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Prostaglandin-induced programmed cell death in *Trypanosoma brucei* involves oxidative stress

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Abstract

Recently, we reported the induction of a programmed cell death (PCD) in bloodstream forms of *Trypanosoma brucei* by prostaglandin D_2 (PGD_2). As this prostanoid is readily metabolized in the presence of albumin, we were prompted to investigate if PGD_2 metabolites rather than PGD_2 itself are responsible for the observed PCD. In fact, J series metabolites, especially PGJ_2 and $\Delta^{12}PGJ_2$, were able to induce PCD more efficiently than PGD_2. However, the stable PGD_2 analog 17phenyl-trinor-PGD_2 led to the same phenotype as the natural PGD_2, indicating that the latter induces PCD as well. Interestingly, the intracellular reactive oxygen species (ROS) level increased significantly under J series metabolites treatment and, incubation with *N*-acetyl-L-cysteine or glutathione reduced ROS production and cell death significantly. We conclude that PGJ_2 and $\Delta^{12}PGJ_2$ formation within the serum represents a mechanism to amplify PGD_2-induced PCD in trypanosomes via ROS production.
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Abbreviations: DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; dUTP, deoxyuridinetriphosphate; EIC, extracted ion chromatogram; Ψm, mitochondrial membrane potential; FACS, fluorescence-activated cell sorting; LC-ESI/MS, liquid chromatography electrospray ionization mass spectrometry; NAC, *N*-acetyl-L-cysteine; PCD, programmed cell death; PG, prostaglandin; ROS, reactive oxygen species; TEM, transmission electron microscopy; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

Introduction

The protozoan hemoflagellate *Trypanosoma brucei* is the causative agent of sleeping sickness in humans and of nagana in domestic animals. This unicellular eucaryote has a complex life cycle involving several morphologically and biochemically distinguishable stages during its development in the mammalian host (bloodstream form) and the tsetse fly vector (procyclic form).[1,2] Trypanosomes evade the host immune system by a combination of antigenic variation[3] and immune suppression.[4,5] The appearance of parasitic waves in blood during the course of infection is attributed to the action of specific antibodies. However, this behavior was also observed *in vitro* if culture medium was regularly replaced by fresh medium.[6] Thus, this parasitemia is controlled by the immune system of the host and is also regulated by the parasite itself.[7]

Untreated trypanosome infections are lethal. Patients with advanced sleeping sickness show an elevated prostaglandin (PG) level in their cerebrospinal fluid.[8] PGs are produced by many cell types in response to a variety of stimuli.[9,10] They are synthesized from arachidonic acid via an unstable endoperoxide intermediate by a cyclooxygenase and are eventually converted by specific synthases to one of several related products, such as PGD_2, PGE_2 and $PGF_{2\alpha}$. PGs have been shown to play a prominent role in inflammatory processes, regulation of vascular tone, infertility, pain, fever, sleep, apoptosis and others.[11–16] The PG production is not restricted to mammals but has also been described in parasites such as cestodes, trematodes, nematodes and protozoa.[17–20] In *Schistosoma mansoni*, the PG production has been associated with the transformation of cercaria into schistosomules,[21] whereas in *Amoeba proteus*, its synthesis plays a role during phagocytosis, eliciting vacuole formation.[22] In the bloodstream form of *T. brucei*, an apoptosis-like programmed cell death (PCD) is induced by prostaglandin D_2 (PGD_2).[23] PGD_2 is the major prostanoid produced and secreted by the stumpy bloodstream form of *T. brucei*,[24] which was shown to be more sensitive to this compound than the slender bloodstream form.[23] In this sense, the physiological function of PGD_2 in trypanosomes can be related to the population density regulation by PCD induction. However, it has already been shown that PGD_2 undergoes dehydration *in vivo* and *in vitro* to additional, biologically active PGs of the J series.[25,26] Metabolites of the J series have their own unique spectrum of biological activities, including inhibition of cell cycle progression, suppression of viral replication, induction of heat-shock proteins, stimulation of osteogenesis and induction of apoptosis in different cell types.[26–28] In this study, we analyzed the PGD_2 metabolism under *in vitro* conditions and evaluated the effect of the produced metabolites on the bloodstream forms of *T. brucei*.

Results

Separation and identification of PGD_2 metabolites in cultures

PGD_2 is known to be spontaneously converted in blood and albumin-containing media to different metabolites, especially to the J derivatives (Figure 1).[25,26,29,30] In order to identify the respective metabolites produced under our experimental

conditions, PGs were analyzed by liquid chromatography electrospray ionization mass spectrometry (LC-ESI/MS) in the negative-ion mode. PGD_2 was metabolized to derivatives of the J series PGJ_2, $\Delta^{12}PGJ_2$ and 15deoxy-PGJ_2 and derivatives of the D series 15keto-PGD_2 and 15deoxy-PGD_2, as detected by their comigration with authentic and commercially available standards and by their ionic masses (*m/z*). Figure 2a shows the extracted ion chromatograms (EICs) of the single charged molecular ion *m/z* 351.1, $[M-H]^-$ of the PGs. PGD_2 could be detected by the formation of an intense peak eluting after 15.8 min in the 0 h sample, which strongly decreased after 20 h and was practically absent after 40 h. Furthermore, the formation of 15k-PGD_2 could be observed by the detection of an additional peak in the *m/z* 351.1 ion trace (14.2 min) after 20 and 40 h (Figure 2a). PGJ_2, $\Delta^{12}PGJ_2$ and 15d-PGD_2 were detected by EIC of the single charged molecular ion *m/z* 333.0, $[M-H]^-$ (Figure 2b). The formation of PGJ_2 and 15d-PGD_2 was detected after 20 h incubation, which further increased after 40 h incubation. In addition, the formation of $\Delta^{12}PGJ_2$ (16.9 min) was observed only after 40 h incubation (Figure 2b). Finally, the PGD_2 metabolite 15d-PGJ_2 was detected as a peak eluting after 24 min in both the 20 and 40 h samples, as monitored by the EIC of the single charged molecular ion *m/z* 315.1 (Figure 2c).

Inhibition of cellular proliferation and induction of morphological changes by PGs of the J_2 series

Following identification of PGD_2 metabolites in culture media, we investigated their effects on cell growth of *T. brucei*. In addition, we used a stable PGD_2 analog, 17phenyl-trinor-prostaglandin D_2 (17phenyl-PGD_2), in order to explore if PGD_2 itself is able to induce PCD.[23] Using a [^{3}H]thymidine incorporation assay, we found that PGs of the J series, PGJ_2 and $\Delta^{12}PGJ_2$, strongly reduced cellular proliferation of *T. brucei* at concentrations significantly lower than those required for PGD_2 (5 μM). 15d-PGJ_2 showed similar effects as PGD_2 in the inhibition of proliferation, whereas metabolites of the D series, 15k-PGD_2 and 15d-PGD_2, remained ineffective (Figure 3). The IC_{50} for each metabolite was determined after 24 h incubation of trypanosomes in culture media. As shown in Table 1, metabolites of the D series were cytotoxic only at concentrations higher than 30 μM, suggesting that they do not play an important role in the PGD_2-induced cell death. In contrast, J series metabolites have similar or lower IC_{50} than that for PGD_2 (Table 1). In addition, 17phenyl-PGD_2 inhibited proliferation with an IC_{50} of 7.7 μM (i.e. at a concentration two-fold higher than that for PGD_2), demonstrating that PGD_2 itself is able to inhibit cellular growth. On the other hand, the results suggest that part of the PGD_2 effects depend on the formation of J series metabolites.

