Available online at www.sciencedirect.com
ScienceDirect
Free Radical Biology & Medicine 44 (2008) 1668–1676
www.elsevier.com/locate/freeradbiomed

Original Contribution

Inhibition of in vivo leishmanicidal mechanisms by tempol: Nitric oxide down-regulation and oxidant scavenging

Edlaine Linares [a], Selma Giorgio [b], Ohara Augusto [a,*]

[a] *Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, CP 26077, CEP 05513-970 São Paulo, SP, Brazil*
[b] *Departamento de Parasitologia, Instituto de Biologia, Universidade Estadual de Campinas, CEP 13083-970 Campinas, SP, Brazil*

Received 22 January 2008; accepted 24 January 2008
Available online 12 February 2008

Abstract

Tempol (4-hydroxy-2,2,6,6-tetramethyl-1-piperidinyloxy) has long been known to protect experimental animals from the injury associated with oxidative and inflammatory conditions. In the latter case, a parallel decrease in tissue protein nitration levels has been observed. Protein nitration represents a shift in nitric oxide actions from physiological to pathophysiological and potentially damaging pathways involving its derived oxidants such as nitrogen dioxide and peroxynitrite. In infectious diseases, protein tyrosine nitration of tissues and cells has been taken as evidence for the involvement of nitric oxide-derived oxidants in microbicidal mechanisms. To examine whether tempol inhibits the microbicidal action of macrophages, we investigated its effects on *Leishmania amazonensis* infection in vitro (RAW 264.7 murine macrophages) and in vivo (C57Bl/6 mice). Tempol was administered in the drinking water at 2 mM throughout the experiments and shown to reach infected footpads as the nitroxide plus the hydroxylamine derivative by EPR analysis. At the time of maximum infection (6 weeks), tempol increased footpad lesion size (120%) and parasite burden (150%). In lesion extracts, tempol decreased overall nitric oxide products and expression of inducible nitric oxide synthase to about 80% of the levels in control animals. Nitric oxide-derived products produced by radical mechanisms, such as 3-nitrotyrosine and nitrosothiol, decreased to about 40% of the levels in control mice. The results indicate that tempol worsened *L. amazonensis* infection by a dual mechanism involving down-regulation of iNOS expression and scavenging of nitric oxide-derived oxidants. Thus, the development of therapeutic strategies based on nitroxides should take into account the potential risk of altering host resistance to parasite infection.

Keywords: Tempol; Nitric oxide; Nitric oxide-derived oxidants; Leishmaniasis; Macrophage microbicidal mechanisms; Protein nitration; Protein nitrosation; Free radicals

Tempol and other stable cyclic nitroxide radicals have long been known to protect laboratory animals, bacteria, and mammalian cells from the injury associated with oxidative stress conditions [1–4]. These protective effects have been mostly attributed to their superoxide dismutase-like activity, although nitroxides are not particularly efficient as superoxide dismutase mimetics [5]. Other nitroxide antioxidant mechanisms include inhibition of Fenton chemistry by the ability to oxidize transition metal ions, termination of radical chain reactions by radical recombination, and acceptance of electrons from the mitochondrial electron transport chain (reviewed in [3,4]). The possibility of cyclic nitroxides interacting with nitric oxide-derived oxidants has been less frequently investigated [6–11]. Nevertheless, this is particularly relevant because tempol has also been shown to protect animals from injuries associated with inflammatory conditions, which are characterized by increased production of nitric oxide and its derived oxidants (reviewed in [12]).

In most studied animal models of inflammation, the protective effects of tempol were paralleled by decreased levels of protein tyrosine nitration [12]. The occurrence of protein nitration represents a shift of nitric oxide actions from physiological

Abbreviations: COX, prostaglandin endoperoxide H synthase; IFN-γ, interferon-γ; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NOX, NADPH oxidase; peroxynitrite, the sum of peroxynitrite anion ($ONOO^-$), oxoperoxonitrate (−1) and peroxynitrous acid (ONOOH, hydrogen oxoperoxonitrate) unless specified otherwise; Tempol, 4-hydroxy-2,2,6,6,-tetramethyl-1-piperidinyloxy.

* Corresponding author. Fax: +55 11 30912186, 55 11 3815 5579.
E-mail address: oaugusto@iq.usp.br (O. Augusto).

0891-5849/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.freeradbiomed.2008.01.027

to pathophysiological and potentially damaging pathways involving its derived oxidants such as nitrogen dioxide and peroxynitrite (reviewed in [13]). Consequently, protein tyrosine nitration is attracting considerable attention from biomedical investigators because of its potential ability to indicate acute and chronic disease states and predict disease risk [13]. In the case of infectious diseases, the occurrence of protein tyrosine nitration in infected tissues and cells has been taken as evidence for the involvement of nitric oxide-derived oxidants in phagocyte microbicidal mechanisms [13–21].

The better elucidated protein nitration mechanism supports a free radical recombination between nitrogen dioxide and protein tyrosyl radicals. Not unexpectedly, efficient tyrosine nitration has been demonstrated so far with systems that directly or indirectly produce tyrosyl radicals in addition to nitrogen dioxide. Examples of such systems are peroxynitrite/carbon dioxide and hemeperoxidases such as myeloperoxidase and eosinophil peroxidase/hydrogen peroxide/nitrite [13,18]. Peroxynitrite is produced from the diffusion-controlled reaction of nitric oxide with the superoxide radical [22], whereas nitrite is a biological product and precursor of nitric oxide [23]. More recently, other hemeproteins such as COX-2 [21] and iNOS [24] have been proposed as catalysts of biological protein tyrosine nitration.

Relevant to this, we showed that tempol efficiently inhibits both myeloperoxidase- [25] and peroxynitrite/carbon dioxide-mediated protein nitration in vitro [10], mainly because of its capability to react rapidly with nitrogen dioxide and carbonate radical. Although these reactions are likely to be relevant to the effects of tempol in reducing tissue injury under inflammatory conditions [12], they may compromise microbicidal mechanisms during infections. For instance, tempol has been shown to increase *Leishmania chagasi* infection of human and mouse macrophages in vitro, and its effects have been attributed to superoxide radical dismutation [26]. Here, to better evaluate tempol influence on macrophage microbicidal mechanisms, we investigated its effects on *Leishmania amazonensis* infection in vivo (C57Bl/6 mice) and in vitro (RAW 264.7 murine macrophages). Previously, based on several lines of evidence, we proposed that C57Bl/6 mice control cutaneous *L. amazonensis* infection mainly because their lesion macrophages are activated at an early infection stage to produce nitric oxide-derived oxidants that are crucial to parasite elimination [16].

Experimental procedures

Materials

All reagents were purchased from Sigma, Merck, or Fischer and were analytical grade or better. All solutions were prepared with distilled water purified with a Barnstead Milli-Q system.

Parasites

L. amazonensis (MHOM/BR/73/M2269) amastigotes were obtained from footpad lesions of BALB/c mice as previously described [15,16].

Animal infection and tempol administration

Female C57Bl/6 mice (6 weeks of age) were obtained from our animal facilities and the experiments performed followed the approved animal guidelines. The animals were injected subcutaneously in the right hind footpad with 2×10^6 *L. amazonensis* amastigotes [15,16]. After infection, the animals were divided into two groups, one of control animals (30 mice) receiving normal drinking water and another (30 mice) receiving 2 mM tempol aqueous solution as the only drinking water throughout the experiment. The course of infection was monitored by measuring the increase in footpad thickness, compared with the contralateral uninfected footpad, with a dial caliper [15].

