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ORIGINAL PAPER

Apoptosis caused by Hsp90 inhibitor geldanamycin in *Leishmania donovani* during promastigote-to-amastigote transformation stage

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Abstract The role of heat shock protein 90 inhibitor geldanamycin (GA) during *Leishmania donovani* promastigote-to-amastigote transformation in axenic conditions was investigated. Promastigotes exhibited apoptotic morphologic changes after GA treatment at a high temperature, and the effect is in a dose- and time-dependant manner. Meanwhile, cell cycle analysis showed a significant increase at the expense of cells in the G0/G1 phase and a decrease in the S and G2/M phases after GA treatment. In addition, cellular glutathione level was reduced and reactive oxygen species content was increased afterwards. Pretreatment with antioxidants reduced the percentage of GA-induced cell apoptosis. After treatment, cultures in pH 5.5 showed a lower percentage of apoptosis than those in pH7.4. The present study showed that GA could cause apoptosis in *L. donovani* but could not cause stage differentiation in high temperature and that acidic conditions were likely to be crucial for the transformation and survival of the parasite within its human host.

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Introduction

Leishmania donovani, whose life cycle is characterized by the presence of a flagellated promastigote stage in the sand fly (Rioux et al. 1979) and a nonmotile amastigote stage within a mammalian host (Alexander and Vickerman 1975; Chang and Dwyer 1976), is the major causative agent of visceral leishmaniasis. The promastigote-to-amastigote cytodifferentiation is a profound morphological and physiological transformation. This not only results in morphologic transformation but also allows survival within the parasitophorous vacuole. During the process of differentiation, the parasite loses its flagellum, rounds up, changes its glycoconjugate coat, and begins to express a set of metabolic enzymes which are optimally active at a low pH. However, the biochemical changes related to this process are not yet fully understood.

In higher eukaryotes, heat shock protein 90 (Hsp90) is known to associate with cell cycle regulators and with transcription factors (Buchner 1999). Hsp90 is the most abundant class of protein in *Leishmania* and is involved in a variety of cellular processes (Brandau et al. 1995). However, only little is known about the function of *Leishmania* Hsp90. Sufficient Hsp90 overexpression level and viable Hsp90-null mutants could not be obtained although the function of this protein had been investigated in different ways (Wiesgigl and Clos 2001a, b). Recently, benzoquinoid ansamycins, originally developed as antitumor drugs (Sakagami et al. 1999), were found to specifically inhibit members of the Hsp90 family of chaperones. Of these compounds, geldanamycin (GA) is widely used to study the effects of functional Hsp90 depletion (Pratt 1998).

Previous study demonstrated that promastigote-to-amastigote differentiation could be induced at a low temper-