Electron microscopic studies of the ultrastructure of J series metabolites-treated cells showed that these PGs induced typical morphological changes of PCD. After an incubation time of 20 h, control cells show nuclei with a prominent central nucleolus and equally distributed chromatin, a not dilated mitochondrion and characteristic organelles (Figure 4a–c). In contrast, most of the treated cells displayed a fragmented nucleus with condensed chromatin (Figure 4d–g), a swollen mitochondrion (Figure 4k, m and n) and an increase of

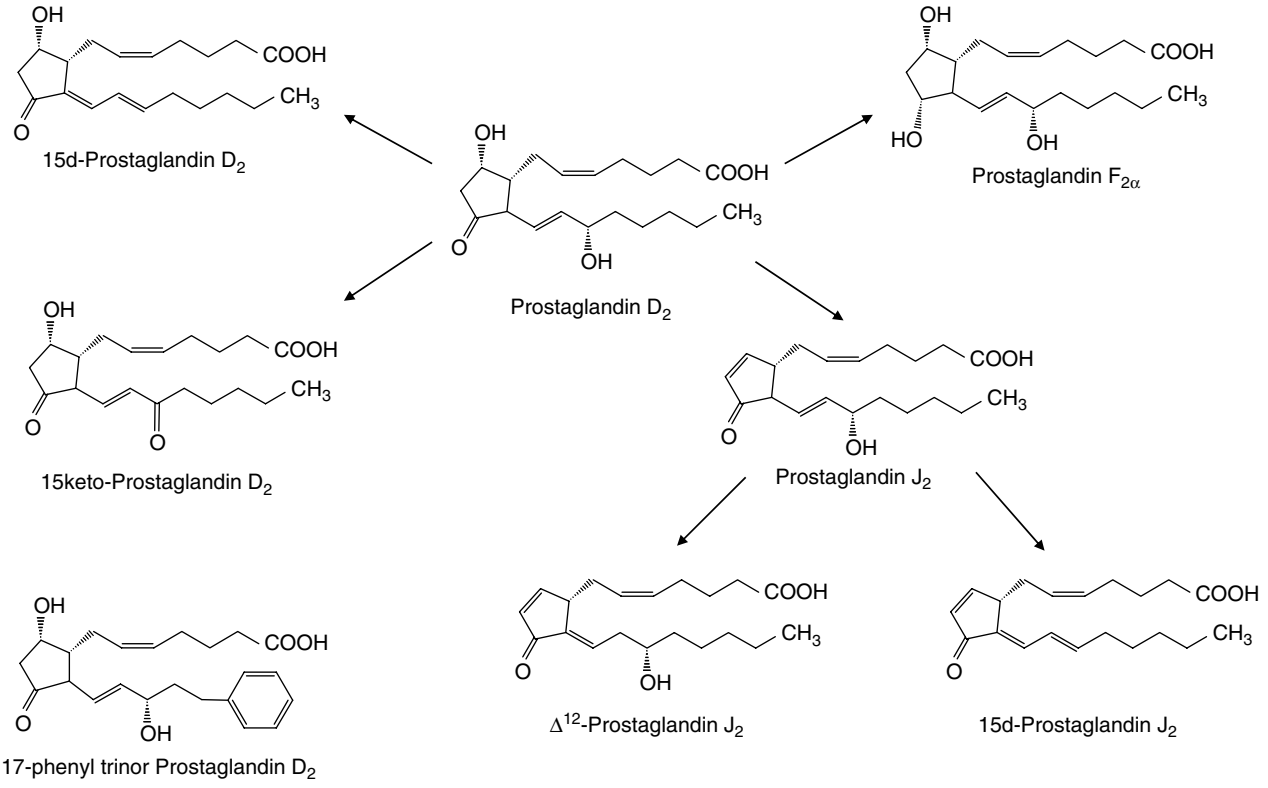

Figure 1 Structures of the more important PGD_2 metabolite. The inset at the left bottom shows the structure of the PGD_2 analog, 17phenyl-PGD_2

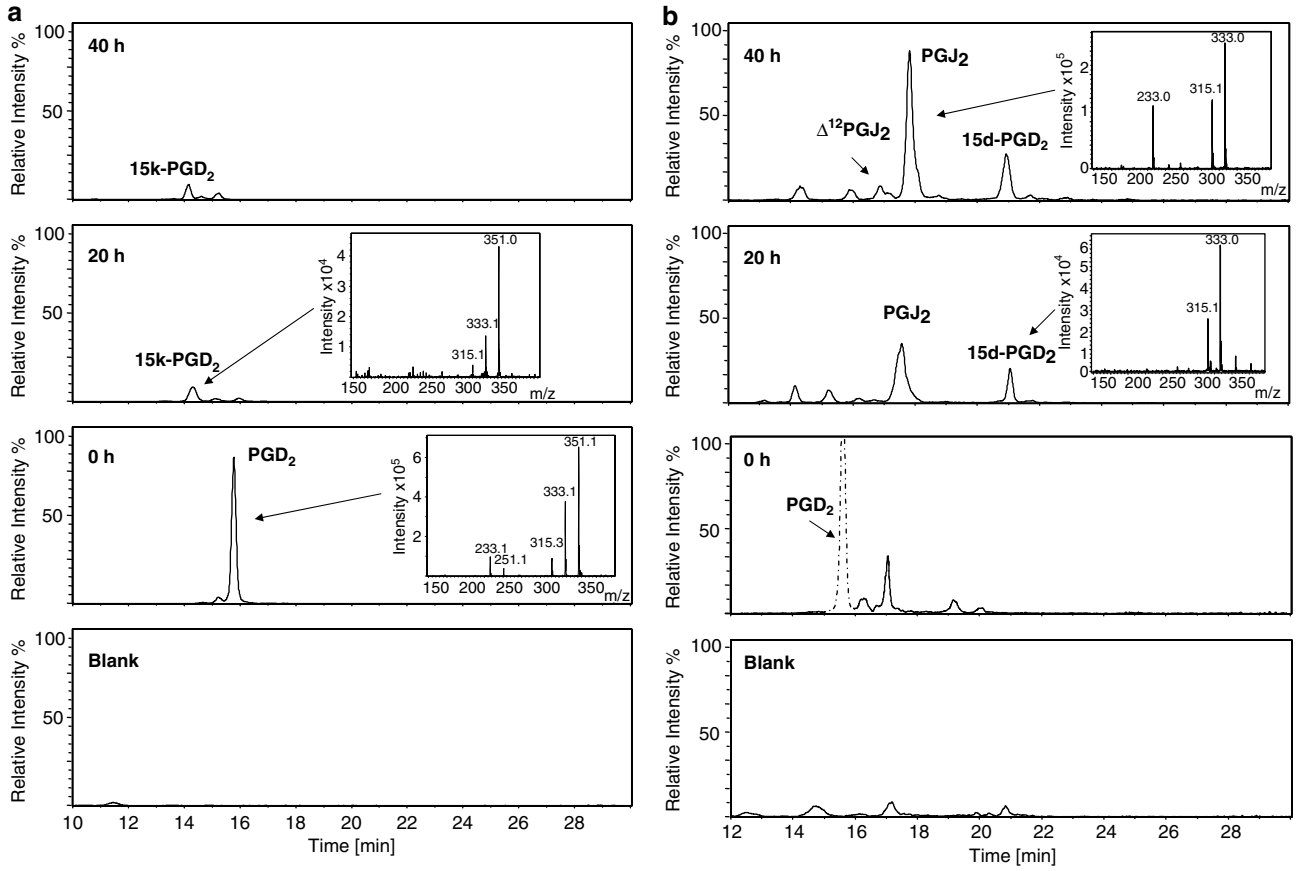

Figure 2 LC-ESI/MS analysis of PGD_2 metabolites. PGD_2 and its metabolites were separated on a 0.5 × 50 mm Saphire 110 C18 (5 μm) capillary column eluted with a gradient of 0–5 min 10% mobile phase B and 5–30 min 10–40% mobile phase B at a flow rate of 15 μl/min, where mobile phase A was 10 mM triethylamine in water and mobile phase B was 65 : 35 (v/v) acetonitrile : methanol. Elution profiles (150–750 *m/z*) were monitored online by negative-ion mode ESI mass spectrometry. (**a**) EIC of 351.1 *m/z* (PGD_2 and 15k-PGD_2). The inset shows the averaged negative-ion mass spectra acquired during the elution of PGD_2 (*m/z* 351.1 $[M-H]^-$, *m/z* 333.1 $[M-H-H_2O]^-$, *m/z* 315.1 $[M-2H_2O-H]^-$). (**b**) EIC of 333.0 *m/z* (PGJ_2, $\Delta^{12}PGJ_2$, 15d-PGD_2). The dashed peak (15.8 min) in the 0-h trace originates from a 18 Da loss from the single charged molecular ion ($[M-H]^-$, *m/z* 351.1) of PGD_2 during the ionization process, leading to a $[M-H_2O-H]^-$ neutral loss fragment ion at *m/z* 333.1. (**c**) EIC of 315.1 *m/z* (15d-PGJ_2). The negative-ion mass spectra of the 15d-PGJ_2 peak are shown as an inset (*m/z* 315.1 $[M-H]^-$). In the medium, a compound with *m/z* 315.2 was detected, leading to additional peaks (labeled with an asterisk) in the 315.1 *m/z* EICs. All other signals in the EICs are due to a neutral loss (18 Da) from the molecular ions of PGD_2 ($[M-2H_2O-H]^-$ *m/z* 315.1), PGJ_2 and 15d-PGD_2 ($[M-H_2O-H]^-$ *m/z* 315.1) occurring during the ionization process. Each extracted ion chromatogram was normalized to the highest peak. Incubation of PGD_2 (5 μM) was carried out for 0, 20 and 40 h at 37°C in culture medium

lysosomal structures (Figure 4g–j). Additionally, we observed a dilated flagellar pocket and the appearance of two or more flagellae in the same flagellar pocket (Figure 4o and p).