Parasite burden

At different infection times, mice were sacrificed and the footpad lesion tissue was excised and homogenized in 1 ml saline using a T8 Ultra-Turrax homogenizer (IKA Works). The suspension was poured through sterile gauze to eliminate debris and the filtrate disrupted by five passages through a 26-gauge needle to release parasites from tissue macrophage vacuoles. Parasite number was recorded by microscopic observation [15,16].

Extraction of footpad lesion tissue for biochemical analysis

Six weeks after infection, mice were sacrificed and the footpad lesion tissue was homogenized in lysis buffer (2.5 ml/mg tissue) containing 50 mM Tris (pH 7.4), 0.5% Triton X-100, 5 mM EDTA, 10 mM *N*-ethylmaleimide, 2 μg/ml aprotinin, 2 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate [10,27]. The homogenate was centrifuged at 4°C and 1000*g* for 5 min and the supernatant was stored at −80°C until analysis of total protein, protein 3-nitrotyrosine, iNOS expression, and nitric oxide products. Total protein in the samples was determined by the Bradford method with a Bio-Rad kit.

Chemiluminescence assay of nitric oxide products

Determination of nitric oxide products in the lesion tissue was performed with a chemiluminescence nitric oxide analyzer (NOA^{280}; Sievers Instruments) following the procedure optimized by Feelisch and co-workers [27]. Footpad lesion tissue extracts of each animal were divided into four aliquots. The first aliquot was directly injected into a vessel containing a solution of 45 mM potassium iodide and 10 mM iodine in glacial acetic acid that was maintained at 60°C and continuously purged with nitrogen. Under these conditions, nitrite, nitrosothiol, and mercury-resistant nitroso compounds (nitrosamines and iron–nitrosyl complexes) are reduced to nitric oxide gas, which is carried on the stream of nitrogen into the chemiluminescence detector. The second aliquot was incubated with 10% (v/v) sulfanilamide solution (5% sulfanilamide in 1 M HCl) for 30 min at room temperature in the dark to use up the present nitrite and then injected into the analyzer. The third aliquot was incubated with 10% (v/v) sulfanilamide solution containing 2%

mercurium chloride for 30 min at room temperature in the dark to use up the present nitrite and nitrosothiols and then injected in the analyzer. The fourth aliquot was directly injected into a vessel containing a saturated solution of vanadium(III) chloride in 1 N HCl maintained at 90°C. Under these conditions, all nitric oxide-derived products (nitrate, nitrite, nitrosothiol, nitrosamines, and iron–nitrosyl complexes) are reduced to nitric oxide. The peak areas of the samples were calculated with the instrument software and compared with those of standard solutions of nitrate and nitrite analyzed under the same experimental conditions. The amount of nitrate, nitrite, and nitrosothiol present in the samples was given by the concentration difference between the fourth and the first aliquot, the first and the second aliquot, and the second and the third aliquot, respectively [27].

Analysis of protein nitration by immunoblotting

Samples of lesion tissue extracts (5 μg protein) were transferred onto a nitrocellulose membrane by vacuum for dot blots, and the membrane was blocked with 5% nonfat dried milk (2 h) before exposure to primary antibody solution for 2 h (sheep anti–nitrotyrosine, 1/2500; Oxis) [10]. Primary antibody was detected by 1 h incubation with secondary antibody (anti-sheep IgG peroxidase conjugated, 1/2500; Calbiochem). Dot blots were revealed with chemiluminescent reagents (Amersham) and relative quantification of 3-nitrotyrosine was performed by densitometry (ImageQuant v5.2; Molecular Dynamics). Bovine serum albumin (BSA) and peroxynitrite/carbon dioxide-treated BSA were employed as negative and positive controls, respectively [10].

Analysis of iNOS expression by Western blot

Samples of lesion tissue extracts (30 μg protein) were incubated with Laemmli's buffer for 5 min at 100°C and subjected to SDS–PAGE with 8% gel slabs. Separated proteins were transferred onto nitrocellulose membrane [28]. Nonspecific binding sites were blocked with 5% nonfat dried milk in Tris-buffered saline (90 min) before exposure to primary antibody solution for 1 h (rabbit anti-iNOS, 1/1000; Alexis). Primary antibody was detected by incubation with secondary antibody for 1 h (anti-rabbit IgG peroxidase conjugated, 1/2000; Amersham). Western blot was revealed with chemiluminescent reagents (Amersham) and relative quantification of iNOS was performed by densitometry (ImageQuant v5.2; Molecular Dynamics).

Macrophage infection

RAW 264.7 macrophages ($\sim 1.5 \times 10^5$) were allowed to attach to round 13-mm-diameter glass coverslips placed in 24-well plates (Costar) containing 1 ml of DME medium supplemented with 10% inactivated fetal calf serum (Cultilab, Brazil), 25 mg/ml ampicillin, 100 mg/ml streptomycin, and 3.7 mg/ml sodium bicarbonate and maintained in 5% CO_2 at 37°C [29]. After 30 min incubation, the nonadherent cells were removed by two washes with saline. Then, the adherent macrophages were treated with 100 U/ml IFN-γ and 1 μg/ml LPS. After 7 h incubation, macrophages were treated with tempol (0–100 μM) and infected with *L. amazonensis* (10 amastigotes/macrophage). After 17 h (total incubation 24 h), the glass coverslips were washed to remove extracellular parasites, stained with Giemsa, dried, mounted on glass slides, and examined microscopically [29]. For each glass coverslip, about 250 cells were examined to count the number of infected macrophages and the average number of parasites per macrophage. The results were expressed as the infection index, which is the product of the percentage of infected macrophages times the number of amastigotes per macrophage [29].

Analysis of tempol and its hydroxylamine derivative by EPR

EPR spectra were recorded at room temperature on a Bruker ER 200 D-SRC upgraded to an EMX instrument. In the case of C57Bl/6 mice experiments, footpad lesion tissues were vigorously homogenized in saline (2.5 volumes/mg tissue) and subjected to EPR analysis before (to determine tempol levels) and after addition of 1 mM ferricyanide (to determine tempol plus hydroxylamine levels). Indeed, ferricyanide oxidizes cellular reductants and the hydroxylamine derivatives to nitroxides, permitting detection of the latter by EPR [30]. In the macrophage experiments, cell culture, activation, and infection in the presence of 100 μM tempol were performed as above except for the use of proportionally higher numbers of cells and parasites (~5 times higher) and higher total volumes (8 ml). After 24 h incubation, the culture medium was aspirated and saved, and adherent cells were washed two times with PBS. Cells were resuspended in 0.5 ml PBS (thus, concentrated 60 times compared with culture conditions). Both culture media and resuspended cells were subjected to EPR analysis before and after 30 min incubation with 1 mM ferricyanide at room temperature [30]. Tempol concentration in the footpad lesions or in culture media was estimated by double integration of the EPR spectrum and comparison with that of a standard tempol solution scanned under the same conditions.

Statistical analyses

Data are expressed as means ± standard error. The differences between experimental groups were examined by using ANOVA.

