inhibitors do

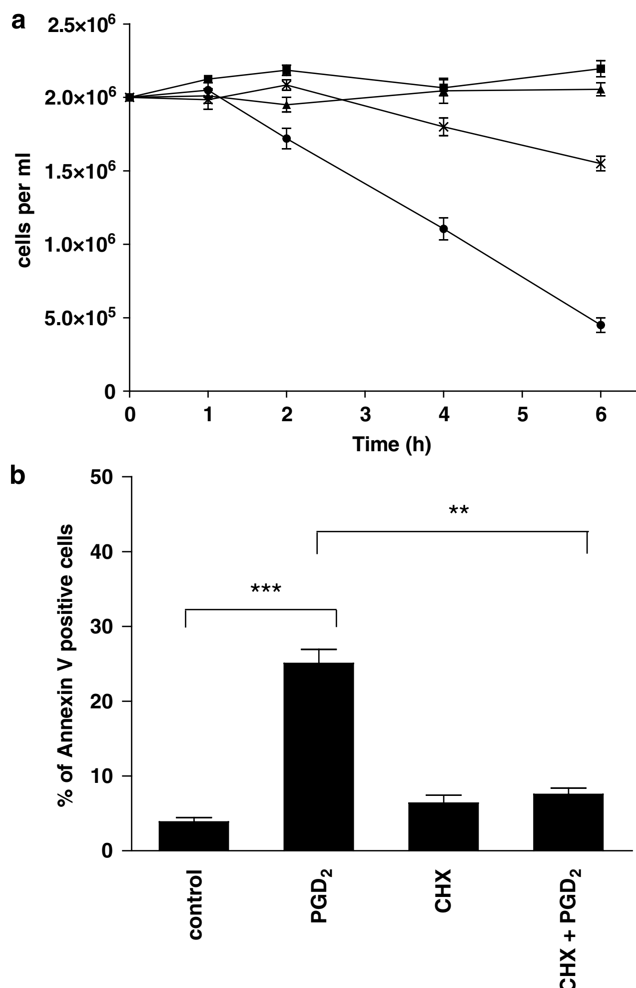

Figure 8 Protein synthesis is required for PGD_2-induced cell death. Cells from the stationary phase were incubated for 1 h at 37°C with or without 10 μg/ml cycloheximide (CHX) and then treated with PGD_2. (**a**) Using control conditions, cell density remained stable during the incubation time of 6 h (■). In the presence of 5 μM, PGD_2 cell death occurred after 2 h (●). Addition of 10 μg/ml cycloheximide 1 h prior to PGD_2 treatment abolished cell death induced by the prostaglandin (**X**). Cycloheximide alone (▲) had no effect on cell growth during the incubation time used. (**b**) Phosphatidyl serine exposure measured by double staining with annexin V-FLUOS and propidium iodide was also inhibited by cycloheximide. The bars represent the S.D. of three independent experiments. The asterisks indicate significant differences calculated by student's *t*-test using the GraphPad Software (***$P<0.001$; **$P<0.01$)

not prevent apoptosis in most mammalian models either, and that the morphology of dying cells in these cases resembled that of unicellular eukaryotes, which lack caspases.[60]

Our findings suggest that PGD_2 secretion by trypanosomes is involved in cell cycle regulation and provide evidence for the existence of a caspase-independent PCD.

Bloodstream forms of African trypanosomes differentiate from dividing slender to nondividing stumpy forms, with the latter preadapted for fly transfection. If stumpy forms are not taken up by a tsetse fly during a blood meal, these cells survive for only 2 to 3 days,[61] especially the stumpy forms produce PGD_2,[11] which – as we describe here – will lead to PCD. Taken these data together, we propose a two-step mechanism to control cell density in infected animals: (1) slender forms produce a so far unknown differentiation factor,[6,62] which, depending on cell density, induces differentiation to stumpy forms.[9,63] (2) Stumpy forms produce PGD_2, which induces cell death primarily of stumpy form cells. Since PGs have a short half-life and thus work as local mediators only, this model provides a mechanism for a sustained infection, because it would certify that cell density is locally regulated, leading to a controlled parasite density throughout the host. On the other hand, a sustained infection would never be in question, because dividing slender forms would not be affected by PCD. The appearance of specific antibodies would thus provide an additional mechanism to keep the parasitemia low. It should be kept in mind, however, that cell density regulation also occurs in cell culture in the absence of antibodies.[6]

Materials and Methods

Chemicals

PGs of the highest analytical grade were obtained either from Sigma Chemicals (Deisenhofen, Germany) or from Cayman Chemicals (Ann Arbor, MI, USA). Methyl-[^{3}H]-thymidine was purchased from Hartmann Analytic (Braunschweig, Germany). All other chemicals used were of the highest analytical reagent grade and obtained from Sigma Chemicals (Deisenhofen, Germany). PGs have been used as a 4 mM stock solution in ethanol and diluted to the respective concentration using media.