Analysis of the mitochondrial membrane potential and the DNA content

PGD_2-induced cell death of *T. brucei* includes a set of different cellular changes such as loss of mitochondrial membrane potential (Ψm), DNA degradation and phosphatidylserine exposure in the outer leaflet. To investigate these parameters individually, exponentially growing cells were treated for 24 h with either J series or D series metabolites before they were sorted according to size and fluorescent label using a FACScalibur® cell sorter (BD Biosciences). Using propidium iodide staining as a control for necrosis, we could never detect a significant staining (data not shown). As shown in Figure 5a, treated cells were generally smaller in size than control cells. In addition, cell death was associated with a loss of the Ψm, as reflected by a leftward shift of the histograms obtained using tetramethylrhodamine (TMRE) staining (Figure 5b). After 24 h, 24.5% of PGJ_2-treated cells had a low Ψm, as compared with 6.1% of control cells. Cells treated with $\Delta^{12}PGJ_2$ and 15d-PGJ_2 showed 18.3 and 12.8% Ψm disruption, respectively. Treatment of cells with 17phenyl-PGD_2 depolarized the mitochondrial membrane, which is consistent with the results obtained after PGD_2 treatment.[23] Control experiments using valinomycin completely abolished the Ψm (Figure 5b).

We also observed that cell death induced by J series metabolites was associated with a loss of DNA content. As shown in Figure 5c, a significant percentage of the nuclei of treated cells had a low DNA content: 47.1% for PGJ_2- and 14.5% for $\Delta^{12}PGJ_2$-treated cells *versus* 3.9% for control cells. $\Delta^{12}PGJ_2$ treatment increased the percentage of nuclei with low DNA content to 36.9% after 32 h (data not shown). Interestingly, 15d-PGJ_2 was not able to induce DNA degradation, even after 32 h treatment with concentrations up to 5 μM.

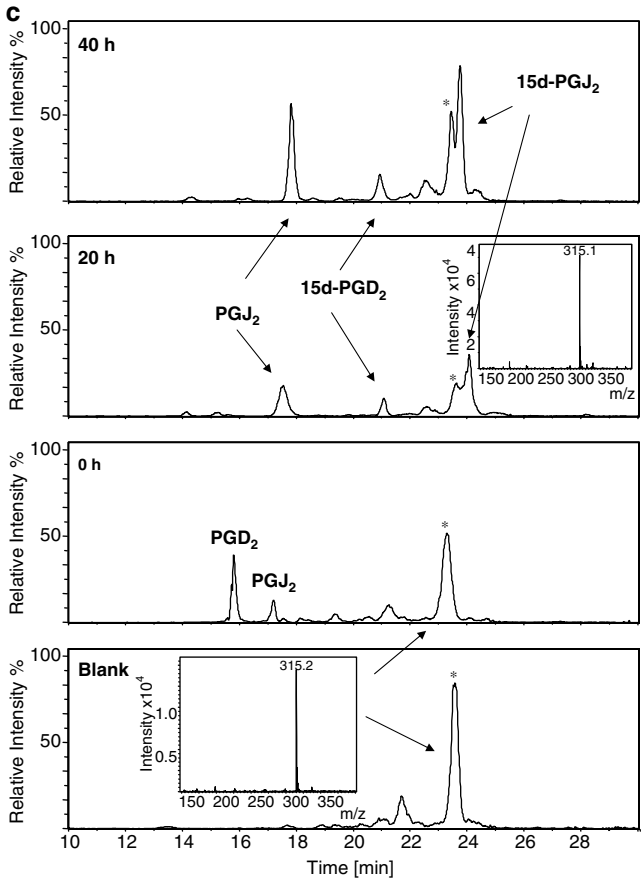

Figure 2 (Continued)

Table 1 IC_{50} values for the different prostaglandins

	Prostaglandin	IC_{50} (μM)
D series	PGD_2	3.7±0.6
	15deoxy-PGD_2	31.6±4.4
	15keto-PGD_2	104±8.7
J series	PGJ_2	2.7±0.3
	$\Delta^{12}PGJ_2$	0.7±0.4
	15deoxy-PGJ_2	3.5±0.3
	$PGF_{2\alpha}$	199±19
	17phenyl-PGD_2	7.7±1.3

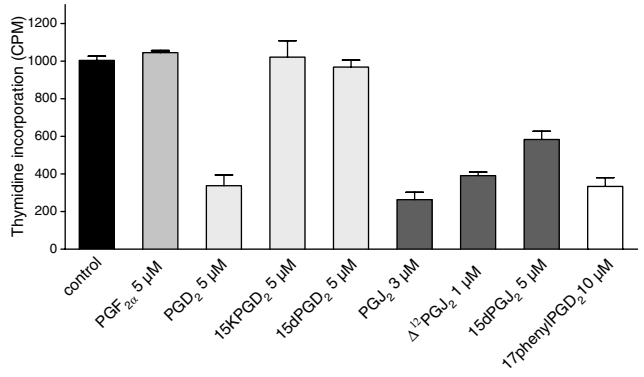

Figure 3 [^{3}H]thymidine incorporation after 24 h treatment with the different metabolites. Control cells were treated with ethanol. The bars represent the mean and S.D. of three independent experiments

However, when this experiment was carried out with cells of the stationary phase, which had been shown to be more sensitive to the action of PGs than logarithmically growing cells,[23] DNA degradation reached 16.2 *versus* 2.5% for control cells after only 4 h of incubation with 15d-PGJ_2.

In addition, cell cycle analysis of treated cells clearly indicated an increase of cells in the G1 phase, which was paralleled by a decrease of cells remaining in the G2 and S phases (Figure 5d). After a 32 h treatment, the level of cells in the G1 phase was about 20% increased as compared with control cells. The cell cycle arrest induced by 15d-PGJ_2 was rather moderate, with only a 10% difference as compared with cells incubated under control conditions, which did not increase further with time.

Detection of DNA disruption following J series PG treatment

In another set of experiments, we examined the incorporation of deoxyuridinetriphosphate (dUTP) using terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay to investigate if J series metabolites are able to induce DNA degradation. This assay was performed using cells from the exponential growing phase after 24 h of incubation with the respective PGs. Figure 6 shows the incorporation of dUTP into nuclei of control and treated cells. Only samples that were treated with PGJ_2, $\Delta^{12}PGJ_2$, or 17phenyl-PGD_2 showed a nuclear staining, which was not seen in control cell and scarcely observed in cells treated with 15d-PGJ_2 (data not shown). Quantification was visually monitored by counting of 100 labeled and unlabeled cells each in three independent experiments. After 24 h, PGJ_2-, $\Delta^{12}PGJ_2$- and 17phenyl-PGD_2-treated cells showed 40, 21 and 39% positive staining, respectively (Figure 6). These results are consistent with those obtained by fluorescence-activated cell sorting (FACS) analysis.