Results

Leishmania infection in mice

The course of *L. amazonensis* infection in C57Bl/6 mice proceeded as previously described [15,16]. Cutaneous footpad lesions reached maximum size at week 6 and then declined during the 15-week period (Fig. 1A). Likewise, the number of parasites in the lesions increased up to 6 weeks after infection and decreased thereafter, with the animals becoming clinically cured (Fig. 1B). Mice that received continuous tempol (2 mM) in the drinking water showed an increase in lesion size compared to controls at week 6 (~120%) and thereafter (Fig. 1A). Parasite burden in the lesions showed that the tempol-treated

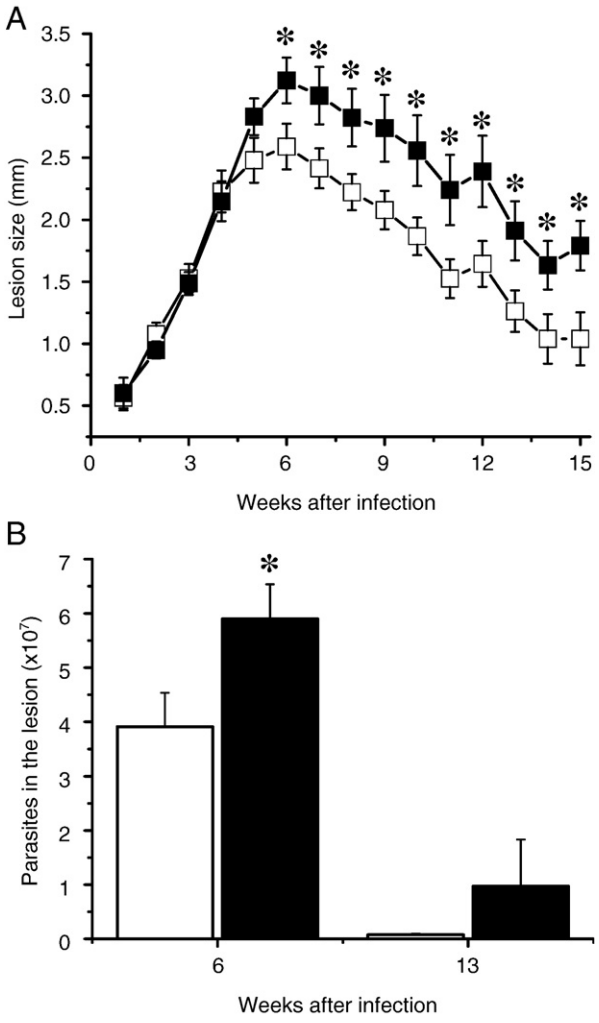


Fig. 1. Course of *L. amazonensis* infection in control (□) and tempol-treated (■) C57Bl/6 mice monitored by (A) lesion size and (B) parasite burden. The animals (30 per group) were injected with 2×10^6 amastigotes and received normal water or 2 mM tempol as the drinking water. Measurements were performed as described under Experimental procedures. The values shown correspond to the means ± standard error of lesion size of 30 animals (A) and of the parasite burden in the lesions of 7 animals (B); $*p \leq 0.05$, ANOVA.

group fared worse than the controls by week 6 (~150%); at week 13, parasite counts were also higher than those of control animals but the difference was not significant (Fig. 1B). These results indicate that tempol-treated C57Bl/6 mice became less resistant to *L. amazonensis* infection than control mice.

To elucidate the mechanisms by which tempol increased lesion size and parasite burden in infected animals (Fig. 1), we first examined whether the nitroxide administered in the drinking water reached the infected footpads of treated animals by EPR analysis of their homogenates at week 6. As shown in Fig. 2, tempol was detectable in the lesions of treated animals but the dominant EPR signal is the one attributed to the ascorbyl radical (Fig. 2A). The intensity of the tempol signal increased upon addition of 1 mM ferricyanide to the homogenates (Fig. 2B), indicating that a considerable fraction of tempol was present in the lesions in the reduced form, that is, as the hydroxylamine derivative [30]. Although the maximum tempol plus hydroxylamine derivative level detected in the lesions at week 6 was around 1 μM (Fig. 2B), these experiments indicated that the nitroxide and its redox-active derivatives are likely to be responsible for the worsening of *L. amazonensis* infection (Fig. 1).

In the case of C57Bl/6 mouse infection, we showed that control of parasite burden starts at 6 weeks, concomitant with maximum nitric oxide synthesis and 3-nitrotyrosine protein levels in the animal footpads [16]. Thus, it was important to examine the levels of nitric oxide-derived products in footpad homogenates collected at 6 weeks from control and tempol-treated animals. Nitric oxide-derived products were quantified by specific reduction and chemiluminescence as previously described (see Experimental procedures) [27]. Footpad homogenates of animals receiving tempol presented a decreased level of the sum of all chemiluminescence-detectable nitric oxide products compared with control animals (about 80%) (Fig. 3A). Likewise, nitrate (Fig. 3A) and nitrite (Fig. 3B) decreased to 79 and 76%, respectively. Less abundant nitric oxide-derived products such as nitrosylheme plus nitrosamines (100%) and nitrosothiol (40%) remained the same or decreased by a considerable extent, respectively (Fig. 3B). Tempol administration also decreased the levels of nitrated proteins in the footpad homogenates to about 40% of control animals, as demonstrated by parallel experiments of immunoblotting monitoring 3-nitrotyrosine protein levels (Fig. 4). Previously, we showed by confocal

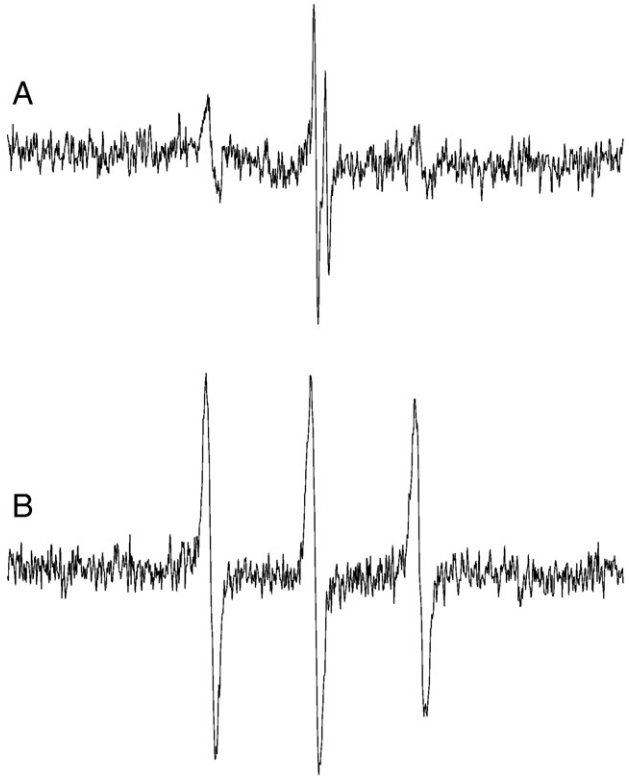


Fig. 2. Representative EPR spectrum of footpad homogenates of *L. amazonensis*-infected C57Bl/6 mice at week 6 treated with tempol (A) before and (B) after addition of 1 mM ferricyanide. The central doublet observed in (A) corresponds to the EPR spectrum of the ascorbyl radical. Instrumental conditions: microwave power, 10 mW; modulation amplitude, 0.1 mT; time constant, 327 ms; scan rate, 0.029 mT/s.