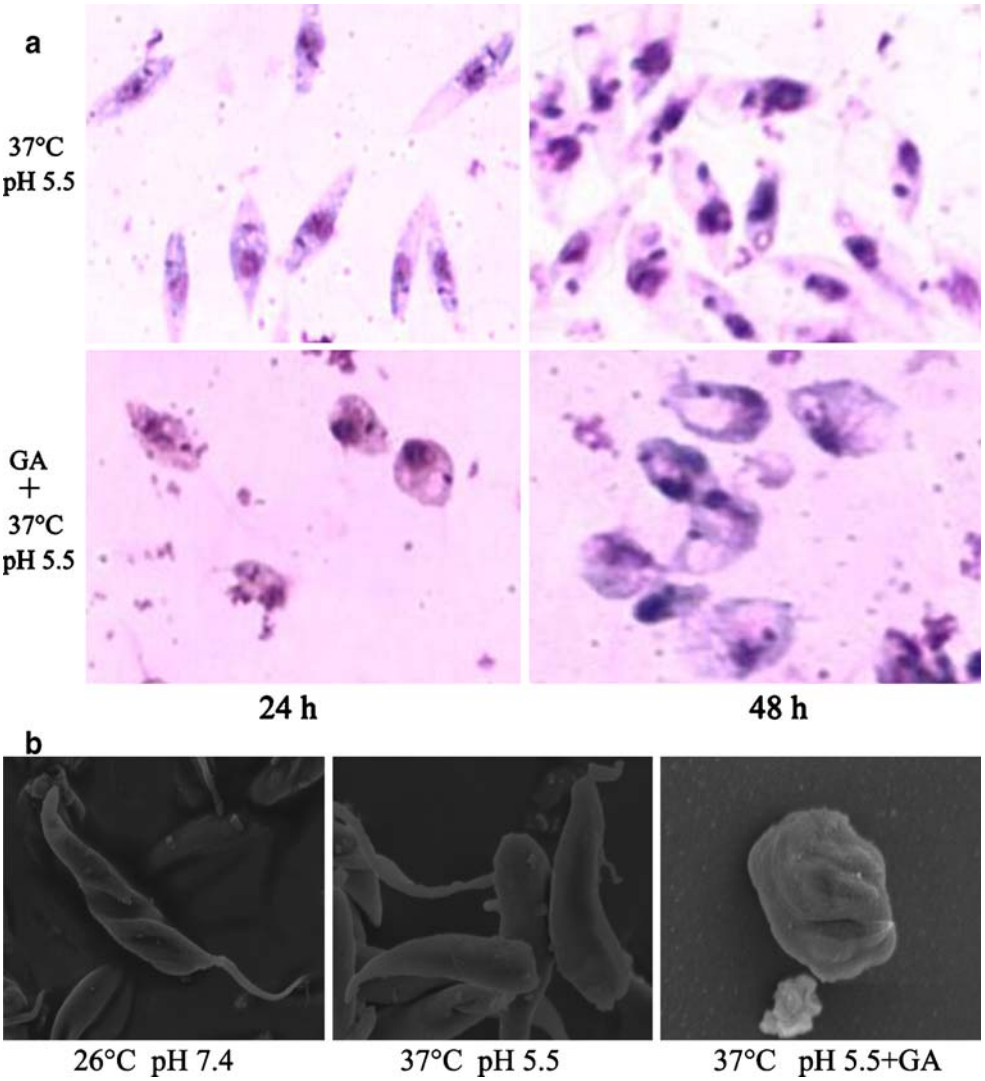

Fig. 1 Apoptosis induced by GA in *L. donovani*. Promastigotes growing logarithmically were incubated with 100 ng ml^{-1} of GA (GA group) or without GA (untreated group) at 37°C in pH 5.5. **a** Light micrograph of Giemsa-stained preparation of treatment promastigotes. Cells were axenically incubated for up to 48 h and subsequently adhered onto slides. They were stained with Giemsa for light microscopy. The images show representative results for each day and culture. **b** Scanning electron microscope images of treatment promastigotes. *Left*: Typical normal promastigote; *Middle*: untreated group at 24 h, cells were spindle-shaped still; *Right*: GA treatment after 24 h, cells with shrinkage and without flagellum. **c** In situ analysis of apoptosis in *L. donovani* by TUNEL. Cells incubated for different time periods as indicated were subjected to TUNEL staining as described in experimental procedures. Pictures show immunofluorescent (TUNEL) image of *L. donovani* promastigotes. *Green color* indicates TUNEL-positive cells

ature (25°C) and at neutral pH by pharmacological inhibition of Hsp90 using GA and related compounds (Wiesgigl and Clos 2001a, b). Curiously, another study showed that heat stress could trigger a process of programmed cell death in *Leishmania infantum* promastigotes (Raina and Kaur 2006). Incubation of *L. donovani* promastigotes at 37°C combined with a subsequent acidification of the culture medium could induce a reversible in vitro promastigote-to-amastigote differentiation (Debrabant et al. 2004). During this transformation, more detailed evidence of the exact effects of Hsp90 had to be found. In light of this, we think it necessary that the effects of GA and pH of media during promastigote-to-amastigote transformation be studied. The primary interest is to find out whether GA can induce apoptosis-like death or stage transformation in *L. donovani* promastigote at a high temperature and a low pH (i.e., promastigote-to-amastigote stage transformation condition) and to discover the possible evidence of Hsp90 protection pathway and the effects of media pH during GA treatment.

Materials and methods

Chemicals and reagents

GA (InvivoGen, USA) was kept as a stock concentration of 1 mg ml^{-1} in dimethyl sulfoxide at −20°C. Glutathione (GSH) and *N*-acetyl-cysteine (NAC) were dissolved in sterile water as a stock concentration of 0.5 M at 4°C.

Parasites culture conditions and treatment

The promastigotes of an *L. donovani* (MHOM/CN/Gansu-8801) clone were used. The promastigotes were cultured in medium 199 (GIBCO) supplemented with 10% (*v/v*) fetal bovine serum at 26°C and pH7.4. These cultures were maintained in an exponential growth phase by passages every 2 to 3 days.

Promastigotes harvested from log-phase cultures and resuspended in medium 199 at pH5.5 and 7.4, respectively,