Detection of phosphatidylserine exposure after treatment with PGs of the J series

One of the first phenotypic changes associated to PCD is the exposition of phosphatidylserine residues on the surface of the plasma membrane.[31] In order to assay this parameter and, if observed, to confirm that it is an early event in *T. brucei* PCD that requires protein biosynthesis, we used cells from the stationary phase (i.e. stumpy cells) as they are more sensitive than dividing parasites.

As shown in Figure 7a, J series metabolites were able to induce phosphatidylserine exposure after only a few hours of treatment. This phenotype was prevented by treatment with cycloheximide before the incubation with PGs (Figure 7b). Likewise, cycloheximide delayed the cell death. The results indicate that phosphatidylserine exposure and the subsequent cell death caused by the administered PGs are processes that depend on protein biosynthesis.

Taken together, all these results indicate that the J series metabolites, mainly PGJ_2, are able to induce cell death more rapidly and at lower concentrations than their precursor PGD_2.

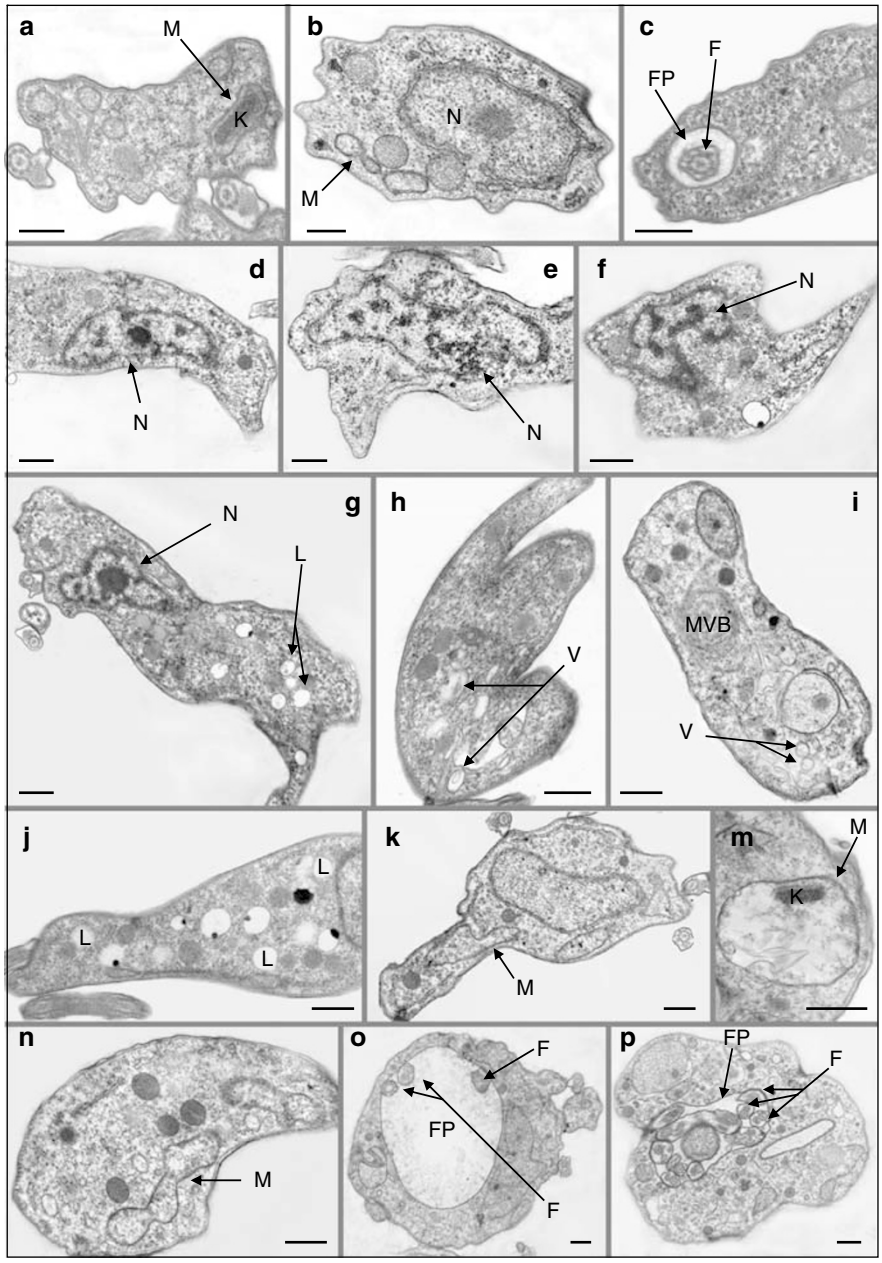

Figure 4 Transmission electron microscopy (TEM) of Epon-embedded cells. The most obvious alterations in J series metabolites-treated cells are as follows: (1) structures of condensed chromatin near the nuclear envelope (**d**–**e**); (2) dilated flagellar pockets and appearance of two or more flagellae in the same flagella pocket (**o**, **p**); (3) dilated mitochondria (**k**–**n**); and (4) an elevated amount of lysosomes (**g**, **j**) as compared with control cells. Note the usual appearance of mitochondria, nucleus and flagella pocket in control cells (**a**, **b** and **c**, respectively). Bars in TEM represent 0.5 μm each. Abbreviations used: lysosome (L); nucleus (N); flagellum (F); flagella pocket (FP); mitochondrion (M)

Measurement of reactive oxygen species after treatment with PGD_2 and their active metabolites

Previous studies have demonstrated that PGs are potential inducers of intracellular stress via production of reactive oxygen species (ROS).[32,33] In order to investigate if this phenomenon is involved in the PCD induced by PGD_2 metabolites in *T. brucei*, we used the sensitive probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). This non-fluorescent dye diffuses across cell membranes and is hydrolyzed to dichlorohydrofluorescein (DCFH) intracellularly. In the presence of ROS, DCFH is rapidly oxidized to highly fluorescent dichlorofluorescein. For this assay, we used cells from the stationary phase (after about 40 h), again because they are more sensitive to the apoptosis-like effects of PGs than the slender form of parasites.[23] As shown in Figure 8, treatment of the bloodstream forms of trypanosomes with PGJ_2 for 2 h evoked an abrupt ROS generation. This effect was also induced by $\Delta^{12}PGJ_2$, 15d-PGJ_2 and 17-phenyl-PGD_2, although after a much longer incubation time.