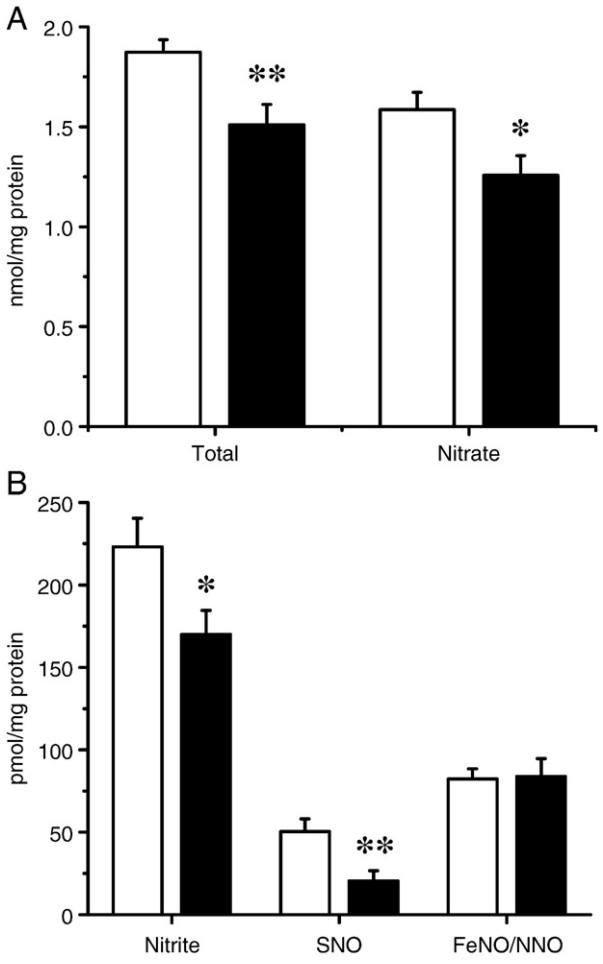


Fig. 3. Levels of nitric oxide products detected in lesion extracts of *L. amazonensis*-infected C57Bl/6 mice untreated (□) and treated with tempol (■). (A) The total corresponds to the sum of all specified products. (B) Nitrosothiols (SNO), nitrosylheme (FeNO), and nitrosamines (NNO) are shown. Analysis was performed as described under Experimental procedures. The values shown correspond to the means ± standard error obtained in lesion extracts of six to nine mice; $^{**}p \leq .01$ and $^{*}p \leq 0.05$, ANOVA.

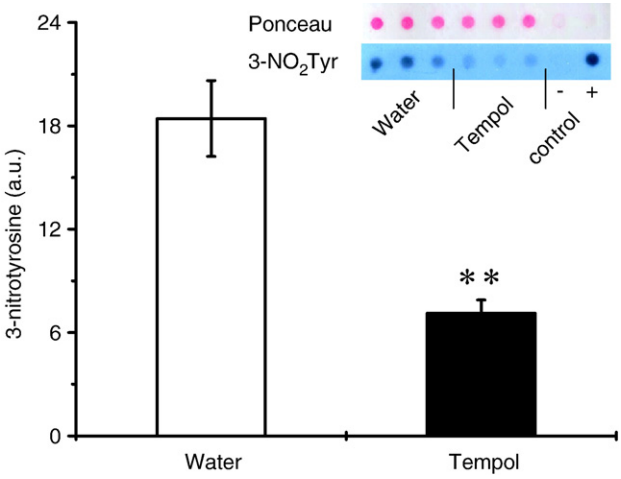


Fig. 4. Levels of 3-nitrotyrosine residues in lesion extracts of *L. amazonensis*-infected C57Bl/6 mice untreated (□) and treated with tempol (■). Inset: Staining of protein and 3-nitrotyrosine; BSA and peroxynitrite/carbon dioxide-treated BSA correspond to negative and positive control, respectively. Analysis and quantification were performed as described under Experimental procedures. The values shown correspond to the means ± standard error obtained in lesion extracts of three mice; $^{**}p \leq 0.01$, ANOVA.

microscopy of the lesions that proteins from both parasites and hosts were nitrated at 6 weeks of infection [16].

The levels of nitric oxide-derived products measured in the lesions of mice infected with *L. amazonensis* were about 10 times higher than those reported in most tissues of naive rats [31], attesting to the intensive activity of iNOS in the infected footpad macrophages [16]. On the other hand, tempol decreased the overall yield of nitric oxide-derived products to about 80% of control animals (Fig. 3), suggesting that it could be modulating iNOS expression. In agreement, the levels of inducible nitric oxide synthase in the footpad homogenates of tempol-treated mice were about 80% of those of untreated animals (Fig. 5).

Leishmania infection in activated macrophages

RAW 264.7 macrophages became infected even when activated with interferon-γ (100 U/ml) and LPS (1 μg/ml) 7 h before the addition of a 10-fold excess of *L. amazonensis* amastigotes (Fig. 6) [30]. Under our experimental conditions, about 50% of the macrophages housed parasites, with an average of seven parasites per infected macrophage. These values provided an infection index of about 350 (Fig. 6). In parallel experiments, parasites and tempol (10–100 μM) were applied concomitantly to activated macrophages and infection parameters were monitored. It was observed that 100 μM tempol significantly increased the infection index and, thus, enhanced parasite replication (Fig. 6). However, no significant decrease in iNOS expression was detectable with tempol concentrations lower than 0.5 mM (data not shown). Previously, 0.4 mM tempol was shown to increase *L. chagasi* infection in mouse and human

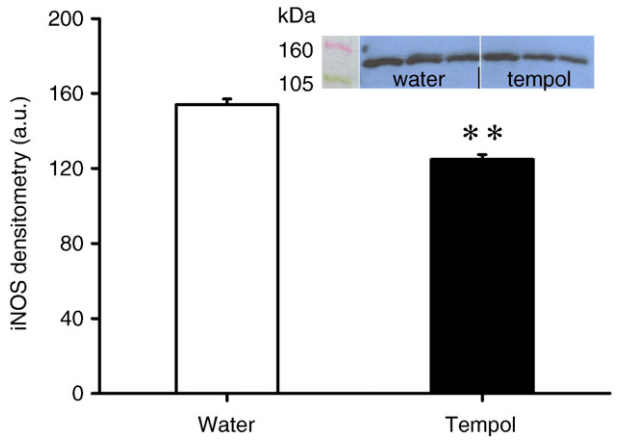


Fig. 5. Levels of iNOS expression in lesion extracts of *L. amazonensis*-infected C57Bl/6 mice untreated (□) and treated with tempol (■). Inset: Molecular weight marker and representative staining of iNOS. Analysis and quantification were performed as described under Experimental procedures. The values shown correspond to the means ± standard error obtained in lesion extracts of four to six mice; $^{**}p \leq 0.01$; ANOVA.

macrophages without presenting toxicity to the host cells [26]. However, due to the potential cytotoxic effects of tempol at millimolar concentration [3,4,32,33] and the low concentration of tempol plus its hydroxylamine derivative (ca. 1 μM) detected in footpad homogenates of treated mice (Fig. 2), we did not pursue in vitro experiments with tempol concentrations higher than 100 μM. Nevertheless, our results clearly show that tempol increases *L. amazonensis* infection in macrophage cultures (Fig. 6).