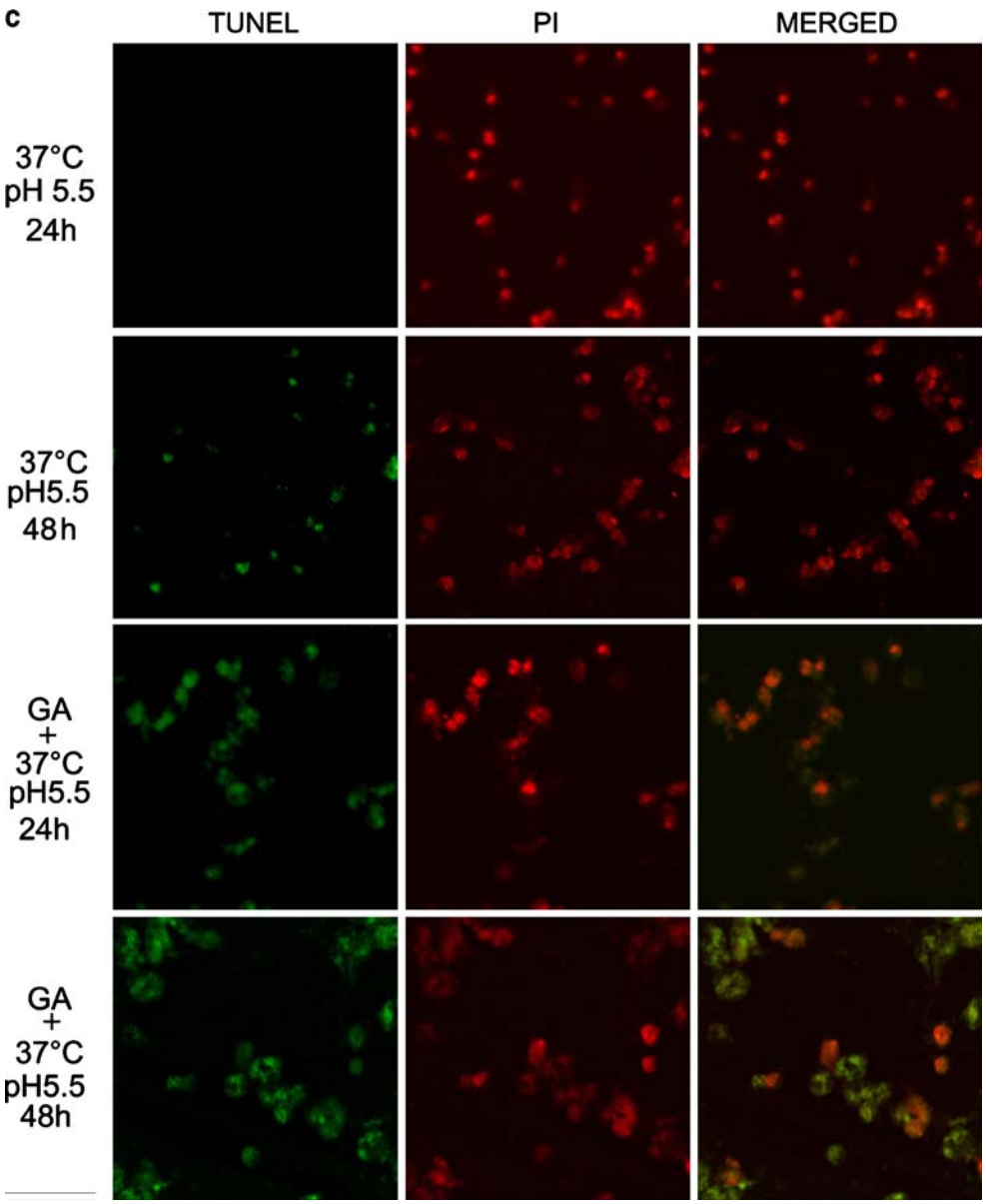

Fig. 1 (continued)

were incubated with 50, 100, and 200 ng ml^{-1} GA (GA-treated group) or without GA (untreated group) at 37°C. GSH and NAC were used at a concentration of 1 mM, with 1-h preincubation preceding drug treatments.

Light microscopy

For Giemsa staining, parasites were spread on glass slides, air-dried, and fixed for 10 min with methanol. Afterwards, the preparations were examined under a light Olympus microscope made in Japan.

Scanning electron microscopy

The parasites were harvested by centrifugation, washed twice in a phosphate buffer, and fixed for 2 to 3 h at 4°C in 2.5% (*v*/*v*) glutaraldehyde in phosphate buffer, with pH7.4. Meanwhile, the cells were pelleted, postfixed with 1% (*w*/*v*) osmium tetroxide for 30 min, and then processed in ethanol propylene oxide series. Finally, the samples were point-dried utilizing liquid CO_2 and coated with gold particles. They were observed and imaged through a JSM-5610LV scanning electron microscope.

In situ labeling of DNA fragments by terminal deoxyribonucleotide transferase-mediated dUTP nick end labeling

In situ detection of DNA fragments by terminal deoxyribonucleotide transferase (TdT)-mediated dUTP nick end labeling (TUNEL) was performed using the one-step TUNEL apoptosis assay kit produced by Beyotime Institute of

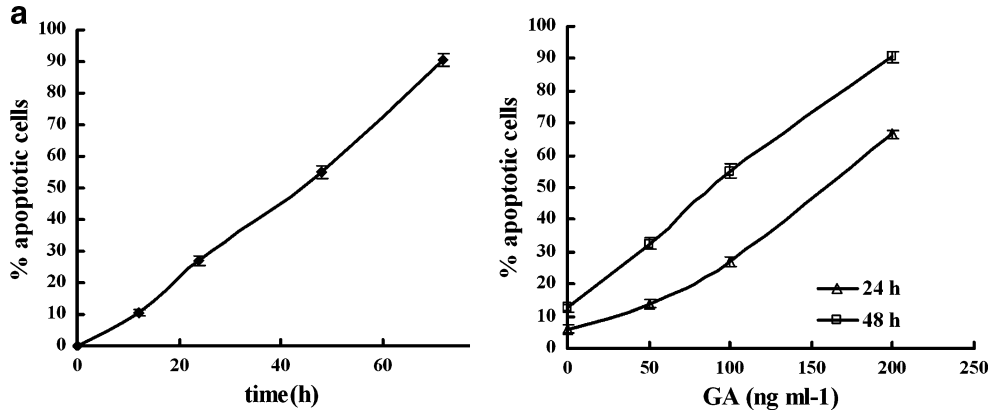

Fig. 2 Flow cytometric analysis of TUNEL-labeled cells at different time points. Promastigotes growing logarithmically were incubated with 50, 100, and 200 ng ml^{-1} of GA at 37°C in pH 5.5. The percentage of apoptotic cells was analyzed at different time points by TUNEL flow cytometry examination. All the analyses and graphical representations were carried out with CELLQUEST PRO (BD) package version 4. **a** GA induced a dose-dependent and time-dependant apoptosis. **b** Representative histograms of TUNEL analysis. (*a*) *Left*: negative control. *Right*: untreated group at 24 h. (*b*) *Left*: negative control. *Right*: untreated group at 48 h. (*c*) *Left*: negative control. *Right*: GA group at 24 h. (*d*) *Left*: negative control. *Right*: GA group at 48 h. *M1*, average of TUNEL population unlabeled; *M2*, average of TUNEL population labeled. (*e*) Histograms are the means of six experiments with at least 200,000 cells analyzed, grouped, and experimented. Data are means±SE (n=6). *p<0.01, compared with untreated group at 24 h

Biotechnology in China. The cells treated as indicated were fixed in 4% paraform/PBS onto poly-(L-lysine)-coated slides, rinsed with PBS, then permeabilized by 0.1% Triton X-100; slides were washed with PBS, and cells were added with 50 µl of TUNEL reaction mixture, then incubated for 1 h at 37° C in the dark. Negative controls were incubated in the absence of the enzyme terminal transferase. Slides were washed and incubated with RNase A (50 µg ml^{-1}) for 1 h at room temperature. Finally, propidium iodide (PI; 0.5 µg ml^{-1}) was added for 1 h at room temperature. Slides were observed under an LSM 510 META Olympus Confocal Laser Scanning Microscope by using 488-nm excitation and 530-nm emission, and the images were captured and digitized by image analysis software.