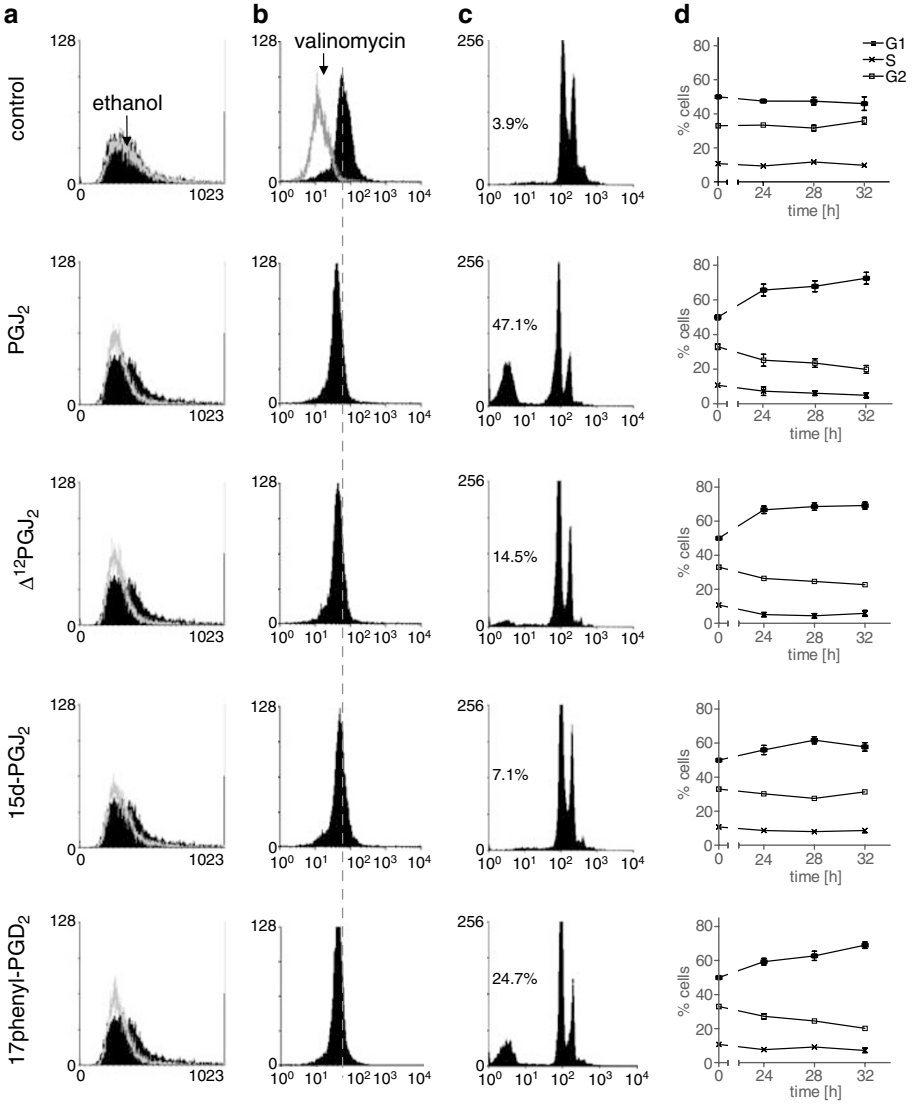

Figure 5 Propidium iodide or TMRE staining of bloodstream forms in FACS analysis. Cells were treated for 24 h with different metabolites (PGJ_2 3 μM, $\Delta^{12}PGJ_2$ 1.5 μM, 15d-PGJ_2 5 μM and 17phenyl-PGD_2 10 μM) and prepared for analysis of DNA content and Ψm. From left to right, cell size (**a**), Ψm(**b**), DNA content (**c**) and the phases of cell cycle (**d**) are shown. (**a**) The black histograms represent control cells and the gray line corresponds to cells treated with ethanol or metabolites. A positive control was performed with valinomycin for Ψm. Percentages of cells in G1, S and G2 phase at 0, 24, 28 and 32 h were determined using the CellQuest® software

Furthermore, ROS production induced either by PGD_2 or its metabolites was completely abolished by pretreatment with the thiol compounds NAC or glutathione. Likewise, NAC was also able to inhibit the PG effect on cellular growth, and occurrence of PCD phenotype markers such as morphological changes, phosphatidylserine exposure and DNA degradation (Figure 9), suggesting that the mechanism by which PGs mediate their effects involves alteration of the cellular redox status. Interestingly, untreated trypanosomes from the late stationary phase showed also an increased intracellular ROS level ($25.1 \pm 3.5\%$) as compared with NAC-treated parasites from the same phase ($7.9 \pm 1.0\%$). These parasites died more rapidly than those treated with NAC (data not shown).

On the other hand, ROS formation has been shown to require an upregulation of protein biosynthesis, in particular of enzymes involved in their formation.[34,35] Interestingly, pretreatment of trypanosomes with cycloheximide led to a significant inhibition of ROS production, suggesting that protein synthesis is a prerequisite for PCD (Figure 8).

Discussion

In a previous study, we reported the capacity of PGD_2 to induce PCD in the bloodstream forms of trypanosomes, which appeared to be a caspase-independent apoptosis.[23] However, the instability of this prostanoid in albumin-containing aqueous solutions and its subsequent conversion to different metabolites has been already demonstrated.[25] In addition, it was reported earlier that growth inhibition induced in murine leukemia cells after exposition to PGD_2 was in fact due to an active metabolite, $\Delta^{12}PGJ_2$, and that PGD_2 exerted no growth inhibition by itself.[36] We thus analyzed the metabolic conversion of PGD_2 under our experimental conditions in order to

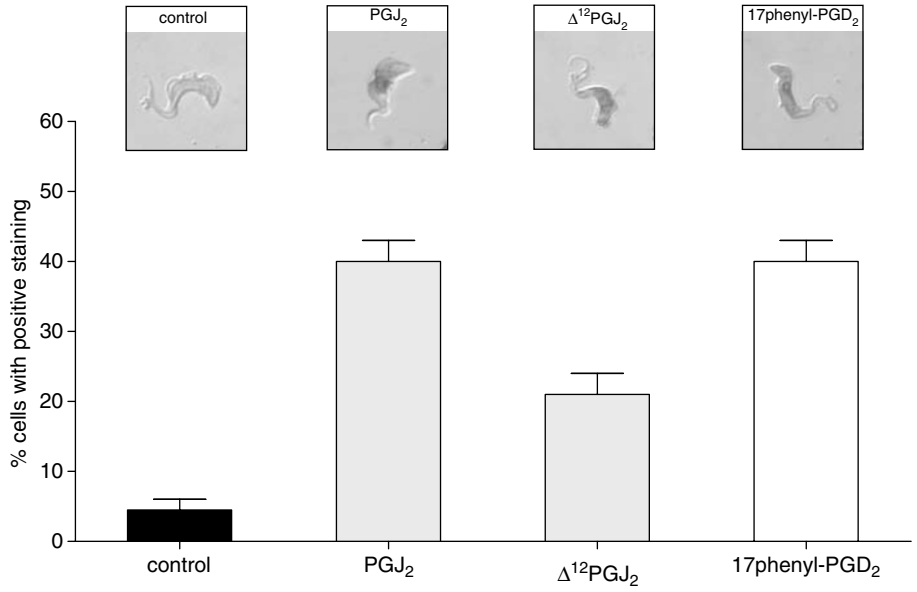

Figure 6 DNA double strand nicks staining using the TUNEL assay. Quantification was performed by counting 100 cells in three independent experiments