Because cell cultures are usually deprived of ascorbate [34], which is an important biological reductant of nitroxides (Fig. 2) [30], it was important to examine tempol distribution and fate in RAW 264.7 macrophages infected or not with *L. amazonensis.* Activation, infection, and incubation were performed as described above except for the use of a proportionally higher number of cells and parasites and only one tempol concentration (100 μM); at the end of the incubation (24 h), culture media and cells were isolated and analyzed by EPR as described under Experimental procedures. Tempol and its hydroxylamine derivative were detected in the culture media and in the cell fractions, particularly in the medium, as expected from the ratio between total and cell volumes (Fig. 7, inset). Thus, tempol fate was analyzed by the determination of tempol and hydroxylamine plus tempol levels in the culture media from different incubations (Fig. 7). Tempol (100 μM) remained as the nitroxide (92 μM) in 24 h incubation with culture medium or culture medium containing *L. amazonensis* amastigotes. In incubations containing RAW 264.7 macrophages, tempol appeared in the

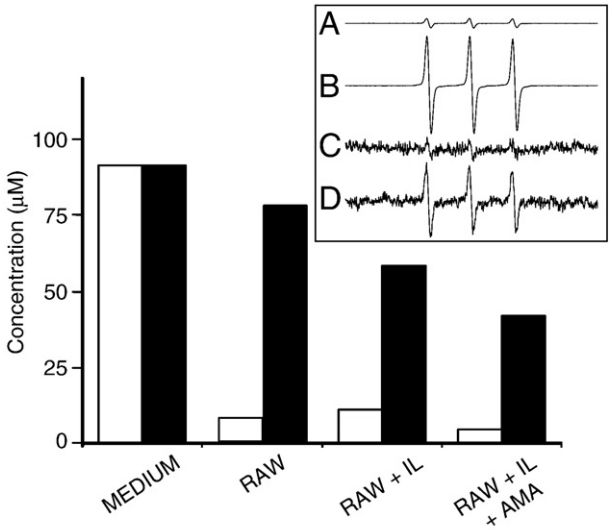


Fig. 7. Levels of tempol (□) and tempol plus its hydroxylamine derivative (■) present in culture medium after incubation of tempol with macrophages under different conditions. Tempol (100 μM) was added to culture medium (MEDIUM), RAW 264.7 macrophages (RAW), macrophages activated with interferon-γ and LPS (RAW + IL), and activated macrophages infected with *L. amazonensis* amastigotes (RAW + IL + AMA). Incubation, activation, infection, and EPR analysis were performed as described under Experimental procedures. The values shown correspond to the means of two or three independent experiments. Representative data obtained in the case of RAW + IL + AMA are shown in the inset. The shown EPR spectra correspond to those of culture medium before (spectrum A) and after addition of 1 mM ferricyanide (spectrum B) and of the concentrated cell fraction (60 times) before (spectrum C) and after addition of 1 mM ferricyanide (spectrum D).

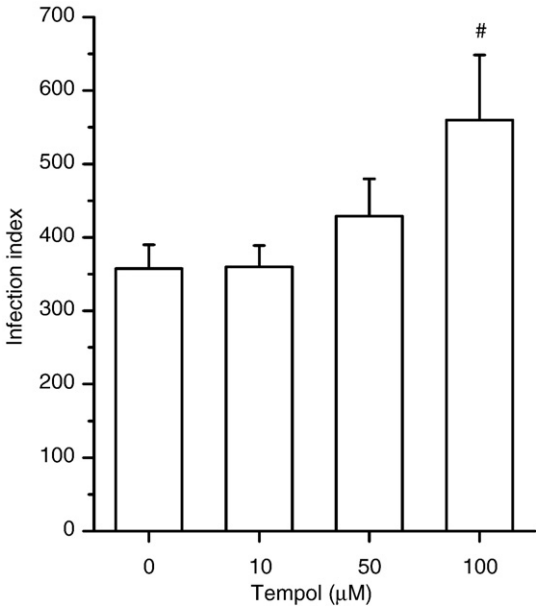


Fig. 6. Effects of tempol on macrophages infected by *L. amazonensis.* RAW 264.7 macrophages were activated with interferon-γ (100 U/ml) and LPS (1 μg/ml) 7 h before the addition of a 10-fold excess of *L. amazonensis* amastigotes. Treatment with tempol and analysis were performed as described under Experimental procedures. The infection index is the product of the percentage of infected macrophages times the average number of amastigotes per macrophage. The values shown correspond to the means ± standard error obtained from three independent experiments; $\#p \leq 0.1$; ANOVA.

culture media as the nitroxide (~10 μM) and as the hydroxylamine derivative (~70 μM) (Fig. 7). Because the initial tempol concentration was 100 μM, the difference (~20 μM) was metabolized to products other than the hydroxylamine derivative. These results confirmed that tempol is quite stable in culture media but it is reduced to the hydroxylamine by cell cultures [35] despite their ascorbate deprivation. Tempol metabolization to products other than the hydroxylamine derivative increased upon macrophage activation and infection (Fig. 7). In the case of activated macrophages, the culture medium contained tempol (~10 μM) and hydroxylamine (~50 μM); about 40 μM tempol was metabolized to other products. The culture medium of activated macrophages infected with *L. amazonensis* amastigotes contained tempol (~4 μM) and hydroxylamine (~40 μM); about 60 μM tempol was metabolized to other products (Fig. 7). These results are consistent with the fact that macrophage activation and infection led to production of free radicals, some of which are able to react with tempol to generate products that are redox inactive (see also Discussion) [10,36]. As was the case in mouse infection (Figs. 1 and 2), tempol and its hydroxylamine derivative were detected in macrophage cultures whose parasite burden they increased (Figs. 6 and 7).

Discussion

Even though leishmaniasis is endemic in developing countries, economic globalization, environmental changes, military

activity, and immune depression caused by medication and viral infections have extended its reach to people in developed countries in recent years [37]. Thus, infection of C57Bl/6 mice with *L. amazonensis* seemed to us an interesting model to explore tempol effects on microbicidal mechanisms in vivo. Murine leishmaniasis has been useful not only for the understanding of leishmaniasis, but also for that of other diseases caused by intracellular pathogens, such as tuberculosis, listeriosis, and toxoplasmosis. Although most studies to elucidate cell types and signal transduction cascades necessary to control cutaneous infection in mice have been performed with *L. major,* the rule of $CD4^+$ T cell- and IFN-γ-mediated macrophage activation holds for most species [38–41]. *L. amazonensis* is one of the causative agents of cutaneous and diffuse cutaneous leishmaniasis in the Americas [42]. In murine models, it leads to uncontrolled cutaneous lesions in BALB/c mice, whereas relatively resistant C57Bl/6 mice are able to control infection. Relevant to this, we showed that infection control relied on lesion macrophage activation with the production of nitric oxide and its derived oxidants, which promoted extensive nitration of parasite and host proteins [16]. Thus, tempol interaction with nitric oxide-derived oxidants [6–11] could affect infection control. This was tested by continuous treatment of infected C57Bl/6 mice with tempol in the drinking water at a dose (2 mM) low enough to be innocuous to the animals [3], which we confirmed by observing them and monitoring their water consumption. We also demonstrated that oral tempol was distributed to the tissues and reached infected footpads by EPR analysis of their homogenates (Fig. 2).