Flow cytometry data acquisition and analysis

The apoptosis percentage of *L. donovani* cells was observed by TUNEL flow cytometry. After being fixed and treated with 0.1% Triton X-100, the cells were incubated with TUNEL reaction mixture for 1 h and then washed twice by PBS for flow cytometric analysis. In addition, the TUNEL assay consisting of a treatment without TdT but with biotinylated dUTP was performed as a negative control.

The PI flow cytometric analysis was performed for cell cycle. Briefly, cells were washed with PBS and fixed with 4% paraform/PBS, then permeabilized by 0.1% Triton X-100. After being incubated with RNase A (50 µg ml^{-1}), cells were stained with PI (50 µg ml^{-1}) for 1 h, and the parasites were immediately analyzed for PI fluorescence. The parasites were analyzed in a FACScalibur flow cytometer equipped with a 488-nm Ar laser. All the analyses were made with CELLQUEST PRO package version 4.

Assay of intracellular reactive oxygen species

To monitor the level of reactive oxygen species (ROS), the nonfluorescent probe 2′, 7′-dichlorofluorescein diacetate (DCFH-DA) was used as illustrated previously (Tian et al. 2006). Briefly, 10^7 cells were washed three times with PBS and incubated with 10 µM DCFH-DA (or without as the negative control) for 20 min at 37°C. The fluorescence was read at 485 nm for excitation and 530 nm for emission with a fluorescence plate reader and flow cytometer. The relative fluorescence unit of each sample was calculated relative to the fluorescence of negative control cells. This increase in value compared to the untreated promastigotes was viewed as an increase in intracellular ROS.

Determination of GSH level

Glutathione was determined by 5,5′-dithiobis (2-nitrobenzoic acid; DTNB) method (Mehta and Shaha 2004) using the glutathione detection kit produced by Nanjing Jiancheng Bioengineering Institute, China.

Statistics

The statistical significance was analyzed using Statistical Package for Social Sciences (SPSS) 15.0 software. To compare the two groups, p values were calculated by a paired two-tailed t test.

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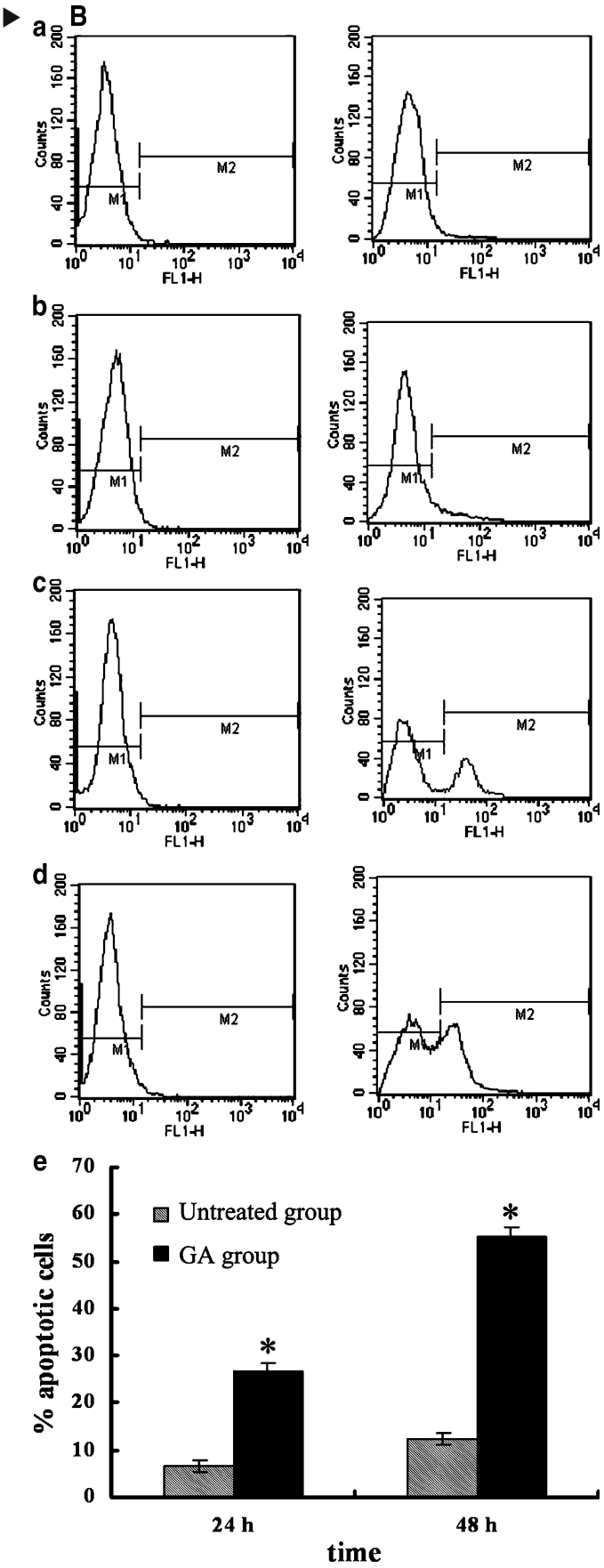

Results

GA-induced apoptosis in *L. donovani*

The morphology of treated cells was observed with Giemsa staining and scanning electron microscopy. Meanwhile, the apoptotic proportion of *L. donovani* cells was observed by TUNEL flow cytometry.

