Original Paper

Journal of Biomedical Science

J Biomed Sci 2004;11:847–854
DOI: 10.1159/000081832

Received: June 25, 2004
Accepted: July 19, 2004

Hypoxia Modulates Expression of the 70-kD Heat Shock Protein and Reduces *Leishmania* Infection in Macrophages

Adriana Degrossoli Marcelle Carolina Colhone Wagner Welber Arrais-Silva
Selma Giorgio

Department of Parasitology, Biology Institute, Universidade Estadual de Campinas, Campinas, Brazil

Key Words
Macrophage · Hypoxia · HSP70 · *Leishmania*

Abstract
Hypoxia, a microenvironmental factor present in diseased tissues, has been recognized as a specific metabolic stimulus or a signal of cellular response. Experimental hypoxia has been reported to induce adaptation in macrophages such as differential migration, elevation of proinflammatory cytokines and glycolytic enzyme activities, and decreased phagocytosis of inert particles. In this study we demonstrate that although exposure to hypoxia (5% O_2, 5% CO_2, and balanced N_2) did not change macrophage viability, or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cleavage and proliferation, it significantly reduced expression of the 70-kD heat shock protein (HSP70), which was restored to prehypoxia levels after reoxygenation. The influence of low oxygen tension on macrophage functional activity was also studied, i.e. the ability of these cells to maintain or resist infection by a microorganism. We demonstrate that macrophages from two different sources (a murine cell line and primary cells) exposed to hypoxia were efficiently infected with *Leishmania amazonensis*, but after 24 h showed a reduction in the percentage of infected cells and of the number of intracellular parasites per macrophage, indicating that hypoxia induced macrophages to kill the intracellular parasites. These results support the notion that hypoxia, a microenvironmental factor, can modulate macrophage protein expression and functional activity.

Introduction

Macrophages are cells involved in the clearance of microorganisms, antigen processing/presentation, angiogenesis, and many other functions. In various disease states, such as fibrosis, dermal wounds, and neoplasias, macrophages accumulate in or adjacent to poorly vascularized hypoxic sites [19]. Experimental hypoxia has been reported to induce phenotypic adaptations in macrophages, including differential migration, elevation of proinflammatory cytokines and glycolytic enzyme activities, and decreased phagocytosis of inert particles [7, 10, 19]. Although these modulations induced by hypoxia may be particularly important in bacterial and parasitic infections that accompany tissue ischemia [19] and macrophages are known to play an important role in host defense, no study has been conducted to establish the effects of hypoxia on macrophage infection by microorganisms.

KARGER
Fax + 41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1021–7770/04/0116–0847$21.00/0
Accessible online at:
www.karger.com/jbs

Selma Giorgio, PhD
Department of Parasitology, Biology Institute, Universidade Estadual de Campinas
Caixa Postal 6109
Cep 13083-970, Campinas, SP (Brazil)
Tel. +55 19 37886287, Fax +55 19 32393124, E-Mail sgiorgio@unicamp.br

Recent reports have proposed that cells, such as cultured rat cardiomyocytes and astrocytes, and human hepatoma cells, protect against hypoxia by enhanced synthesis of heat shock proteins, mainly the 70-kD heat shock protein (HSP70) [13, 30, 35]. Interestingly, hypoxia reduces the expression of HSP70 in human endothelial cells and atrial tissue [25, 27]. In the only study performed with macrophages, low levels of HSP70 were observed in cells exposed to a single period of hypoxia when compared with cells subjected to repeated cycles of hypoxia/reoxygenation [38].

Thus, to gain more insights into the influence of low oxygen tension on macrophage phenotypes, we determined whether hypoxia alters the expression of HSP70 in these cells and also examined whether hypoxia affects macrophage susceptibility to an intracellular pathogen. We used *Leishmania amazonensis*, an intracellular parasite that causes human diseases ranging from localized to diffuse cutaneous infections [11], as an in vitro model to test macrophage host defense. During the life cycle of *L. amazonensis*, the parasite replicates within macrophages [12].

Methods

Cell Culture and Parasites

The murine macrophage cell line, J774, obtained from the American Type Culture Collection (Rockville, Md., USA) was maintained in RPMI medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma Aldrich, St. Louis, Mo., USA), and 10% fetal calf serum (Cultilab, Campinas, Brazil) at 37°C in 5% CO_2, 5% O_2 and balanced N_2 [20]. Primary mouse macrophages were obtained from normal BALB/c mice by peritoneal lavage [3, 9]. *Leishmania amazonensis* (MHOM/BR/73/M2269) amastigote forms were isolated from active skin lesions of BALB/c mice as described previously [3].