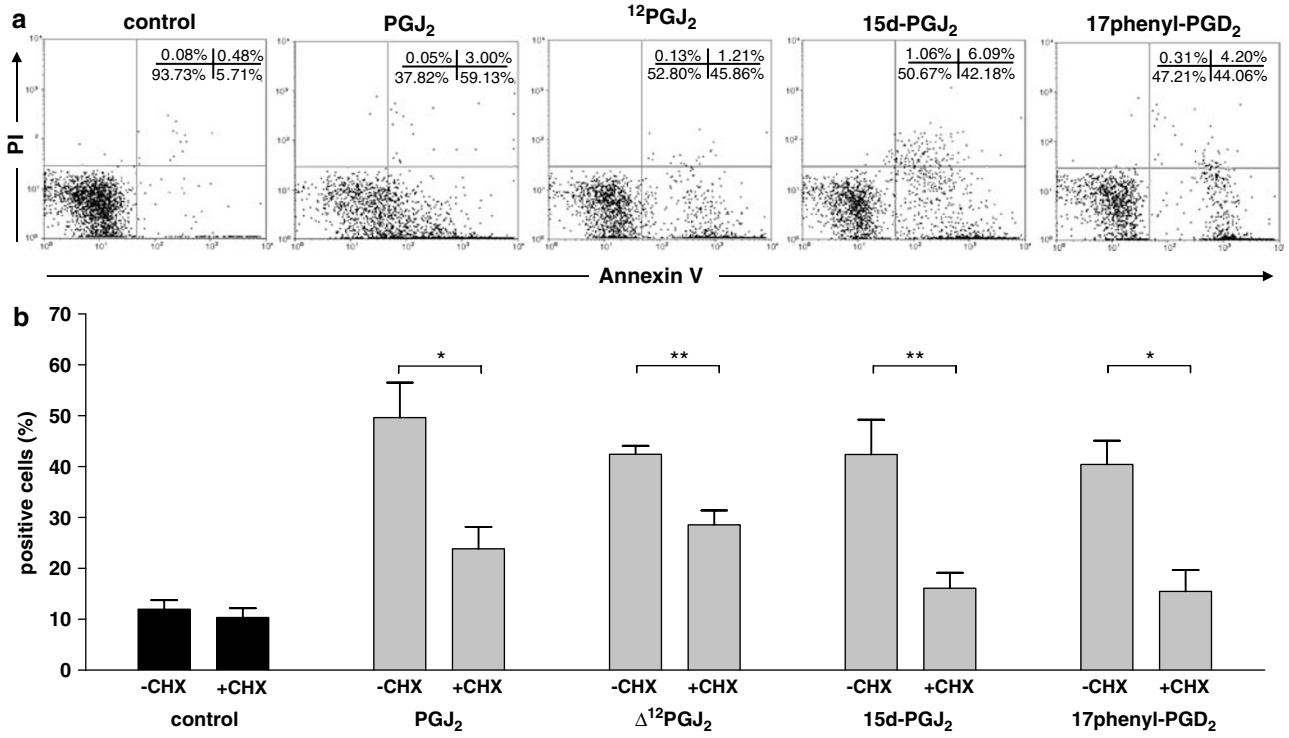

Figure 7 Determination of phosphatidylserine exposition. (**a**) Phosphatidylserine exposure measured by double staining with Annexin-V-Fluos and propidium iodide. Cells from the stationary phase were treated during 2 h (PGJ_2 2 μM), 4 h ($\Delta^{12}PGJ_2$ 1.5 μM, 15d-PGJ_2 5 μM) and 6 h (17phenyl-PGD_2 10 μM) with the indicated PGs. The dot plots represent the results of a typical experiment. (**b**) Phosphatidylserine exposure was inhibited by cycloheximide (CHX). The bars represent the mean and S.D. of three independent experiments. The asterisks indicate significant differences calculated by Student's *t*-test, using the GraphPad Software (**$P<0.01$; *$P<0.05$)

determine if the induced cell death was indeed due to the formation of more potent products. PGD_2 metabolization was a quick event, as it was reduced to about 80% after 12 h of incubation (data not shown). Using LC-ESI/MS, we identified various metabolites of the D and J series. Addition of D series metabolites had no effects on trypanosomes. The IC_{50} values for these metabolites were 10- to 30-fold higher than those for PGD_2. In contrast, addition of J series metabolites led to a significant inhibition of cellular proliferation. The IC_{50} values for PGJ_2 and $\Delta^{12}PGJ_2$ were significantly lower than those for

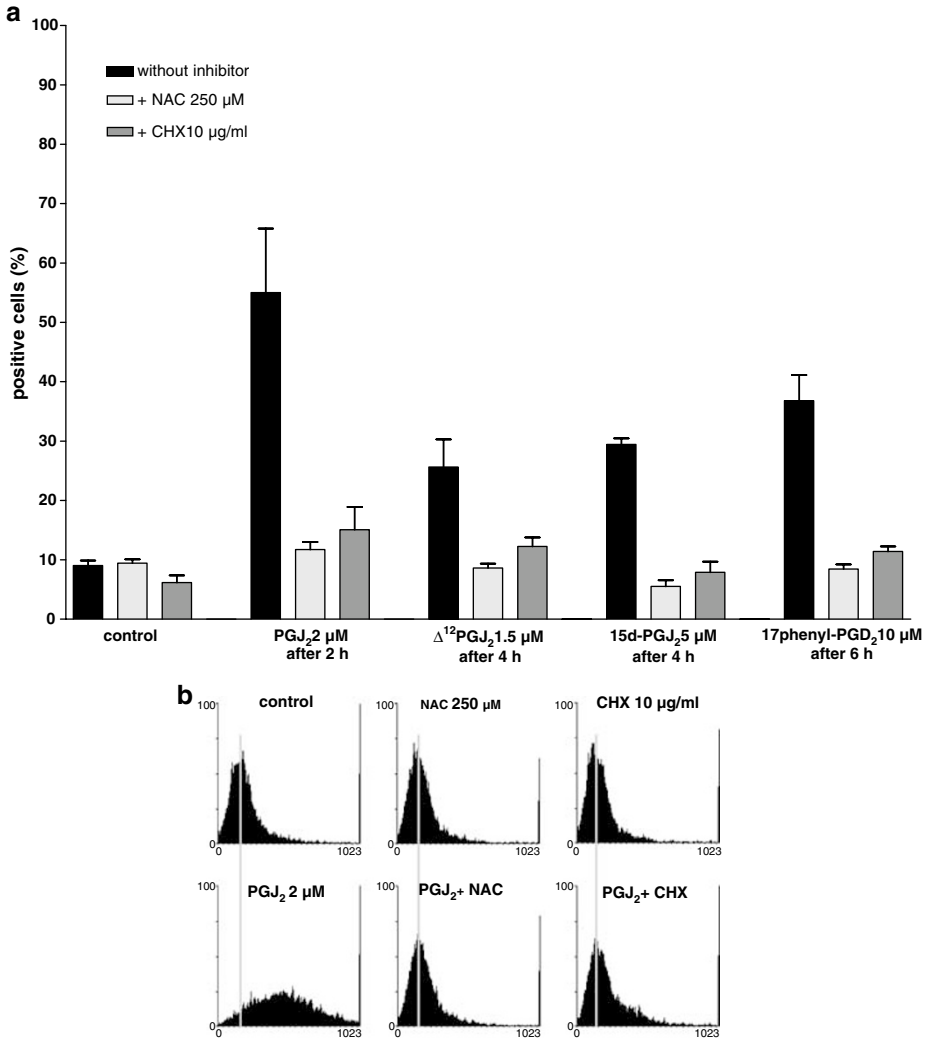

Figure 8 Measurement of ROS generation after PG treatment using DCFH-DA. Cells from the stationary phase were treated for 2 h (PGJ_2 2 μM), 4 h ($\Delta^{12}PGJ_2$ 1.5 μM, 15d-PGJ_2 5 μM) or 6 h (PGD_2 7.5 μM, 17phenyl-PGD_2 10 μM) with the indicated PGs and then prepared to measure intracellular ROS. (**a**) Intracellular ROS values for control and PG-treated cells. Addition of *N*-acetyl-L-cysteine (NAC) or cycloheximide (CHX) significantly attenuated the ROS production. (**b**) Representative FACS histograms showing the ROS-sensitive fluorescence of control and treated trypanosomes after 2 h incubation

PGD_2, whereas 15d-PGJ_2 inhibited cell growth at concentrations similar to those of PGD_2. Treatment with these metabolites led to cell cycle arrest in the G1 phase, as evidenced by FACS analysis and morphological changes, suggesting that this phenomenon is the principal effect exerted by J series metabolites on the slender form. The PG concentrations used in the described experiments are in the low micromolar or nanomolar range and thus, comparable and often lower than those used in similar studies with different mammalian cell types.[15,16,32] In addition, it is widely described in the literature that the effective PG concentration is only a small fraction of the total, as they bind readily to albumin.[37] At our conditions, at least 80% of PGD_2 was sequestered owing to albumin binding.[23] On assuming that this is also the case for the J metabolites, the effective PG concentrations have been in the low nanomolar range. On the other hand, the PGs are local mediators, and it has been reported that PG concentrations increase to the micromolar range during inflammation.[14] Local events may play an important role in the parasite's life, because bloodstream forms shuttle between blood and extravascular tissue. Indeed, it has been demonstrated that differentiation from slender to stumpy form increased owing to local effects.[38] Therefore, the actual PG concentrations may well be of physiological significance.

Similar to PGD_2, J series metabolites induced characteristic features of PCD in trypanosomes. Using electron microscopy, FACS analysis and TUNEL test, we observed an increase of vacuolization in the cytoplasm, nuclear segmentation, chromatin condensation, DNA degradation and phosphatidylserine exposition. In addition, the recorded loss of Ψm correlates with the microscopic observation of dilated mitochondria.