Visual inspection by light microscopy revealed the onset of cell shrinkage and cytoplasmic condensation, which could be observed in *L. donovani* promastigotes under treatment with 100 ng ml^{-1} GA for 24 h, while the group remained elongated and motile, as shown in Fig. 1a. Similarly, membrane blebbing in the parasite could be observed by scanning electron microscopy, as shown in Fig. 1b. Moreover, TUNEL-positive apoptotic cells (green) were obviously higher than the untreated group, 24 and 48 h after GA treatment, as shown in Fig. 1c.

With regard to the proportion of apoptotic cells, TUNEL flow cytometry examination also showed a significant increase in cells after 24 and 48 h following GA application. Hence, it was found that GA induced a dose- and time-dependant apoptosis to *L. donovani* promastigotes when cultures were in a high temperature and a low pH, as shown in Fig. 2a. It was found that there was an increased apoptosis for *L. donovani* promastigotes induced by GA, in which 50 ng ml^{-1} of GA leads to a 32.49% percentage of apoptosis at 48 h, whereas 100 and 200 ng ml^{-1} of GA leads to a 55.09% and 90.45%, respectively. According to the results and previous reports (Wiesgigl and Clos 2001a, b), 100 ng ml^{-1} of GA was used as treatment dose in the following experiments.

The proportion of apoptotic-positive cells treated with 100 ng ml^{-1} GA at 24 and 48 h was 26.84% and 55.09%, respectively, while the untreated group was 6.60% and 12.52%, as shown in Fig. 2b. The percentage of apoptotic-positive cells in GA-treated group was significantly higher than its counterpart induced by heat treat ($P<0.01$).

The data provided strong evidence that GA was able to induce an active apoptotic-like death process in *L. donovani* during its transformation stage.

GA arrests cell cycle progression of *L. donovani* promastigotes in G0/G1 phase

The changes in cell cycle progression of synchronized *L. donovani* promastigotes were examined by flow cytometry analysis with PI-stained cells after exposure to GA for 24 h at 37°C in pH5.5. After 24 h of GA treatment, most of the

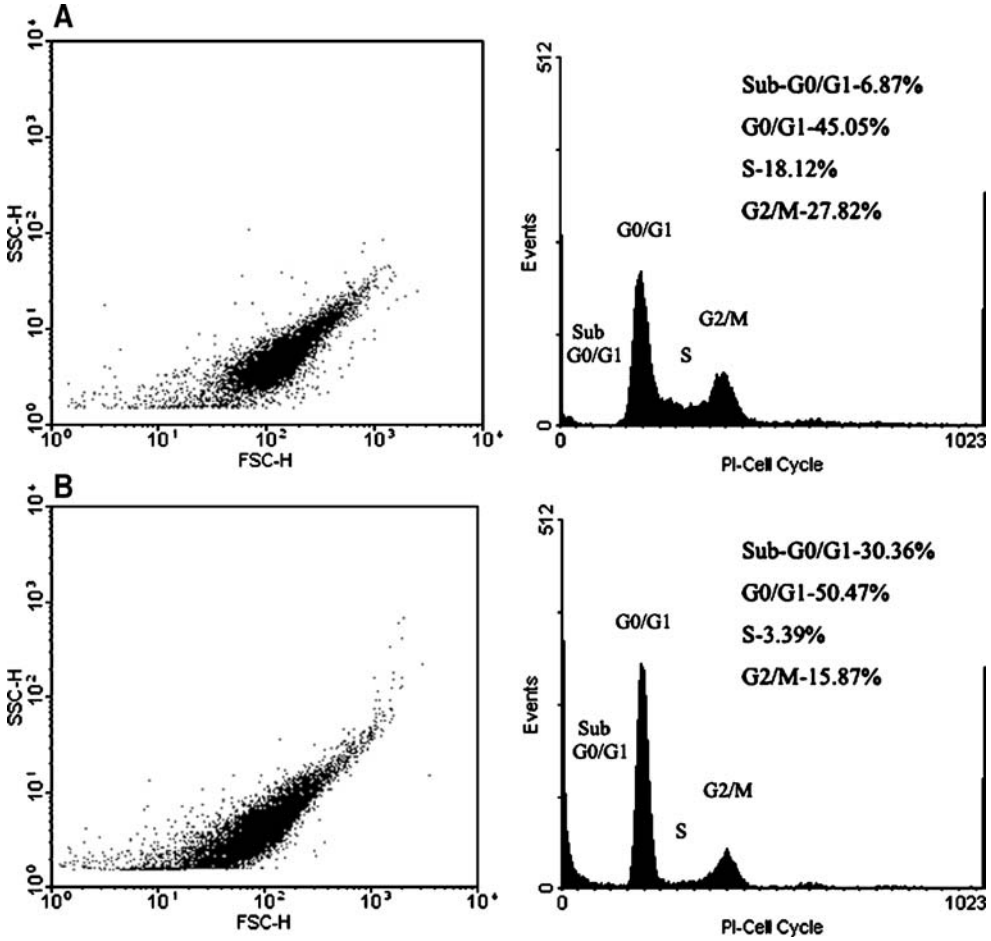

Fig. 3 Representative dot plots and histograms of cell cycle analysis by PI. Promastigotes growing logarithmically were incubated with 100 ng ml^{-1} of GA (GA group) or without GA (untreated group) at 37°C in pH5.5. The changes in cell cycle progression of synchronized *L. donovani* promastigotes were examined by flow cytometry analysis with PI-stained cells after treatment for 24 h. After 24 h of GA treatment, most of the cells were found to be arrested at the G0/G1 phase of the cell cycle. **a** *Left*: the dot plot of untreated group. *Right*: the histogram of untreated group. **b** *Left*: the dot plot of GA group. *Right*: the histogram of GA group. Percentages of cells in respective cell cycle stages are provided

cells were found to be arrested at the G0/G1 phase of the cell cycle. Thus, there was a significant increase at the expense of cells in the G0/G1 phase and a decrease in the S and G2/M phases when treated by a 100 ng ml^{-1} GA in contrast to untreated group, as shown in Fig. 3.