Normoxic and Hypoxic Conditions

Macrophages ($1–5 \times 10^5$/ml) were added to 25-cm^2 culture flasks or 24-well culture plates containing 13-mm-diameter glass coverslips. The cell cultures were then placed in a gas-tight modular chamber (Billups-Rothenberg, Del Mar, Calif., USA). The chamber was gassed for 15 min at a flow rate of 2 liters/min using certified gases containing O_2, CO_2, and N_2 (White Martins, Campinas, Brazil), and placed in a 37°C temperature-controlled incubator. The percentage of O_2 was verified by measuring the outflow of gas at the end of the initial flushing period and then at 24-hour intervals using a Fyrite apparatus (Bacharach, Pittsburgh, Pa., USA). The oxygen tension in the culture medium was 37 mm Hg under hypoxic conditions and 150 mm Hg under the normoxic condition (O_2 analyzer YSI/53, Yellow Springs Instruments, Yellow Springs, Ohio, USA). The pH of the medium was 7.4, and it did not change significantly during the course of the experiments. In all experiments, exposure of cells to 5% O_2, 5% CO_2, and balanced N_2 is referred to as hypoxia and exposure of cells to 21% O_2, 5% CO_2, and balanced N_2 is referred to as normoxia. Cell viability was tested by the trypan blue exclusion method, the adherence ability, cell growth, and the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye reduction assay, which results in the production of formazan by mitochondria (Sigma Aldrich) [26]. The viability of *Leishmania* amastigotes exposed to normoxic and hypoxic conditions was determined by erythrosine B staining (Sigma Aldrich) [20], the MTT assay, and by the ability to transform amastigotes into promastigote forms at 26°C [18, 26].

Macrophage Infection, and Activation and Assessment of Intracellular Parasites

J774 and peritoneal macrophages were infected by adding to the cell cultures a suspension of living *Leishmania* amastigotes in RPMI supplemented with antibiotics with 10- and 3-fold excesses of parasites for 24 and 1 h, respectively. After the infection period, cultures were washed to remove extracellular parasites and replaced with fresh medium (150 mm Hg oxygen tension) for the time of each experiment in the normoxic condition and with fresh medium (37 mm Hg oxygen tension) for the time of each experiment in the hypoxic condition. Intracellular parasite destruction was assessed by morphological examination. Briefly, for evaluating the percentage of infected macrophages and the number of amastigotes per macrophage, cells on coverslips were stained with Giemsa [9]. Intracellular amastigotes, which are exclusively localized in parasitophorous vacuoles [5], were examined microscopically at a magnification of 600× and photographed with Image Pro Plus 2000 (Media Cybernetics, Silver Spring, Md., USA). About 600 cells were counted per triplicate coverslip. The killing of intracellular parasites was also quantified by a slight modification of a described technique [23]. By this method, macrophage cultures were washed and then lysed with 0.01% sodium dodecyl sulfate (SDS), which released the amastigotes. Viability was determined by erythrosine B staining [21]. Amastigotes were also left to transform into promastigote forms, and after 3 days at 26°C, the number of promastigotes was recorded by microscopic observation. For the experiments of macrophage activation, cells were treated with 20 ng/ml recombinant mouse gamma interferon (IFN-γ) and 10 ng/ml *Escherichia coli* lipopolysaccharides (L4391; LPS; Sigma Aldrich), 8 h before the *Leishmania* infection [20].

Immunoblotting Analyses

After different treatments, cells were scraped from culture flasks, checked for viability, and then rinsed twice with PBS. Lysis buffer (62.5 m*M* Tris-HCl, pH 6.8, 69 m*M* SDS, 10% glycerol, 2% 2-mercaptoethanol, 34 m*M* ethylenediaminetetraacetic acid, 2 μg/ml pepstatin, and 1 m*M* phenylmethylsulfonyl fluoride; Amersham Pharmacia Biotech, Piscataway, N.J., USA) was added to the cell pellets. Proteins were denatured at 95°C for 3 min, eletrophoresed on a 10% SDS-PAGE (polyacrylamide) gel system (Thermo EC, Holbrook, N.Y., USA) and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech). After blotting, membranes were incubated with mouse monoclonal anti-HSP70 antibody (Sigma Aldrich). The secondary antibody consisted of peroxidase-conjugated rabbit anti-mouse IgG (Sigma Aldrich), and it was developed with 3,3-diaminobenzidine. Immunoreaction images were scanned, and the densitometric value of each band was determined using Image Master Total Lab version 1 software (Amersham Pharmacia Biotech). For heat shock induction, macrophages were incubated in a water bath at 44°C for 30 min, followed by a 6-hour recovery period at 37°C [34].

Statistical Evaluation

All experiments were repeated at least 3 times, and the results are expressed as the mean ± SD. Data obtained under different conditions (hypoxia and normoxia; noninfection and infection) were analyzed statistically by Student's t test, with levels of significance set at $p \leq 0.01$ for in vitro assays and $p \leq 0.05$ for immunoblotting analyses.

Results

Effect of Hypoxia on Macrophage Viability

Macrophage viability as tested by the trypan blue exclusion method, cell growth, and the colorimetric MTT dye reduction assay was determined under normoxic and hypoxic conditions. Viability was approximately 90%, and the cell growth rates were similar in J774 macrophage cultures under both conditions (fig. 1a). Formazan production by macrophage mitochondria evaluated by the MTT assay was also similar in cells exposed to normoxia and hypoxia (fig. 1b). These data indicate that macrophages not only tolerate hypoxia but also proliferate and have normal mitochondrial function in the presence of 5% oxygen tension.