Using cells from the stationary phase, which represent stumpy-like cells[39] and are more sensitive to PGs than the slender form of parasites,[23] confirmed that phosphatidylserine exposure is an early event in *T. brucei* PCD. This phenotype

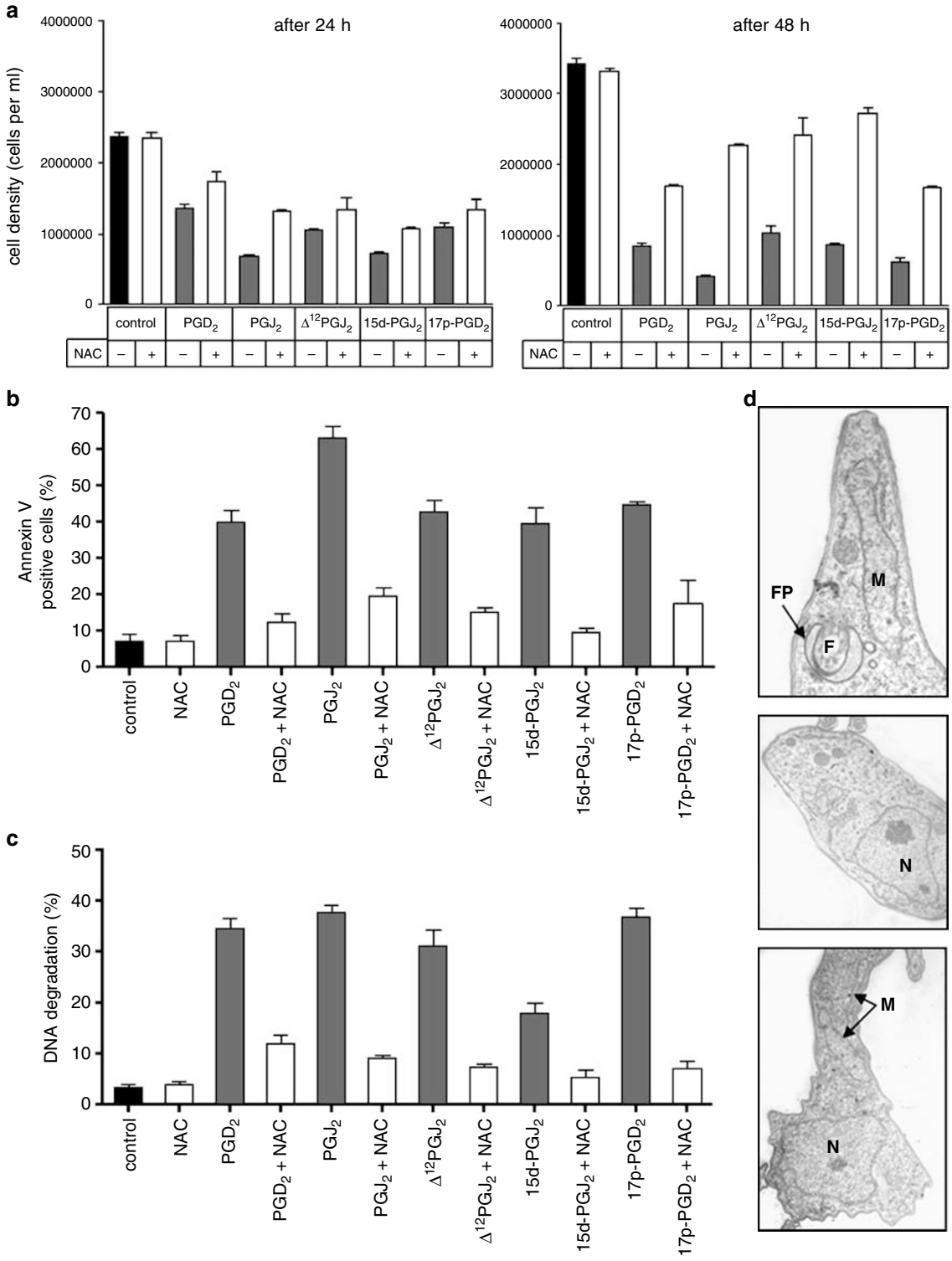

Figure 9 Inhibition of the cell death phenotype by incubation with NAC. Cells were treated with the indicated PGs in the presence or absence of NAC (250 μM). (**a**) After 24 and 48 h, cell density was determined using a hemocytometer. NAC protects the trypanosomes, as shown by the increase of the cell density in these cultures. Pretreatment of cells from stationary phase with NAC also prevented phosphatidylserine exposure (**b**) and DNA degradation (**c**) induced by PGs. (**d**) Morphology of trypanosomes treated with PGJ_2 plus NAC. Abbreviations used: nucleus (N); flagellum (F); flagella pocket (FP); mitochondrion (M)

depends on protein biosynthesis, because short-time pretreatment with cycloheximide inhibited the phosphatidylserine exposure and significantly delayed PCD.

On the other hand, 17phenyl-PGD_2, which was only slowly metabolized under our experimental conditions (data not shown), led to the same phenotype as PGD_2, indicating that

this latter compound is able to induce PCD itself. However, the concentration required to obtain the same effects was about two-fold higher than that of PGD_2. These results suggest that the induced phenotype is partially owing to the action of PGD_2, and that J series metabolites may be the more physiological inducers of PCD in the bloodstream form of *T. brucei*.

In general, PCD induced by J series metabolites was highly dependent on the growth phase. These PGs trigger cell death more rapidly and efficiently in the stationary than in the exponential growth phase. Similar results were found during PGD_2 treatment or pretreatment with a membrane-permeable cAMP analog, which induced differentiation from slender to stumpy form, and enhanced the parasite's sensitivity for PGD_2.[23] Therefore, our data indicate that these PGs primarily exert their effects on the stumpy form of parasites.

Despite the well-documented action of ROS as harmful molecules, they have also been shown to play an important role in signal transduction in innumerable physiological events.[35,40–44] In addition, ROS have also been described to participate in the signal cascades associated to PCD.[28,32,33,45–49] Likewise, PGs are potent inductors of oxidative stress in different cell types. This alteration of the redox state is known to be the basis of many of their biological effects and was recently discovered to be involved in the activation of PCD.[28,32,33] 15d-PGJ_2 has been reported to cause apoptosis in human hepatic myofibroblasts and breast cancer cells via oxidative stress.[28,33] PGD_2 and PGJ_2 as well as their metabolites (but not PGE_2 or $PGF_{2\alpha}$) induce apoptosis in human leukemia cells through ROS production and caspase 3 activation.[49] Therefore, we investigated if PCD in *T. brucei* is also induced by PGs via ROS generation. FACS analyses using DCFH-DA showed that all three J metabolites assayed stimulated ROS production. PGJ_2 was the most potent ROS inductor, followed by $\Delta^{12}PGJ_2$ and 15d-PGJ_2. Interestingly, the potency to induce ROS was always closely related with the capacity to cause PCD. In addition, PGD_2 and its analog 17phenyl-PGD_2 also generated high ROS levels. Interestingly, ROS appearance was always the first event observed after treatment of the parasites with prostanoids. As shown earlier, different biological effects induced by PGs via ROS production (even PCD in other cells) were prevented by treatment with antioxidants.[28,32] In trypanosomes, pretreatment with two ROS scavengers, NAC or glutathione, completely inhibited the ROS production and occurrence of the other PCD phenotype markers induced by PGs and led to a significant reduction of cell death. Taken together, we propose that ROS are implicated as intermediates in the PCD signaling pathway induced by J series PGs. These results are consistent with the data published by Ridgley *et al.*,[50] who stated that the increase of ROS led to PCD-like phenomena in procyclic trypanosomes. In addition, Fang and Beattie[51] showed that the trypanosome alternative oxidase (TAO) decreases the mitochondrial ROS production, and recently Tsuda *et al.*[52] demonstrated that TAO overexpression prevents PCD-like phenomena in the bloodstream form of *T. b. rhodesiense*.

The sensibility of PCD to protein synthesis inhibitors is an important feature, which clearly links this phenomenon to gene expression. Similarly, other compounds involved in the ROS signal pathway could require an upregulation of protein biosynthesis. This was the rationale to examine if ROS generation by PGs depends on this process. Parasites pretreated with cycloheximide and exposed to the different prostanoids showed no elevated ROS levels or phosphatidylserine exposition and survived a much longer time. These results are consistent with those reported for different neuronal cell types, in which apoptosis is triggered by ROS; their formation was totally or partially blocked by transcription and translation inhibitors.[53,54] We thus conclude that PG-induced ROS formation is a prerequisite for PCD in *T. brucei*.

Oxidative stress is considered to be an important condition to promote cell death in response to a variety of signals. Our results provide evidences for the existence of an active process of PCD in *T. brucei*, which involves *de novo* protein synthesis and oxidative stress.