GA induced formation of ROS and diminished GSH content

Since GA treatment had an inhibitory effect upon cell cycle arrest and apoptotic-like death, the level of ROS production in promastigotes was investigated. Promastigote treatment by way of 100 ng ml^{-1} GA for different periods revealed a rise in ROS generation compared with untreated group, as shown in Fig. 4a.

Having found that a considerable generation of ROS took place after GA treatment, it was applicable to check GSH levels. Under normal physiological conditions, cellular ROS generation was offset by the action of antioxidant enzymes and other redox molecules like GSH. Surprisingly, a drop in GSH level was recorded after GA treatment compared with untreated group, as shown in Fig. 4b.

Antioxidants prevent GA-induced apoptosis

With the evidence that the ROS level in GA-treated parasites was considerably higher than those of untreated parasites and that GSH content decreased at 37°C and pH 5.5, it was compelling enough to test the possible role of oxidative stress in the induction of the GA-induced apoptotic-like death. Pretreatment of promastigotes with GSH (1 mM) or NAC (1 mM) for 1 h reduced the proportion of GA-induced apoptosis, as shown in Fig. 5a. Moreover, the cellular ROS level was lower while GSH

content was higher than that of the GA-treated group, and their difference was significant ($P<0.05$). These results suggested an important mediating role of ROS in cell death.

Role of pH during GA treatment

The percentage of TUNEL-positive cells were analyzed at 24 and 48 h in pH5.5 and 7.4, post-GA treatment, respectively, to test the effect of acidic conditions on GA-induced cell apoptotic process. The cellular level of ROS and GSH was also investigated. The results, as shown in Fig. 5b, indicated that parasites incubated at acidic conditions had lower apoptosis percentage at 37°C, and the ROS level of pH5.5 was lower than that of pH7.4. In contrast, GSH content was found to be higher. These data suggested that acidic environments having a high temperature might play a protective role during the GA-induced apoptotic process.

Discussion

Hsp90 is the most abundant heat shock protein in *Leishmania* and is involved in a variety of cellular processes (Brandau et al. 1995). In higher eukaryotes, Hsp90 is known to associate with cycle regulators and with transcription factors (Buchner 1999). GA is an Hsp90 binding drug which inhibits the specific chaperone function of this protein. The present study aims to find out more details of Hsp90 function in *L. donovani* promastigotes differentiation.

In *Leishmania*, the elevated temperature encountered during the transmission from a sand fly to a mammalian host is a key trigger of life cycle progression from the promastigote to the amastigote stage (Debrabant et al. 2004). In vitro, this effect can be mimicked by a pharmacological inhibition of Hsp90 with drugs such as GA and radicicol at 25°C (Wiesgigl and Clos 2001a, b). In contrast, *Trypanosoma cruzi* cells treated with GA were arrested in the G1 phase of the cell cycle but had not begun differentiating into relevant life cycle stages (Graefe et al. 2002). Previous studies showed that heat stress could trigger a process of programmed cell death in *L. infantum* promastigotes (Raina and Kaur 2006). Hence, the present research intended to analyze whether apoptosis or differentiation in *L. donovani* promastigotes could be induced by GA at a temperature of 37°C. The parasites, when treated with GA at 37°C, showed a round shape, shrinkage, and no motility. This change was different from the promastigotes. The results showed that GA could induce an apoptotic-like death to promastigotes but could not induce stage transformation at high temperature and that the effect was in a dose- and time-dependant manner. Moreover, it was found that the percentage of cell apoptosis generated by 100 ng ml^{-1} GA was significantly higher than that induced by heat stress at 37°C. Cell cycle analysis with PI showed a significant increase at the expense of cells in the G0/G1 phase when treated with GA. These various results indicated that the function of Hsp90 might vary in the two stages of *Leishmania*, i.e., in the different culture conditions. This finding further indicated that efforts should be made to develop drugs designed to target proteins of these parasites, which implied that the drug effect in different stage culture temperature should be considered.

Apoptosis involves a series of morphological and biochemical changes in which mitochondria serves as a major regulator of apoptosis (Hengartner 2000). ROS are important protozoal infection regulators. A previous study reported that heat-induced apoptotic-like death in *L. infantum* was mediated through mitochondrial superoxide (Alzate et al. 2007). Others showed that under oxidative stress, treatment with drugs led to apoptotic death in *Leishmania* (Luque-Ortega et al. 2001; Mukherjee et al. 2002). Furthermore, miltefosine treatment caused apoptosis in arsenite-resistant *L. donovani* through mitochondrial dysfunction (Verma et al. 2007). Das et al. (2008) reported that ROS and the imbalance of calcium homeostasis contributed to curcumin-induced apoptosis in *L. donovani.* The results of the present study indicated that GA had the ability to cause elevated ROS levels while GSH content decreased during *L. donovani* promastigote transformation. Treating promastigotes with GSH or NAC before GA resulted in the reduction of apoptotic cells. Consequently,