Effect of Hypoxia on Macrophage HSP70 Expression

To examine a possible role of HSP70 in hypoxia-induced tolerance in macrophages, we analyzed the HSP70 expression level of cells exposed to both normoxia and hypoxia. As shown in the Western blot presented in figure 2, HSP70 was constitutively expressed in J774 cells, and, as expected, a 2-fold increase in HSP70 was observed after heat stress (fig. 2a, lanes 1 and 2). In contrast to the effect of heat stress, exposure of J774 cells to hypoxia for 24 h significantly reduced HSP70 expression (about 50% of the normoxic control) (fig. 2a, lane 3; fig. 2b). HSP70 downregulation was due to its reduced production rather than to its release in the medium, since we did not detect HSP70 by Western blot in supernatants of macrophages cultured under hypoxia (data not shown). In addition, cell preparations obtained from macrophages cultured under normoxia and those under hypoxia contained the same amount of proteins and showed a closely similar protein pattern as analyzed by SDS-PAGE (data not shown).

To test whether the reduction in HSP70 expression found in hypoxia-treated J774 macrophages was due to the general inability of these cells to express HSP70 after a hypoxic period, we analyzed the expression of this heat shock protein in macrophages cultured in hypoxia for 24 h and then incubated in normoxia for an additional 24 h. Cells subjected to this condition recovered and reached prehypoxia levels of HSP70 (fig. 2a, lane 4; fig. 2b).

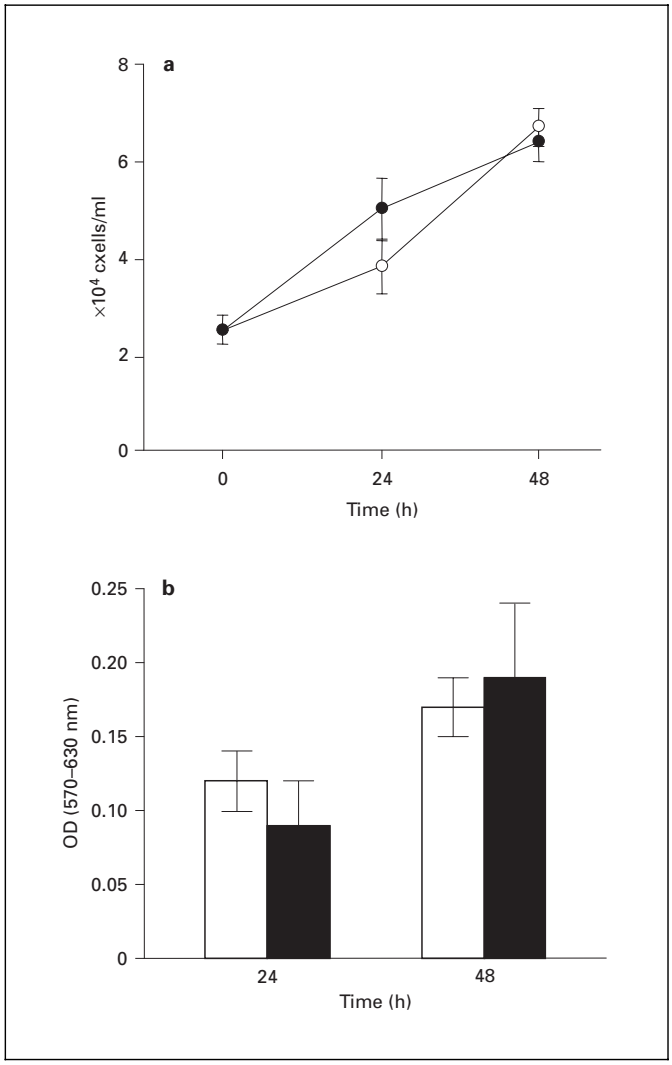

Fig. 1. Effect of hypoxia on J774 macrophages. **a** Numbers of viable macrophages determined in cell cultures maintained in normoxia (●) or hypoxia (○) for the times indicated. **b** MTT production by macrophages determined in cell cultures maintained in normoxia (□) or hypoxia (■) for the times indicated. Results represent the mean ± SD of 1 representative experiment out of 3 independently performed experiments with similar results.