Our results showed that, indeed, tempol worsened *L. amazonensis* infection in C57Bl/6 mice (Figs. 1 and 3–5) and RAW macrophages (Fig. 6), but acted by a dual mechanism involving scavenging of nitric oxide-derived oxidants and down-regulation of iNOS expression. The latter action is directly revealed by the in vivo results (Figs. 3 and 5). Although tempol administration inhibited iNOS expression to a small extent (80% of control animals) the enzyme level decrease was significant and consistent because it was revealed by the level of expressed enzyme (Fig. 5) and the overall yield of enzyme products, that is, of nitric oxide-derived products (Fig. 3A). The effect of tempol in decreasing iNOS expression is probably due to its capability to reduce NF-κB activation as shown before in an acute model of inflammation in mice, carrageenan-induced pleuritis [43]. The NF-κB family of transcription factors is activated by many inflammatory and infectious stimuli. In the case of *Leishmania,* NF-κB activation is required to mount the $CD4^+$ T cell- and IFN-γ-mediated macrophage response required to control parasite proliferation (reviewed in [40]). Because tempol is able to reduce NF-κB activation [43], it is not surprising that it reduced iNOS levels [44–46] in the footpad homogenates of *L. amazonensis*-infected mice (Fig. 2). NF-κB activation depends on cellular redox state [47], and, thus, inhibition by tempol may be due to its antioxidant properties [1–4].

A closer analysis of the obtained results indicates that, in addition to inhibiting iNOS expression and nitric oxide synthesis, tempol also scavenged nitric oxide-derived radicals required to control parasite burden. Indeed, tempol decreased iNOS expression (Fig. 5) and overall yield of nitric oxide-derived products (Fig. 3A) to 80% of control levels, but parasite burden increased to 150% (Fig. 1B), suggesting that the leishmanicidal agent is not nitric oxide itself. This is in agreement with our previous studies showing that nitric oxide is cytostatic to the parasites but not cytotoxic [16]. In addition, tempol decreased the levels of nitric oxide-derived products whose formation depends on a free radical mechanism, such as nitrated proteins (3-nitrotyrosine-protein) (Fig. 3) and nitrosothiols [10,13,18,48] (Fig. 4), to 40% of control levels. Such a decrease is higher than that of iNOS expression and total nitric oxide-derived products, indicating that tempol is also reacting with radical species that can be produced downstream of nitric oxide synthesis (Fig. 8) [10,13,18,48].

The above view is also supported by tempol distribution and fate in our experimental models (Figs. 2 and 7). It should be mentioned that tempol reacts with diverse biological oxidants and reductants while being recycled through the oxoammonium cation and the hydroxylamine derivative, respectively (Fig. 8) [3,4]. Tempol can also be consumed by recombination reactions

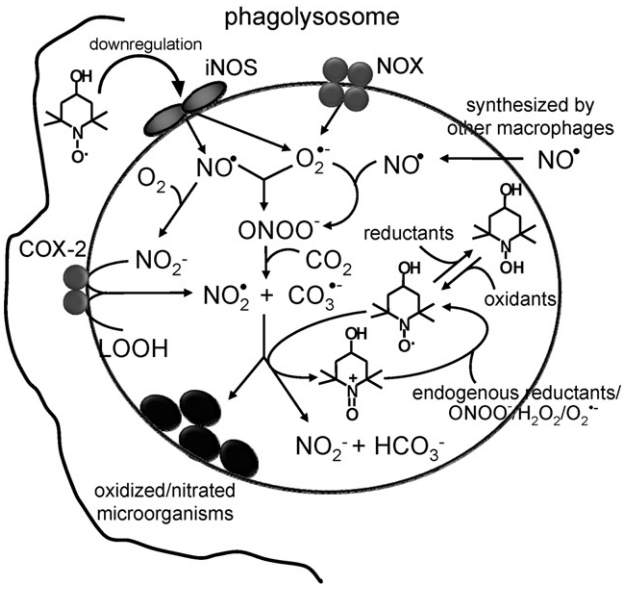
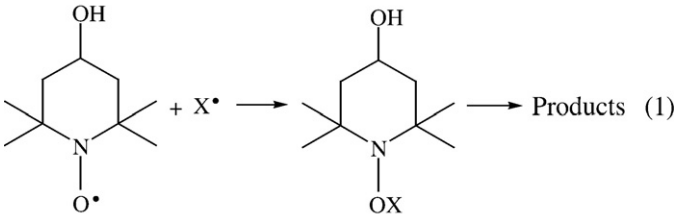


Fig. 8. Schematic representation of the effects of tempol on macrophage production of reactive species proposed to be relevant for defense against microorganisms [16,18–21]. Involvement of iNOS and nitric oxide is established but participation of other enzymes and reactive species remains controversial. Although our results cannot discriminate between the auxiliary enzymes and the reactive species participating in the oxidative/nitrative reactions responsible for parasite elimination, they support a role for radicals produced downstream of nitric oxide synthesis because of their rapid reaction with tempol (see text). Thus, in the scheme, both NOX [16,18] and COX-2 [21] were included as possible auxiliary enzymes. Concomitant production of nitric oxide and superoxide radical anion to produce peroxynitrite ($ONOO^-$/ONOOH) in macrophage cell cultures has not been demonstrated so far [17,21]. In vivo, however, tissue-resident and newly recruited macrophages are being continually invaded by proliferating organisms and superoxide radical and nitric oxide production does not have to occur at the same time in the same cell because nitric oxide diffuses from one cell to another [18].

with certain radicals, such as thiyl radicals, to produce redox-inactive products (Eq. 1) [36].



All of these reactions (Fig. 8, Eq. (1)) are likely to be occurring in the employed experimental models because tempol and the hydroxylamine derivative were detected in the mouse lesions (Fig. 2) and macrophage cultures (Fig. 7) whose parasite burden increased (Figs. 1 and 6). Detection of the oxoammonium cation derivative is precluded because it is a reactive intermediate with short half-life even in chemical systems [9]. In cell cultures, it was possible to demonstrate that part of the tempol was metabolized to products that are redox inactive or, at least, are not oxidized by ferricyanide, particularly in infected macrophages (Fig. 7). This indicates that macrophage infection produces radicals from biotargets such as glutathionyl [36], protein-thiyl [10], and protein-tyrosyl [25], which are able to consume tempol (Eq. (1)). Production of biotarget-derived radicals requires the production of upstream radicals. Thus, it is reasonable to propose that tempol and its redox-active derivatives scavenge species produced downstream of nitric oxide, compromising parasite elimination (Fig. 8). Both tempol and its hydroxylamine derivative scavenge radicals, such as nitrogen dioxide ($k = 8.7 \times 10^8$ and $\leq 10^5$ M^{-1} s^{-1}, respectively) [8] and carbonate radical ($k = 4 \times 10^8$ and 1.7×10^8 M^{-1} s^{-1}, respectively) [9]. Tempol is usually more reactive than the hydroxylamine as exemplified by the above second-order rate constant values. In addition, tempol is oxidized to the oxoammonium cation, which can be recycled back to tempol by reacting with cellular reductants or upstream species, such as hydrogen peroxide, peroxynitrite, and superoxide [7,9,10,23]. This argues for a direct involvement of tempol in scavenging the radicals that contribute to parasite elimination (Fig. 8), although a role for the hydroxylamine derivative cannot be excluded.