Parasites

T. brucei MITat 1.2 (VSG-variant 221) of the monomorphic strain EATRO 427 were used throughout the experiments. Bloodstream forms were grown in axenic culture at 37°C and 5% CO_2 as described earlier.[6,62] For the experiments described here, parasites were taken from frozen stabilates, seeded at a cell density of 2×10^5 cells/ml and grown for about 18 h until a cell density of 8×10^5 cell/ml was reached. By adding fresh media, cells were adjusted to 2×10^5 trypanosomes/ml and split into the desired number of individual culture flasks. At the same time, PGs or ethanol were added at the denoted concentrations and cells were grown at 37°C in a CO_2 incubator. All experiments were performed at least in triplicate.

Proliferation assay

The cellular proliferation was determined by measuring thymidine incorporation. Cells were adjusted to 2×10^5 trypanosomes/ml and seeded in 200 μl aliquots into 96-well microtiter plates. After an 24 h incubation at 37°C and 5% CO_2, cells were pulsed for 4 h with 1 μCi/well of methyl-[^{3}H]-thymidine before they were harvested and counted in a liquid scintillation counter. Incubation of media alone was used to estimate the radioactivity background. Results are represented as counts per minute (c.p.m.).

Cytotoxicity assay

The IC_{50} values were determined according to Bodley *et al.*[64] Exponentially growing parasites were diluted to 2×10^5 cells/ml, placed in a 96-well microtiter plate (199 μl) and grown with or without PGs (1 μl/well) at different concentrations. Plates were incubated for 24 h at 37°C

and 5% CO_2. Cell growth was stopped by the addition of 20 μl lysis buffer containing *p*-nitrophenyl phosphate (20 mg/ml in 1 M sodium acetate, pH 5.5 with, 1% Triton X-100). Finally, following a 6 h incubation at 37°C, the phosphatase activity was measured at 405 nm using an ELISA reader (MRX II, Dynex Technologies, Middlesex, England).

Morphological analysis

Light and fluorescence microscopy

PGD_2-treated or nontreated cells were stained with a fluorescent dye (DAPI; purchased from Sigma). After 24 h, aliquots from each culture were withdrawn and washed twice with phosphate-buffered saline (PBS). Cells were resuspended in 200 μl PBS, placed on a slide and fixed with methanol. DAPI staining was performed for 5 min (0.1 μg/ml final concentration)., Slides were washed three times thereafter and examined using an Olympus BH2 fluorescence microscope.

Transmission electron microscopy

For TEM, at least 10^8 trypanosomes were taken at intervals during *in vitro* cultivation. Fixation was performed in 2% (vol/vol) glutaraldehyde in 0.2 M sodium cacodylate buffer containing 0.12 M sucrose for 1 h at 4°C. After washing four times (10 min each) and storage over night in sodium cacodylate buffer, cells were postfixed in osmium tetroxide (1.5%, wt/vol) and stained in 0.5% uranyl acetate.[65] Dehydration in ethanol, clearing in propylene oxide and embedding in Agar 100 (equivalent to Epon 812) was performed according to standard procedures.[66] Sections were stained in 5% (wt/vol) uranyl acetate and 0.4% (wt/vol) lead citrate.

Scanning electron microscopy

For SEM, the same fixation and in block staining protocol as described above was applied. Cells were sequentially dehydrated in 50, 75, 95 and 100% ethanol. Critical point drying and gold-palladium sputter staining was performed using standard protocols.

TUNEL test

To detect DNA double-strand ruptures, we applied the TUNEL test, using the *in situ* cell death detection kit obtained from Roche, Mannheim. The assay procedure was performed as described previously.[67] The ratio of cell death was determined by counting three times about 100 cells each and dividing the number of cells showing a clearly stained nucleus by the total number of trypanosomes counted, to yield the percentage of stained cells.

FACS analysis

DNA content

In order to determinate the content of DNA within the nucleus, a propidium iodide staining method was used.[40] For this purpose, at least 5×10^5 cells were spun down, washed once with PBS (pH 7.4) and lysed in 100 μl phosphate buffer (10 mM, pH 7.4; containing 6 μM digitonin). Samples were vortexed and incubated for 30 min at room temperature. Nuclei were stained with a propidium iodide solution (10 μg/ml final concentration in 10 mM phosphate buffer) 1 h before measurement of the nuclei in a FACSCalibur® apparatus (Becton Dickinson & Co., NJ, USA) using FACSflow®.

Mitochondrial membrane potential

To determine the mitochondrial membrane potential, 10^6 cells/ml were incubated in MEM containing 25 nM of tetramethylrhodamine (TMRE) for 30 min at 37°C and immediately loaded into the cell sorter for further analysis. The ionophore valinomycin (100 nM) was used as control for a complete depolarization of the inner mitochondrial membrane.

Phosphatidylserine exposure

Exposed phosphatidylserine was detected on the outer membrane of cells using annexin-V-fluos® (Roche, Mannheim). Cells were washed in HEPES buffer (10 mM, pH 7.4; containing 140 mM NaCl and 5 mM $CaCl_2$) and incubated for 15 min with annexin V at 4°C. Fluorescence was measured using FACS analysis as described before.

Cellular permeability

Plasma membrane integrity of PGD_2-treated or nontreated trypanosomes (2×10^6 cells/ml) was assessed using propidium iodide (5 μg/ml) staining. Cells were analyzed by flow cytometry, using digitonin (6 μM) as a positive control to induce necrosis.

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