Effect of Hypoxia on Macrophage Susceptibility to Leishmania

To assess whether hypoxia also has an effect on macrophage susceptibility to an intracellular pathogen, we used cells from two different sources, the J774 cell line and pri-

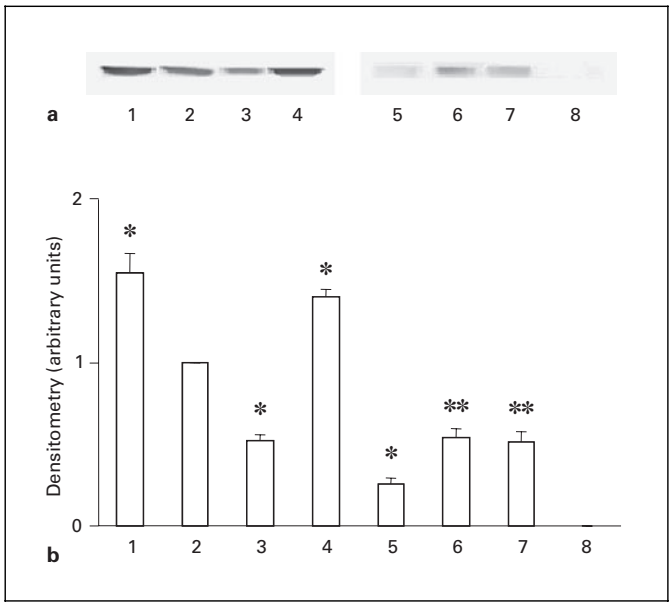

Fig. 2. Western blot analysis of HSP70 in J774 macrophages. **a** Macrophages exposed to heat shock (lane 1), cultured in normoxia for 24 h (lane 2), cultured in hypoxia for 24 h (lane 3), cultured in hypoxia for 24 h and then maintained in normoxia for an additional 24 h (lane 4), infected with *Leishmania* and cultured in normoxia for 24 h (lane 5), infected with *Leishmania* and cultured in hypoxia for 24 h (lane 6), activated with IFN-γ and LPS, infected with *Leishmania* and cultured in normoxia for 24 h (lane 7), and *Leishmania* amastigotes (lane 8). HSP70 was detected by Western blotting as described in Methods. **b** Graph showing the results of Western blots analyzed by densitometry of independent experiments. The numbers on the x-axis correspond to the numbers given in **a**. Bars represent the mean ± SD of the relative optical density normalized to the control (column 2; macrophages cultured in normoxia). * $p \leq 0.05$ compared with the control (column 2); ** $p \leq 0.05$ compared with macrophages infected with *Leishmania* and cultured in normoxia (column 5).

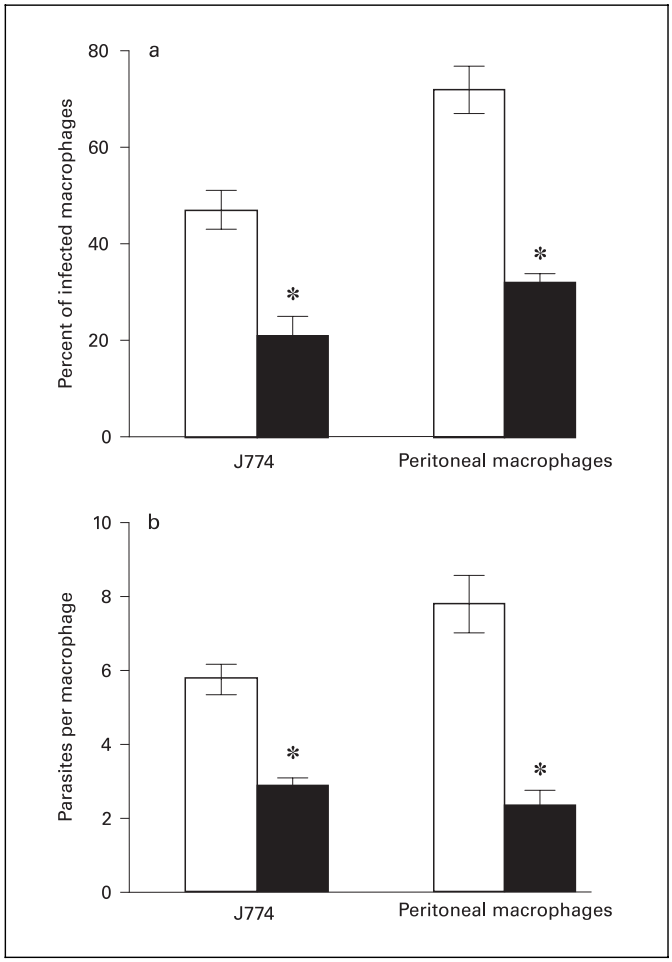

Fig. 3. Effect of hypoxia on *Leishmania* infection in macrophages. Peritoneal and J774 macrophages were infected with *Leishmania* amastigotes and cultured in normoxia (□) or hypoxia (■) for 24 h. The percentage of infected macrophages (**a**) and the number of amastigotes per macrophage (**b**) were determined as described in Methods. The results represent the mean ± SD of 3 experiments. The significance of the difference between cell cultures in hypoxia and normoxia is indicated: * $p < 0.01$.

mary macrophages obtained from the peritoneum of mice, for infection with *Leishmania*. Both cell systems were efficiently infected with *Leishmania* under normoxia or under hypoxia (data not shown). Cell cultures infected with *Leishmania* and maintained under normoxia for 24 h, as expected, showed a significantly high number of infected macrophages (47% for J774 cultures and 72% for peritoneal macrophage cultures) as well as intracellular parasites (5.7 for J774 cells and 7.8 for peritoneal macrophages) (fig. 3). However, in cultures subjected to hypoxia for 24 h, there was a significant reduction in the percentage of infected macrophages (21% for J774 cultures and 32% for peritoneal macrophages) and also in the number of parasites inside the cells (2.8 for J774 cells and 2.3 for peritoneal macrophages) when compared with the normoxic condition (fig. 3). Interestingly the rate of reduction of infection observed in cells under hypoxia was comparable to that obtained for macrophages stimulated with IFN-γ and LPS, a well-known system which activates killing of *Leishmania* in macrophages (data not shown) [6, 20, 29].