The oxidative mechanisms by which macrophages eliminate microorganisms remain unresolved and are an area of active investigation [15–21,38–42,49–52]. The results reported here cannot discriminate between enzymes and reactive species participating in oxidative/nitrative reactions that contribute to parasite elimination, but they support a role for radicals produced downstream of nitric oxide synthesis because of their rapid reaction with tempol (Fig. 8). In addition, tempol decreased iNOS expression. Down-regulation of iNOS expression and reaction with nitric oxide-derived radicals are also likely to be responsible for tempol protecting experimental animals from the injury associated with inflammatory conditions [12]. Thus, the development of therapeutic strategies based on cyclic nitroxides [3,4] should take into account the potential risk of altering host resistance to parasite infection.

Acknowledgments

This work was supported by grants from the Fundação de Amparo à Pesquisa do Estado de São Paulo and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). O. Augusto and E. Linares are also supported by the network Redoxoma (Projeto Instituto do Milênio do CNPq).

References

[1] Krishna, M. C.; Samuni, A. Nitroxides as antioxidants. *Methods Enzymol.* **274:**580–589; 1994.
[2] Offer, T.; Russo, A.; Samuni, A. The pro-oxidative activity of SOD and nitroxide SOD mimics. *FASEB J.* **14:**1215–1223; 2000.
[3] Soule, B. P.; Hyodo, F.; Matsumoto, K.; Simone, N. L.; Cook, J. A.; Krishna, M. C.; Mitchell, J. B. The chemistry and biology of nitroxide compounds. *Free Radic. Biol. Med.* **42:**1632–1650; 2007.
[4] Kagan, V. E.; Jiang, J.; Bayir, H.; Stoyanovsky, D. A. Targeting nitroxides to mitochondria: location, location, location, and … concentration: highlight commentary on "Mitochondria superoxide dismutase mimetic inhibits peroxide-induced oxidative damage and apoptosis: role of mitochondrial superoxide." *Free Radic. Biol. Med.* **43:**348–350; 2007.
[5] Goldstein, S.; Merenyi, G.; Russo, A.; Samuni, A. The role of oxoammonium cation in the SOD-mimic activity of cyclic nitroxides. *J. Am. Chem. Soc.* **125:**789–795; 2003.
[6] Carroll, R. T.; Galatsis, P.; Borosky, S.; Kopec, K. K.; Kumar, V.; Althaus, J. S.; Hall, E. D. 4-Hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (tempol) inhibits peroxynitrite-mediated phenol nitration. *Chem. Res. Toxicol.* **13:**294–300; 2000.
[7] Bonini, M.; Mason, R. P.; Augusto, O. The mechanism by which 4-hydroxy-2,2,6,6-tetramethylpiperidene-1-oxyl (tempol) diverts peroxynitrite decomposition from nitrating to nitrosating species. *Chem. Res. Toxicol.* **15:**506–511; 2002.
[8] Goldstein, S.; Samuni, A.; Russo, A. Reaction of cyclic nitroxides with nitrogen dioxide: the intermediacy of the oxoammonium cations. *J. Am. Chem. Soc.* **125:**8364–8370; 2003.
[9] Goldstein, S.; Samuni, A.; Merenyi, G. Reactions of nitric oxide, peroxynitrite, and carbonate radicals with nitroxides and their corresponding oxoammonium cations. *Chem. Res. Toxicol.* **17:**250–257; 2004.
[10] Fernandes, D. C.; Medinas, D. B.; Alves, M. J.; Augusto, O. Tempol diverts peroxynitrite/carbon dioxide reactivity toward albumin and cells from protein-tyrosine nitration to protein-cysteine nitrosation. *Free Radic. Biol. Med.* **38:**189–200; 2005.
[11] Goldstein, S.; Samuni, A.; Hideg, K.; Merenyi, G. Structure–activity relationship of cyclic nitroxides as SOD mimics and scavengers of nitrogen dioxide and carbonate radicals. *J. Phys. Chem., A* **110:**3679–3685; 2006.
[12] Thiemermann, C. Membrane-permeable radical scavengers (tempol) for shock, ischemia–reperfusion injury, and inflammation. *Crit. Care Med.* **31:** S76–S84; 2003.
[13] Radi, R. Nitric oxide, oxidants, and protein tyrosine nitration. *Proc. Natl. Acad. Sci. USA* **101:**4003–4008; 2004.
[14] Evans, T. J.; Buttery, L. D.; Carpenter, A.; Springall, D. R.; Polak, J. M.; Cohen, J. Cytokine-treated human neutrophils contain inducible nitric oxide synthase that produces nitration of ingested bacteria. *Proc. Natl. Acad. Sci. USA* **93:**9553–9558; 1996.
[15] Giorgio, S.; Linares, E.; Ischiropoulos, H.; Von Zuben, F. J.; Yamada, A.; Augusto, O. In vivo formation of electron paramagnetic resonance-detectable nitric oxide and of nitrotyrosine is not impaired during murine leishmaniasis. *Infect. Immun.* **66:**807–814; 1998.
[16] Linares, E.; Giorgio, S.; Mortara, R. A.; Santos, C. X.; Yamada, A. T.; 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.
[17] Pfeiffer, S.; Lass, A.; Schmidt, K.; Mayer, B. Protein tyrosine nitration in cytokine-activated murine macrophages: involvement of a peroxidase/

nitrite pathway rather than peroxynitrite. *J. Biol. Chem.* **276:**34051–34058; 2001.

[18] Augusto, O.; Bonini, M. G.; Amanso, A. M.; Linares, E.; Santos, C. X. C.; Lopes de Menezes, S. Nitrogen dioxide and carbonate radical anion: two emerging radicals in biology. *Free Radic. Biol. Med.* **32:**841–859; 2002.

[19] Alvarez, M. N.; Piacenza, L.; Irigoin, F.; Peluffo, G.; Radi, R. Macrophage-derived peroxynitrite diffusion and toxicity to Trypanosoma cruzi. *Arch. Biochem. Biophys* **432:**222–232; 2004.

[20] Naviliat, M.; Gualco, G.; Cayota, A.; Radi, R. Protein 3-nitrotyrosine formation during Trypanosoma cruzi infection in mice. *Braz. J. Med. Biol. Res.* **38:**1825–1834; 2005.

[21] Palazzolo-Balance, A. M.; Suquet, C.; Hurst, J. K. Pathways for intracellular generation of oxidants and tyrosine nitration by a macrophage cell line. *Biochemistry* **46:**7536–7548; 2007.

[22] Beckman, J. S.; Beckman, T. W.; Chen, J.; Marshall, P. A.; Freeman, B. A. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. USA* **87:**1620–1624; 1990.

[23] Gladwin, M. T.; Schechter, A. N.; Kim-Shapiro, D. B.; Patel, R. P.; Hogg, N.; Shiva, S.; Cannon III, R. O.; Kelm, M.; Wink, D. A.; Espey, M. G.; Oldfield, E. H.; Pluta, R. M.; Freeman, B. A.; Lancaster Jr., J. R.; Feelisch, M.; Lundberg, J. O. The emerging biology of the nitrite anion. *Nat. Chem. Biol.* **1:**308–314; 2005.

[24] Marechal, A.; Mattioli, T. A.; Stuehr, D. J.; Santolini, J. Activation of peroxynitrite by inducible nitric-oxide synthase: a direct source of nitrative stress. *J. Biol. Chem.* **282:**14101–14112; 2007.

[25] Muntz Vaz, S. M.; Augusto, O. The mechanism by which tempol inhibits peroxidase-mediated protein nitration. *Free Radic. Biol. Med.* **41:**S142; 2006.