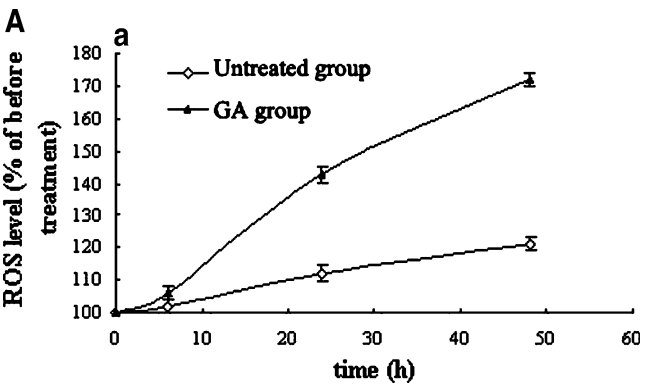

Fig. 4 ROS and GSH level at different time points. Promastigotes growing logarithmically were incubated with 100 ng ml^{-1} of GA (GA group) or without GA (untreated group) at 37°C in pH5.5. **a** (*a*): 107 cells were suspended in medium 199 and incubated with 10 μM DCFH-DA for 20 min at 37°C. The fluorescence was read at 485 nm for excitation and 530 nm for emission. This increase in value compared to untreated group was viewed as an increase in intracellular ROS. (*b*): Representative dot plots of ROS by flow cytometry analysis. *Left*: negative control (without DCFH-DA). *Right*: sample group (with DCFH-DA). **b** Glutathione was determined by DTNB method. Data shown are ratio compared with the content of promastigotes before treatment. Data are means±SE ($n=3$)

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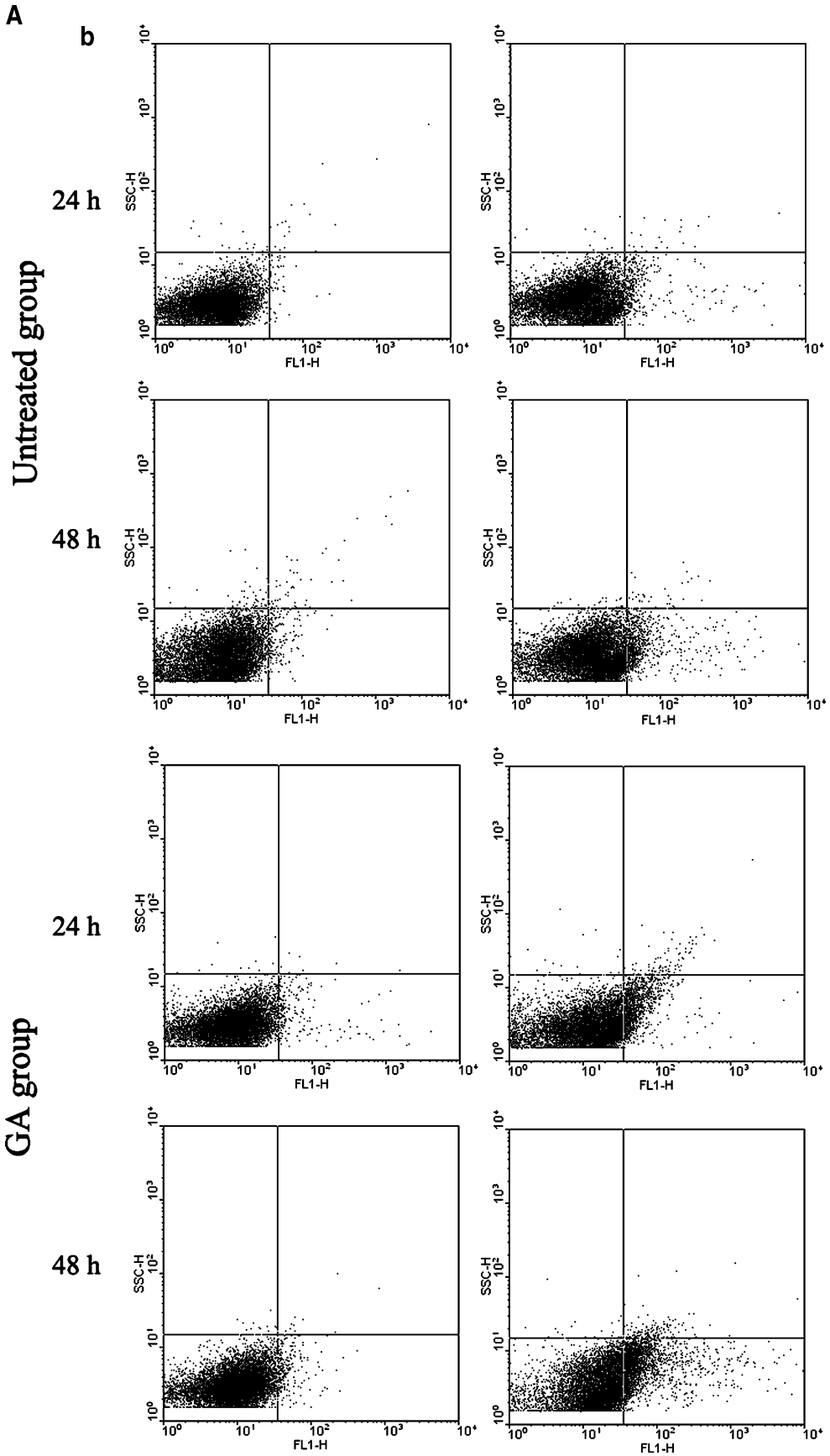

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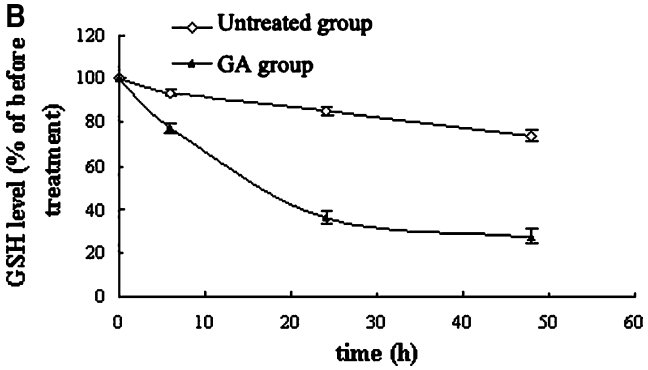

the cellular ROS level was lower, and GSH content was comparatively higher than the GA-treated group. These results suggested that GA might induce *L. donovani* promastigote mitochondria dysfunction at high temperatures.