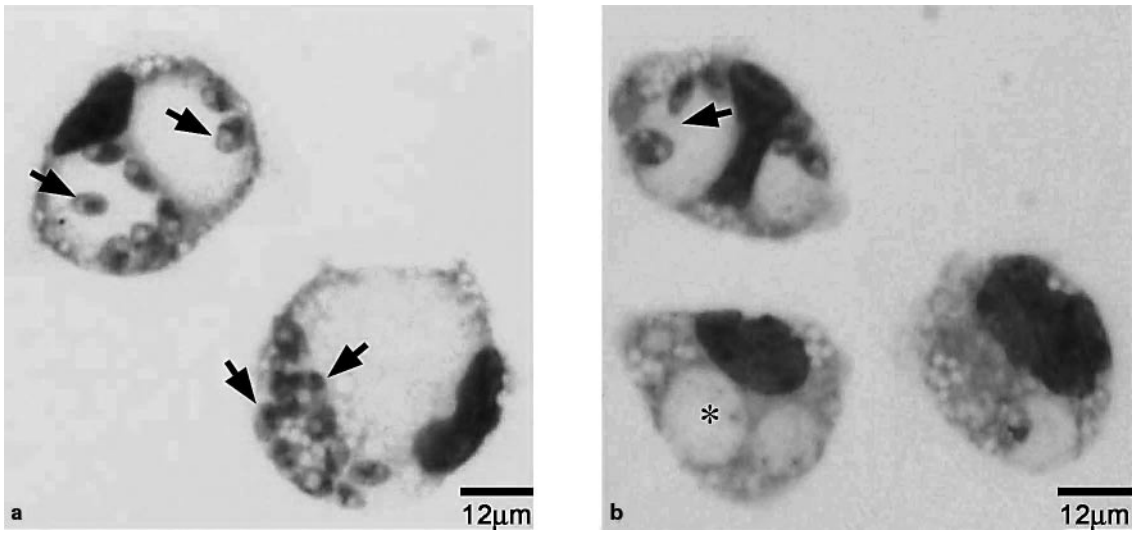

Fig. 4. Photomicrographs of peritoneal macrophages infected with *Leishmania*. **a** Macrophage culture infected with *Leishmania* and maintained in normoxia for 24 h. Arrowheads indicate intracellular parasites inside vacuoles. **b** Macrophage culture infected with *Leishmania* and maintained in hypoxia for 24 h. Arrowheads indicate intracellular parasites inside vacuoles. The asterisk indicates a vacuole containing no parasites.

Light microscopy demonstrated the ability of *Leishmania* to infect macrophages (fig. 4). After 24 h of infection, cells cultured in normoxia had large parasitophorous vacuoles containing intact intracellular parasites, which are characteristics of infection with *L. amazonensis* (fig. 4) [5]. In contrast, most of the macrophages in the population exposed to hypoxia for 24 h had reduced infection and had vacuoles with few intracellular parasites or had cleared their infection and had vacuoles containing no parasites (fig. 4b). In addition, the killing of intracellular parasites which occurred in macrophages could also be assessed by quantification of viable parasites recovered after lysis of infected cells with 0.01% SDS. A significant 30% reduction in the number of viable *Leishmania* released from macrophages under hypoxia was observed when compared with the number of viable parasites released from macrophages under normoxia. These results indicate that hypoxia induces macrophages to kill intracellular parasites.

Effect of Hypoxia on HSP70 Expression of Leishmania-Infected Macrophages

Our results indicated that hypoxia decreased HSP70 expression in macrophages (fig. 2) and induced resistance to parasitic *Leishmania* infection (fig. 3, 4). Previous studies revealed that HSP70 expression declines as the infection progresses in macrophages parasitized with *Leishmania* in a normoxic condition [1, 14]. We thus investigated the effect of hypoxia on HSP70 expression in macrophages infected with *Leishmani*a and its correlation with cellular control of infection. As shown in figure 2, the results confirmed data from the literature [1, 14] showing that expression of HSP70 was significantly reduced in cells infected with the parasite under normoxic conditions (a drop of 80% in HSP70 expression in uninfected cells under normoxia). Interestingly, macrophages maintained in hypoxia and infected with *Leishmania*, which resisted the infection and killed the parasites, showed an HSP70 expression level similar to that of uninfected cells in hypoxia (fig. 2a, lanes 6 and 3). To confirm this hypothesis, i.e., that macrophages resisting infection tended to express normal levels of HSP70, we used Western blotting to analyze *Leishmania*-infected macrophages activated with IFN-γ and LPS, cells that kill intracellular parasites [6, 20, 29]. In fact, the results showed that these cells expressed HSP70 at higher levels than macrophages with progressing infection (fig. 2a, lanes 7 and 5). It should be noted that the antibody against HSP70 used in the Western blot assays did not recognize any *Leishmania* proteins, as shown in figure 2a, lane 8. This antibody thus allowed us to discriminate between macrophage HSP70 and parasitic proteins in *Leishmania*-infected cells.