[26] Gantt, K. R.; Goldman, T. L.; McCormick, M. L.; Miller, M. A.; Jeronimo, S. M.; Nascimento, E. T.; Britigan, B. E.; Wilson, M. E. Oxidative responses of human and murine macrophages during phagocytosis of Leishmania chagasi. *J. Immunol.* **167:**893–901; 2001.

[27] Feelisch, M.; Rassaf, T.; Mnaimneh, S.; Singh, N.; Bryan, N. S.; Jourd'heuil, D.; Kelm, M. Concomitant S-, N-, and heme-nitros(yl)ation in biological tissues and fluids: implications for the fate of NO in vivo. *FASEB J.* **16:**1775–1785; 2002.

[28] Lopes de Menezes, S.; Augusto, O. EPR detection of glutathionyl and protein-tyrosyl radicals during the interaction of peroxynitrite with macrophages (J774). *J. Biol. Chem.* **276:**39879–39884; 2001.

[29] Giorgio, S.; Barao, S. C.; Augusto, O.; Kwee, J. K. Leishmania amazonensis infection is reduced in macrophages treated with guanine ribonucleosides. *Acta Trop.* **70:**119–122; 1998.

[30] Nakao, L. S.; Kadiiska, M. B.; Mason, R. P.; Grijalba, M. T.; Augusto, O. Metabolism of acetaldehyde to methyl and acetyl radicals: in vitro and in vivo electron paramagnetic resonance spin-trapping studies. *Free Radic. Biol. Med.* **29:**721–729; 2000.

[31] Bryan, N. S.; Rassaf, T.; Maloney, R. E.; Rodriguez, C. M.; Saijo, F.; Rodriguez, J. R.; Feelisch, M. Cellular targets and mechanisms of nitros (yl)ation: an insight into their nature and kinetics in vivo. *Proc. Natl. Acad. Sci. USA* **101:**4308–4313; 2004.

[32] Gariboldi, M. B.; Terni, F.; Ravizza, R.; Meschini, S.; Marra, M.; Condello, M.; Arancia, G.; Monti, E. The nitroxide Tempol modulates anthracycline resistance in breast cancer cells. *Free Radic. Biol. Med.* **40:**1409–1418; 2006.

[33] Gariboldi, M. B.; Ravizza, R.; Petterino, C.; Castagnaro, M.; Finocchiaro, G.; Monti, E. Study of in vitro and in vivo effects of the piperidine nitroxide Tempol—a potential new therapeutic agent for gliomas. *Eur. J. Cancer* **39:**829–837; 2003.

[34] Halliwell, B., Gutteridge, J.M.C. (Eds.). *Free radicals in biology and medicine*. Oxford Univ. Press, Oxford; 2007.

[35] Samuni, Y.; Gamson, J.; Samuni, A.; Yamada, K.; Russo, A.; Khrisna, M. C.; Mitchell, J. B. Factors influencing nitroxide reduction and cytotoxicity in vitro. *Antioxid. Redox Signal.* **6:**587–595; 2004.

[36] Borisenko, G. G.; Martin, I.; Zhao, Q.; Amoscato, A. A.; Tyurina, Y. Y.; Kagan, V. E. Glutathione propagates oxidative stress triggered by myeloperoxidase in HL-60 cells: evidence for glutathionyl radical-induced peroxidation of phospholipids and cytotoxicity. *J. Biol. Chem.* **279:**23453–23462; 2004.

[37] Shaw, J. The leishmaniases—survival and expansion in a changing world. *Mem. Inst. Oswaldo Cruz* **102:**541–547; 2007.

[38] Rocha, F. J.; Schleicher, U.; Mattner, J.; Alber, G.; Bogdan, C. Cytokines, signaling pathways, and effector molecules required for the control of Leishmania (Viannia) braziliensis in mice. *Infect. Immun.* **75:**3823–3832; 2007.

[39] Mukbel, R.; Petersen, C. A.; Jones, D. E. Soluble factors from Leishmania major-specific $CD4^+$ T cells and B cells limit L. amazonensis amastigote survival within infected macrophages. *Microbes Infect.* **8:**2547–2555; 2006.

[40] Mukbel, R. M.; Patten, C., Jr.; Gibson, K.; Ghosh, M.; Petersen, C.; Jones, D. E. Macrophage killing of Leishmania amazonensis amastigotes requires both nitric oxide and superoxide. *Am. J. Trop. Med. Hyg.* **76:**669–675; 2007.

[41] Mason, N. J.; Artis, D.; Hunter, C. A. New lessons from old pathogens: what parasitic infections have taught us about the role of nuclear factor-kappaB in the regulation of immunity. *Immunol. Rev.* **201:**48–56; 2004.

[42] Desjeux, P. Leishmaniasis: current situation and new perspectives. *Comp. Immunol. Microbiol. Infect. Dis.* **27:**305–318; 2004.

[43] Cuzzocrea, S.; Pisano, B.; Dugo, L.; Ianaro, A.; Patel, N. S.; Caputi, A. P.; Thiemermann, C. Tempol reduces the activation of nuclear factor-kappaB in acute inflammation. *Free Radic. Res.* **38:**813–819; 2004.

[44] Xie, Q. W.; Kashiwabara, Y.; Nathan, C. Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. *J. Biol. Chem.* **269:**4705–4708; 1994.

[45] Liu, S. F.; Ye, X.; Malik, A. B. In vivo inhibition of nuclear factor-kappa B activation prevents inducible nitric oxide synthase expression and systemic hypotension in a rat model of septic shock. *J. Immunol.* **159:**3976–3983; 1997.

[46] Pahan, K.; Sheikh, F. G.; Namboodiri, A. M.; Singh, I. *N*-Acetyl cysteine inhibits induction of NO production by endotoxin or cytokine stimulated rat peritoneal macrophages, C6 glial cells, and astrocytes. *Free Radic. Biol. Med.* **24:**39–48; 1998.

[47] Flohé, L.; Brigelius-Flohe, R.; Saliou, C.; Traber, M. G.; Packer, L. Redox regulation of NF-kappa B activation. *Free Radic. Biol. Med.* **22:**1115–1126; 1997.

[48] Lancaster Jr., J. R. Nitroxidative, nitrosative, and nitrative stress: kinetic predictions of reactive nitrogen species chemistry under biological conditions. *Chem. Res. Toxicol.* **19:**1160–1174; 2006.

[49] Murray, H. W.; Nathan, C. F. Macrophage microbicidal mechanisms in vivo: reactive nitrogen versus oxygen intermediates in the killing of intracellular visceral Leishmania donovani. *J. Exp. Med.* **189:**741–746; 1999.

[50] Shiloh, M. U.; MacMicking, J. D.; Nicholson, S.; Brause, J. E.; Potter, S.; Marino, M.; Fang, F.; Dinauer, M.; Nathan, C. Phenotype of mice and macrophages deficient in both phagocyte oxidase and inducible nitric oxide synthase. *Immunity* **10:**29–38; 1999.

[51] Peluffo, G.; Piacenza, L.; Irigoin, F.; Alvarez, M. N.; Radi, R. L-Arginine metabolism during interaction of Trypanosoma cruzi with host cells. *Trends Parasitol.* **20:**363–369; 2004.

[52] Tsuhako, M. H.; Augusto, O.; Linares, E.; Dagli, M. L.; Pereira, C. A. Association between nitric oxide synthesis and vaccination-acquired resistance to murine hepatitis virus by SPF mice. *Free Radic. Biol. Med.* **41:**1534–1541; 2006.