PCD in single-cell organisms, from bacteria to unicellular eucaryotes, is well documented.[55–59] To date, the physiological role of PCD in single-cell organisms has not clearly been defined. However, PCD functions similar to those known for metazoa have been suggested for unicellular organisms too,[59,60] because protozoa behave as a population, that is, as a complex community with many cell–cell interactions, which in many ways resemble metazoa.[59,60] Evidence of this crosstalk among trypanosomes is well documented for the differentiation process from the proliferative slender to the non-dividing stumpy form. Differentiation depends on cell density, where local effects like cell-to-cell communication play an important role.[38] Thus, differentiation has been suggested as an autoregulatory process, stopping significantly the cellular proliferation.[38] Although it is known that wave parasitemia can be reproduced *in vitro*,[6] mechanisms of the parasite to regulate the stumpy form density have not been described so far. We propose that PGD_2 secretion by trypanosomes and production of their J series metabolites play a key role as regulators of cell density within their host by PCD induction in the stumpy form of parasites via cell cycle regulation. In this sense, an elevation of the PG levels could arrest or diminish the parasitemia by elimination of the non-dividing form, which represents a transitory stage unable to return to the proliferative slender form. This mechanism of regulation would not compromise the parasites population for two reasons: (1) it would not cause PCD in the proliferative slender form, guaranteeing a persisting parasitemia, and (2) it would be an autolimited process, as PGs involved in PCD are secreted mainly by the stumpy form.

Materials and Methods

Reagents

PGs were purchased from the Caiman Chemical Co. (Ann Arbor, MI, USA). Triethylamine and solvents used were obtained from Merck (Darmstadt, Germany). Methyl-[^{3}H]thymidine was obtained from Hartmann Analytic (Braunschweig, Germany). The TUNEL kit and Annexin-V-Fluos were purchased from Roche Diagnostics GmbH (Mannheim, Germany). NAC and glutathione were obtained from Sigma-Aldrich (Deisenhofen, Germany). DCFH-DA was purchased from Molecular Probes (Karlsruhe, Germany).

PGs were reconstituted in ethanol and diluted to the respective concentrations using culture medium. The respective ethanol concentration was always applied to control cells as well.

Separation and identification of PGD_2 metabolites by LC-ESI/MS

In order to analyze the PGD_2 metabolism, this PG was incubated in medium at 37°C. After different time points (0, 20, 40 h), PGs in the medium were extracted with 4 ml of ethylacetate (1 : 2, v/v) three times. The upper organic phases were pooled and evaporated to dryness under a stream of nitrogen at room temperature. All extraction procedures were performed under conditions of minimal light. Before the LC-ESI/MS analysis, samples (resuspended in 50 μl of acetonitrile : methanol (65 : 35, v/v)) were diluted 1 : 10 (v/v) with 10 mM triethylamine and 10 μl was directly injected onto the column. LC was performed on a 0.5×50 mm Saphire 110 C18 (5 μm) capillary column (Grom, Herrenberg, Germany) attached to an HP1100 capillary HPLC system (Agilent Technologies, Waldbronn, Germany). Mobile phase A was 10 mM triethylamine in water and mobile phase B was 65 : 35 (v/v) acetonitrile : methanol. Samples were separated by a gradient program: 0–5 min 10% mobile phase B and 5–30 min 10–40% mobile phase B at a flow rate of 15 μl/min. The outlet of the column was directly interfaced with the electrospray ionization source of an Esquire 3000plus ion trap mass spectrometer (Bruker-Daltonics, Bremen, Germany). The mass spectrometer was operated in the negative-ion mode. Dry gas (6 l/min) temperature was set to 275°C, nebulizer was set to 20.0 psi and the electrospray voltage was set to $+3000$ V. Maximal accumulation time was set to 200 ms. Loading of the trap was controlled by the instrument (ICC 15000). Mass spectra were acquired from 150 to750 *m/z*, using four averages. All signals were detected as monoisotopic masses.

Cell culture

The monomorphic strain *T. brucei* MITat 1.2 (VSG-variant 221) was taken from frozen stabilates, seeded at a cell density of 2×10^5 cells/ml and grown at 37°C and 5% CO_2 for about 16 h, until a cell density of 8×10^5 cells/ml was reached.[6,39] These precultures were then adjusted to 2×10^5 trypanosomes/ml with fresh medium and incubated again in a CO_2 incubator with PGs at the given concentrations. Experiments were performed with exponentially growing parasites (slender form) to evaluate cellular proliferation, cell cycle and partially PCD. Parasites from the stationary phase, equivalent to stumpy forms, were used to evaluate PCD. For experiments using cycloheximide (an inhibitor of protein synthesis), cells were grown until they reached the stationary phase, preincubated for 1 h with this inhibitor and treated with the respective PG. All experiments were performed at least in triplicate.

Cytotoxicity assay

The IC_{50} values were determined according to Bodley *et al.*[61] Exponentially growing parasites were diluted to 2×10^5 cells/ml, placed in 96-well microtiter plates and grown in the absence or presence of different PG concentrations. Plates were incubated for 24 h at 37°C in a CO_2 incubator. Cell growth was stopped by the addition of lysis buffer containing *p*-nitrophenyl phosphate (20 mg/ml in 1 M sodium acetate, pH 5.5, containing 1% Triton X-100). Likewise, addition of this buffer started the acid phosphatase reaction, which was measured at 405 nm after 6 h incubation by using an ELISA reader (MRX II, Dynex Technologies, Middlesex, England).

Proliferation assay

The cellular proliferation was determined by measuring thymidine incorporation. Cultures were adjusted to 2×10^5 cells/ml and seeded into 96-well microtiter plates. After 24 h incubation at 37°C and 5% CO_2, trypanosomes were pulsed for 4 h with methyl-[^{3}H]thymidine (1 μCi/well), harvested immediately and washed twice in PBS. Cells were lysed in 5% SDS and counted in a liquid scintillation counter.

Transmission electron microscopy

Control or treated cells were harvested after 24 h incubation with PGs and prepared for TEM. The cells were fixed in 2% glutaraldehyde in 0.2 M sodium cacodylate buffer containing 0.12 M sucrose for 1 h at 4°C. After washing and storage overnight in sodium cacodylate buffer, cells were post-fixed in osmium tetroxide (1.5%, w/v) and stained in 0.5% uranyl acetate.[62] Dehydration in ethanol, clearing in propylene oxide and embedding in Agar 100 (equivalent to Epon 812) were performed according to standard procedures.[63] Sections were stained in 5% (w/v) uranyl acetate and 0.4% (w/v) lead citrate.

TUNEL assay

We used the *in situ* cell death detection kit obtained from Roche (Mannheim, Germany) in order to detect DNA double-strand ruptures. The assay procedure was performed as previously described.[55] Briefly, trypanosomes were fixed in 4% paraformaldehyde and 0.2% glutaraldehyde in phosphate buffer for 1 h before they were placed on a slide for staining. The percentage of cells showing a clearly visible nucleus staining was determined in treated and untreated cells by counting three times 100 cells each.

FACS analysis

Mitochondrial membrane potential: This parameter was measured after incubation of PG-treated and untreated trypanosomes with 25 nM TMRE for 30 min at 37°C. Fluorescence analysis was performed in a FACSCalibur® apparatus (Becton Dickinson & Co., NJ, USA). The ionophore valinomycin (100 nM) was used as a control for a complete depolarization of the inner mitochondrial membrane.

DNA content: The assay was performed as previously described.[23] Briefly, following a 30 min permeabilization of trypanosomes in 10 mM phosphate buffer containing 6 μM digitonin, nuclei were stained with a propidium iodide solution (10 μg/ml final concentration in 10 mM phosphate buffer) and kept on ice until measurement in the cell sorter.

Necrosis: In order to analyze disruption of the plasma membrane after PG treatment, nuclei were stained with propidium iodide (5 μg/ml) in the absence of a cell permeant and analyzed by flow cytometry.

Phosphatidylserine exposure: Exposed phosphatidylserine was detected on the outer membrane of cells using Annexin-V-Fluos® (Roche, Mannheim, Germany) according to the manufacturer's protocol. Fluorescence was measured using FACS analysis as described before.

Reactive oxygen species: DCFH-DA was employed to measure intracellular oxidative stress.[34] Control and treated cells were incubated with 10 μM DCFH-DA for 1 h at 37°C. The cellular fluorescence intensity was measured using a flow cytometer as described above.

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