Discussion

In the present study we provide evidence that reduced oxygen tension, although it did not alter macrophage viability, MTT cleavage, or proliferation (fig. 1), significantly affected HSP70 expression and the resistance to *Leishmania* infection (fig. 2–4).

Hypoxia and ischemia regulate a subset of proteins in a cell type- or tissue-dependent manner, mainly enzymes of energy metabolism and stress-related proteins [16]. HSP70, one of the best-characterized heat shock proteins, provides cells with enhanced resistance to heat, hyperosmolarity, exposure to oxidative injury, infection with certain microorganisms, and hypoxia [2, 15]. Recent studies have suggested a positive correlation between HSP70 expression and protection against cellular damage induced by hypoxia in most of the cell systems evaluated [13, 16, 30, 35]. In contrast, we found that HSP70 expression was reduced in macrophages during hypoxia (fig. 2). Our results are consistent with an earlier observation in which RAW murine macrophages exposed to hypoxia were reported to express reduced levels of HSP70 when compared with macrophages exposed to normoxia and selected by hypoxia/reoxygenation cycles [38]. Although those authors suggested that low levels of HSP70 coincide with apoptosis detected in macrophages exposed to hypoxia, we exclude this hypothesis for explaining our results, since a higher tension (5 vs. 2% O_2) than that used by those authors [37] caused no significant changes in J774 macrophage viability, but downregulated HSP70 expression (fig. 2). Similar observations were made in endothelial cells, which are tolerant to hypoxia and which reduce HSP70 to levels observed under low oxygen tension [27]. Endothelial cells as well as macrophages are, in contrast to most other mammalian cell types, well adapted to low oxygen levels by changing their metabolism to anaerobiosis glycolysis [8, 19, 32]. Thus, we may assume that these cells are not stressed by this condition, which may be the reason why they do not stimulate HSP70 in response to hypoxia. It is also possible that HSP70 expression, which is known to exert protective effects against oxidative injury [2, 15], is unnecessary during hypoxia since a cellular defense mechanism may no longer be needed when oxygen is reduced.

The influence of low oxygen tension on macrophage functional activity was also studied, i.e., the ability of these cells to maintain or resist infection by a microorganism. The interaction between macrophages and *Leishmania* is a well-established in vitro model of intramacrophage parasitism [12], and we demonstrated that macrophages from two different sources (a cell line and primary cells) exposed to hypoxia were efficiently infected with *Leishmania*, but after 24 h showed a reduced percentage of infected cells and number of intracellular parasites per macrophage (fig. 3). In addition, a significant reduction was observed in the viability of parasites released from macrophages cultured under hypoxia, and many cells had cleared their infection and had vacuoles containing no parasites (fig. 4). These results indicate that hypoxia induced macrophages to kill intracellular parasites and consequently to control the *Leishmania* infection rate (the percentage of infected cells and the average number of parasites per macrophage). Interestingly, the observations that under normoxia, macrophages infected with *Leishmania* exhibited remarkably diminished expression of HSP70, which is attributed to interference by the parasite with host intracellular signaling pathways [1, 12, 14], were not made in macrophages infected with *Leishmania* under hypoxia or in cells stimulated with IFN-γ and LPS and infected with the parasite (fig. 2). Cells infected with *Leishmania* under hypoxia were capable of killing intracellular parasites (fig. 3, 4) and tended to express HSP70 at levels similar to those expressed by uninfected macrophages cultured under hypoxia (fig. 2). These data confirm previous reports demonstrating that *Leishmania* inhibits HSP70 in macrophages [1, 13] and indicate that under hypoxia, these cells reduce the number of intracellular parasites and consequently reduce the effects of parasites on the host's HSP70-related signaling pathways.