Again, a previous study demonstrated that parasites incubated at acidic conditions are slightly more prone to death after a 12-h heat shock (Alzate et al. 2007). The known data implied that acidic pH does not induce Hsp synthesis in *L. donovani* (Clos et al. 1998). The percentage of TUNEL-positive cells were analyzed at 24 and 48 h in pH5.5 and 7.4, post-GA treatment, respectively, to test the effect of acidic conditions on GA-induced cell apoptotic-like death process. The cellular level of ROS and GSH was also investigated. The result showed that GA-treated parasites incubated at acidic conditions had lower apoptosis percentages at 37°C in 24 and 48 h, respectively. Thus, the

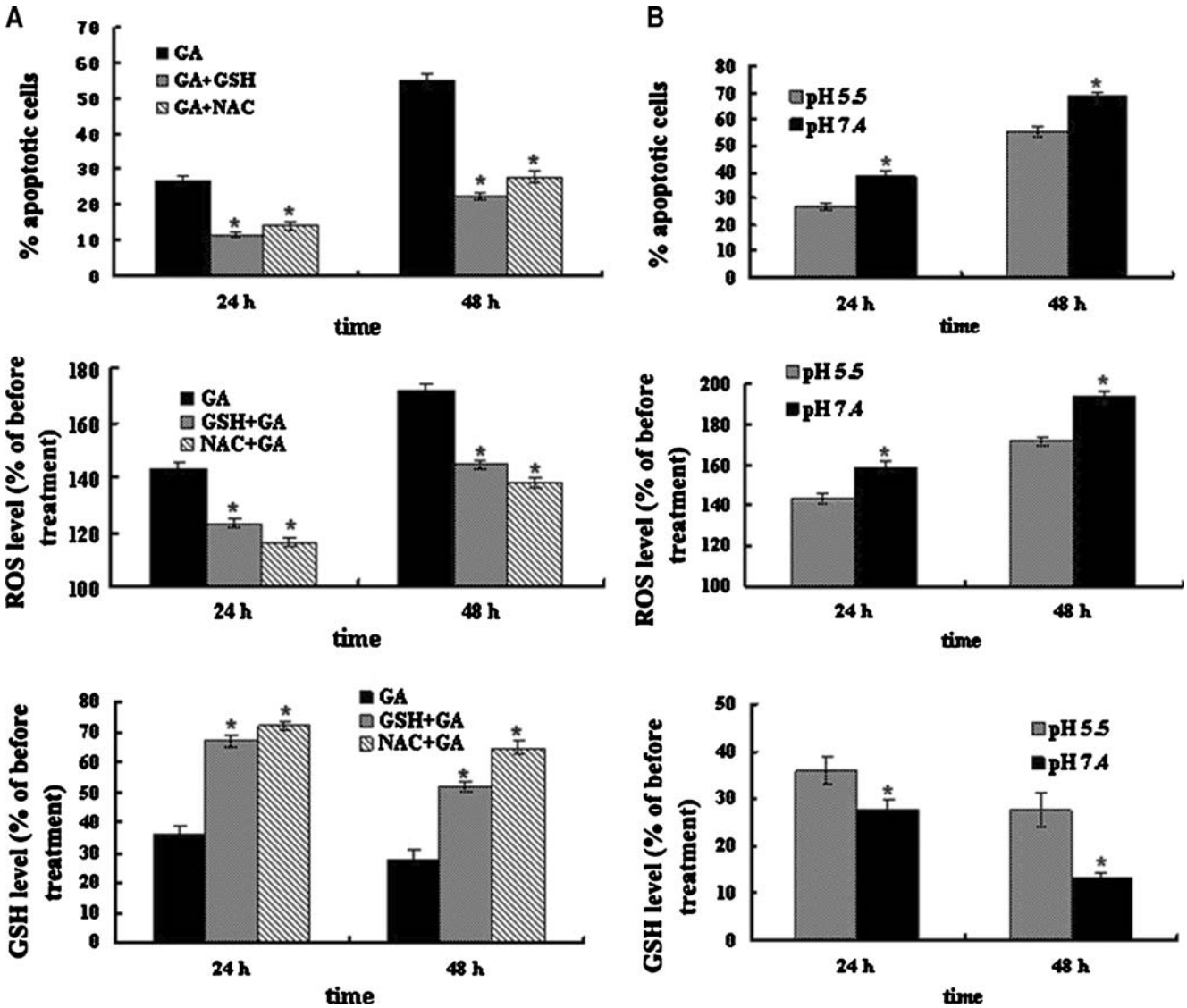

Fig. 5 Effects of antioxidants and pH on the parasite during GA treatment. The percentage of TUNEL-positive cells, ROS level, and GSH content in 24 and 48 h after treatment are shown. Data are means±SE (n=3). *p<0.05 compared with GA pH5.5 group

ROS level of pH5.5 was lower than that of pH 7.4, but the GSH content was higher, which suggested that the decreasing pH during the GA-induced apoptosis could protect the cells. These results also suggested that there is a difference between heat-shock- and GA-induced apoptosis. Perhaps, the pH of the media plays a complex role during cell differentiation.

In conclusion, the present study provides evidence of a connection between ROS level, GSH content, and cell apoptosis during GA treatment. In addition, GA may induce *L. donovani* promastigotes apoptotic-like death through mitochondria dysfunction at a high temperature. The pH of the media may play an important role during this process. These results provide further evidence that both Hsp90 and acidic conditions are likely to be essential to the transformation and survival of the parasite within its human host.

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