There are no studies on the effect of hypoxia on macrophage infection by a microorganism available for comparison with our results. However, various studies have evaluated the effect of hypoxia on phagocytic activity of macrophages. For example, alveolar macrophages showed reduced retention of red blood cells; peritoneal macrophages reduced phagocytosis of zymozan particles and latex beads under hypoxia, and reperfusion after hypoxia caused a 3-fold induction in colloidal carbon uptake by Kupffer cells [17, 22, 31]. Although the mechanism by which macrophages alter the uptake and clearance of particles under hypoxia is not known, it has been hypothesized that metabolic alterations such as changes in intracellular ATP lead to the loss of previously phagocytosed inert particles and the release of lysosomal contents [17, 19]. The data presented here do not explain the mechanism by which macrophages are able to control *Leishmania* infection in hypoxia. Various antimicrobial mechanisms of macrophages that have been well described in normoxia could be involved in macrophage activation in hypoxia. These mechanisms are the production of degra-

dative enzymes such as proteases, nucleases, and lipases, highly basic antimicrobial peptides, respiratory bursts, reactive nitrogen intermediates, and a deprivation mechanism that starves pathogens of essential nutrients [33]. The main effectors responsible for killing *Leishmania* in macrophages activated with cytokines and LPS are reactive oxygen intermediates and reactive nitrogen intermediates [12, 33]. Although we cannot exclude the participation of nitric oxide in the anti-*Leishmania* activities of macrophages under hypoxia, this seems unlikely since nitric oxide synthesis decreases in proportion to oxygen tension [24, 28]. However, it is tempting to speculate that under hypoxia, oxygen reactive species are involved in anti-*Leishmania* activities of macrophages since other investigators have demonstrated that hypoxia itself increases the production of oxygen-reactive species in J774 macrophages, cardiac myocytes, and neuronal-like cells [4, 36, 37]. Further investigations are needed to clarify which mechanisms contribute to the resistance of macrophages to *Leishmania* infection under hypoxic conditions.

We conclude that macrophages are tolerant to 5% O_2 hypoxia, which reduces HSP70 expression. Furthermore, we show that hypoxia acts on macrophages by inducing the killing of the *Leishmania* parasite. These results support the notion that hypoxia, a microenvironmental factor present in diseased tissues, modulates macrophage protein expression and functional activity.

Acknowledgments

This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo and Coordenadoria de Aperfeiçoamento de Pessoal de Nivel Superior, Brasil. The authors thank Ms. E. Green for reviewing the English of the manuscript.

References

1 Adhuna PS, Mukhopadhyay B, Bhatnagar R. Modulation of macrophage heat shock proteins (HSPs) expression in response to intracellular infection by virulent and avirulent strains of *Leishmania donovani*. Biochem Mol Biol Int 43:1265–1275;1997.
2 Bachelet M, Adrie C, Polla BS. Macrophages and heat shock proteins. Res Immunol 149: 727–732;1998.
3 Barbieri CL, Giorgio S, Merjan AJ, Figueiredo EN. Glycosphingolipid antigens from *Leishmania (Leishmania) amazonensis* amastigotes identified by use of a monoclonal antibody. Infect Immun 61:2132–2137;1993.
4 Chandel NS, Trzyna WC, McClintock DS, Schumacker PT. Role of oxidants in NF-kB activation and TNF-α gene transcription induced by hypoxia and endotoxin. J Immunol 165:1013–1021;2000.
5 Chang K-P. Human cutaneous *Leishmania* in a mouse macrophage line: Propagation and isolation of intracellular parasites. Science 209: 1240–1242;1980.
6 Corradin SB, Mauel J. Phagocytosis of *Leishmania* enhances macrophage activation by IFN-γ and lipopolysaccharide. J Immunol 146: 279–285;1991.
7 Crowther M, Brown NJ, Bishop ET, Lewis CE. Microenvironmental influence on macrophage regulation of angiogenesis in wounds and malignant tumors. J Leukoc Biol 70:478–490; 2001.
8 Firth J, Ebert B, Ratcliffe P. Hypoxic regulation of lactate dehydrogenase A. J Biol Chem 270:21021–21027;1998.
9 Giorgio S, Barão SC, Augusto O, Kwee JK. *Leishmania amazonensis* infection is reduced in macrophages treated with guanine ribonucleosides. Acta Trop 70:119–122;1998.
10 Griffiths L, Binley K, Iqball S, Kan O, Maxwell P, Ratcliffe P, Lewis C, Harris A, Kingsman S, Naylor S. The macrophage: A novel system to deliver gene therapy to pathological hypoxia. Gene Ther 7:255–262;2000.
11 Grimaldi G Jr, Tesh RB. Leishmaniases of the new world: Current concepts and implications for future research. Clin Microbiol Rev 6:230–250;1993.
12 Handman E. Cell biology of *Leishmania*. Adv Parasitol 44:1–39;1999.
13 Iwaki K, Chi S, Dillmann W, Mestril R. Induction of HSP70 in cultured rat neonatal cardiomyocytes by hypoxia and metabolic stress. Circulation 87:2023–2032;1993.
14 Kantengwa S, Muller I, Loius J, Polla B. Infection of human and murine macrophages with *Leishmania major* is associated with early parasite heat shock protein synthesis but fails to induce a host cell stress response. Immunol Cell Biol 73:73–80;1995.
15 Kiang JG, Tsokos GC. Heat shock protein 70 kDa: Molecular biology, biochemistry and physiology. Pharmacol Ther 80:183–201; 1998.
16 Kumar GK, Klein JB. Analysis of expression and posttranslational modification of proteins during hypoxia. J Appl Physiol 96:1178–1186; 2004.
17 Leeper-Woodford SK, Mills J. Phagocytosis and ATP levels in alveolar macrophages during acute hypoxia. Am J Respir Cell Mol Biol 6: 326–334;1992.
18 Lemesre J-P, Sereno D, Dauloude S, Veyret B, Brajon N, Vincendeau P. *Leishmania* spp.: Nitric oxide-mediated metabolic inhibition of promastigote and axenically grown amastigote forms. Exp Parasitol 86:58–68;1997.
19 Lewis JS, Lee JA, Underwood JAC, Harris A, Lewis CE. Macrophage responses to hypoxia: Relevance to disease mechanisms. J Leukoc Biol 66:889–900;1999.
20 Linares E, Augusto O, Barão SC, Giorgio S. *Leishmania amazonensis* infection does not inhibit systemic nitric oxide levels elicited by lipopolysaccharide in vivo. J Parasitol 86:78–82;2000.
21 Linares E, Giorgio S, Mortara RA, Santos CXC, Yamada AT, Augusto O. Role of peroxynitrite in macrophage microbicidal mechanisms in vivo revealed by protein nitration and hydroxylation. Free Radic Biol Med 30:1234–1242;2001.
22 Lindert KA, Caldwell-Kenkel C, Nukina S, Lemasters JJ, Thurman RG. Activation of Kupffer cells on reperfusion following hypoxia: Phagocytosis in a low-flow, reflow model. Am J Physiol 262:G345–G350;1992.
23 Mauel J. Intracellular parasite killing induced by electron carriers. I. Effect of electron carriers on intracellular *Leishmania* spp. in macrophages from different genetic backgrounds. Mol Biochem Parasitol 13:83–96;1984.
24 McCormick CC, Li WP, Calero M. Oxygen tension limits nitric oxide synthesis by activated macrophages. Biochem J 350:709–716;2000.

25 Mohan RM, Golding S, Paterson DJ. Intermittent hypoxia improves atrial tolerance to subsequent anoxia and reduces stress protein expression. Acta Physiol Scand 172:89–95;2001.
26 Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63;1983.
27 Oehler R, Schmierer B, Zellner M, Prohaska R, Roth E. Endothelial cells downregulate expression of 70 kDa heat shock protein during hypoxia. Biochem Biophys Res Commun 274: 542–547;2000.
28 Otto CM, Baumgardner JE. Effect of culture PO_2 on macrophage (RAW 264.7) nitric oxide production. Am J Cell Physiol 280:C280–C287;2001.
29 Panaro MA, Acquafreda A, Lisi S, Lofrumento DD, Trotta T, Satalino R, Saccia M, Mitolo V, Brandonisio O. Inducible nitric oxide synthase and nitric oxide production in *Leishmania infantum*-infected human macrophages stimulated with interferon-gamma and bacterial lipopolysaccharide. Int J Lab Res 29:122–127; 1999.
30 Patel B, Khaliq A, Jarvis-Evans J, Boulton M, Arrol S, Mackness M, McLeod D. Hypoxia induces HSP70 gene expression in human hepatoma (HEP G2) cells. Biochem Mol Biol Int 36:907–912;1995.
31 Reichner JS, Fitzpatrick PA, Wakshull E, Albina A. Receptor-mediated phagocytosis of rat macrophages is regulated differentially for opsonized particles and non-opsonized particles containing beta-glucan. Immunology 104:198–206;2001.
32 Simon LM, Axline SG, Horn BR, Robin ED. Adaptations of energy metabolism in the cultivated macrophage. J Exp Med 138:1413–1425; 1973.
33 Stafford JL, Neumann NF, Belosevic M. Macrophage-mediated innate host defense against protozoan parasites. Crit Rev Microbiol 28: 187–248;2002.
34 Szabo C, Wong HR, Salzman AL. Pre-exposure to heat shock inhibits peroxynitrite-induced activation of poly(ADP) ribosyltransferase and protects against peroxynitrite cytotoxicity in J774 macrophages. Eur J Pharmacol 315:221–226;1996.
35 Uehara T, Kaneko M, Tanaka S, Okuma Y, Nomura Y. Possible involvement of p38 kinase in HSP70 expression induced by hypoxia in rat primary astrocytes. Brain Res 823:226–230; 1999.
36 Waypa GB, Chandel NS, Schumacker PT. Model for hypoxic pulmonary vasoconstriction involving mitochondrial oxygen sensing. Circ Res 88:1259–1266;2001.
37 Yermolaieva O, Xu R, Schinstock C, Brot N, Weissbach H, Heinemann SH, Hoshi T. Methionine sulfoxide reductase A protects neuronal cells against brief hypoxia/reoxygenation. Proc Natl Acad Sci USA 101:1159–1164;2004.
38 Yun JK, McCormick TS, Villabona C, Judware RR, Espinosa MB, Lapetina EG. Inflammatory mediators are perpetuated in macrophages resistant to apoptosis induced by hypoxia. Proc Natl Acad Sci USA 94:13903–13908;1997.