Phenotypical characteristics, biochemical pathways, molecular targets and putative role of nitric oxide-mediated programmed cell death in *Leishmania*

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SUMMARY

Nitric oxide (NO) has been demonstrated to be the principal effector molecule mediating intracellular killing of *Leishmania*, both *in vitro* and *in vivo*. We investigated the type of cell death process induced by NO for the intracellular amastigote stage of the protozoa *Leishmania*. Specific detection methods revealed a rapid and extensive cell death with morphological features of apoptosis in axenic amastigotes exposed to NO donors, in intracellular amastigotes inside *in vitro* – activated mouse macrophages and also in activated macrophages of regressive lesions in a leishmaniasis-resistant mouse model. We extended our investigations to the dog, a natural host-reservoir of *Leishmania* parasites, by demonstrating that co-incubation of infected macrophages with autologous lymphocytes derived from dogs immunised with purified excreted-secreted antigens of *Leishmania* resulted in a significant NO-mediated apoptotic cell death of intracellular amastigotes. From the biochemical point of view, NO-mediated *Leishmania* amastigotes apoptosis did not seem to be controlled by caspase activity as indicated by the lack of effect of cell permeable inhibitors of caspases and cysteine proteases, in contrast to specific proteasome inhibitors, such as lactacystin or calpain inhibitor I. Moreover, addition of the products of two NO molecular targets, *cis*-aconitase and glyceraldehyde-3-phosphate dehydrogenase, also had an inhibitory effect on the cell death induced by NO. Interestingly, activities of these two enzymes plus 6-phosphogluconate dehydrogenase, parasitic enzymes involved in both glycolysis and respiration processes, are overexpressed in amastigotes selected for their NO resistance. This review focuses on cell death of the intracellular stage of the pathogen *Leishmania* induced by nitrogen oxides and gives particular attention to the biochemical pathways and the molecular targets potentially involved. Questions about the role of *Leishmania* amastigotes NO-mediated apoptosis in the overall infection process are raised and discussed.

Key words: *Leishmania*, nitrogen oxides, programmed cell death, proteasome, calpain, aconitase.

INTRODUCTION

Leishmaniasis paradox is to be considered as a neglected disease and to be the second-most dreaded parasitic disease in the modern world. There are an estimated 12 million cases worldwide with an annual incidence of about 2 million new cases and more than 350 million women, men and children in 97 countries of the world are at risk of infection (WHO. Leishmaniasis Control home page: http://www.who.int/ctd/html/leish.html). Protozoan trypanosomatids of the genus *Leishmania* cause a wide spectrum of human diseases in many tropical and subtropical regions of the world that range from a self-healing cutaneous ulcer to a potentially fatal visceral infection, depending on the parasite species and host immune responses. The differentiation from metacyclic promastigotes to amastigotes is the first crucial step that determines *Leishmania* pathogenesis (Mallinson and Coombs, 1989). The second step is the adaptation of amastigotes to diverse hostile host environmental conditions and the selection of the fittest individuals to continue the infectious process (Alexander and Russell, 1992).

The activation of the host immune system as a consequence of *Leishmania* infection implies expansion of various cell types; from dendritic cells favouring *Leishmania* dissemination and antigen presentation to lymphocytes that will determine the different possible outcomes of leishmanial infection (Fig. 1; Scott, 1991; Sacks and Noben-Trauth, 2002). In fact, macrophages play a central role in determining *Leishmania* control or multiplication (Fig. 1). Depending on the cytokine environment, macrophages can differentiate into distinct subpopulations, depending on their classical or alternative activation pathway, playing opposite but complementary immunological roles (Fig. 1; Bogdan and Rollinghoff, 1998; Noel *et al*. 2004). Finally, immune control of leishmaniasis involves a dominant

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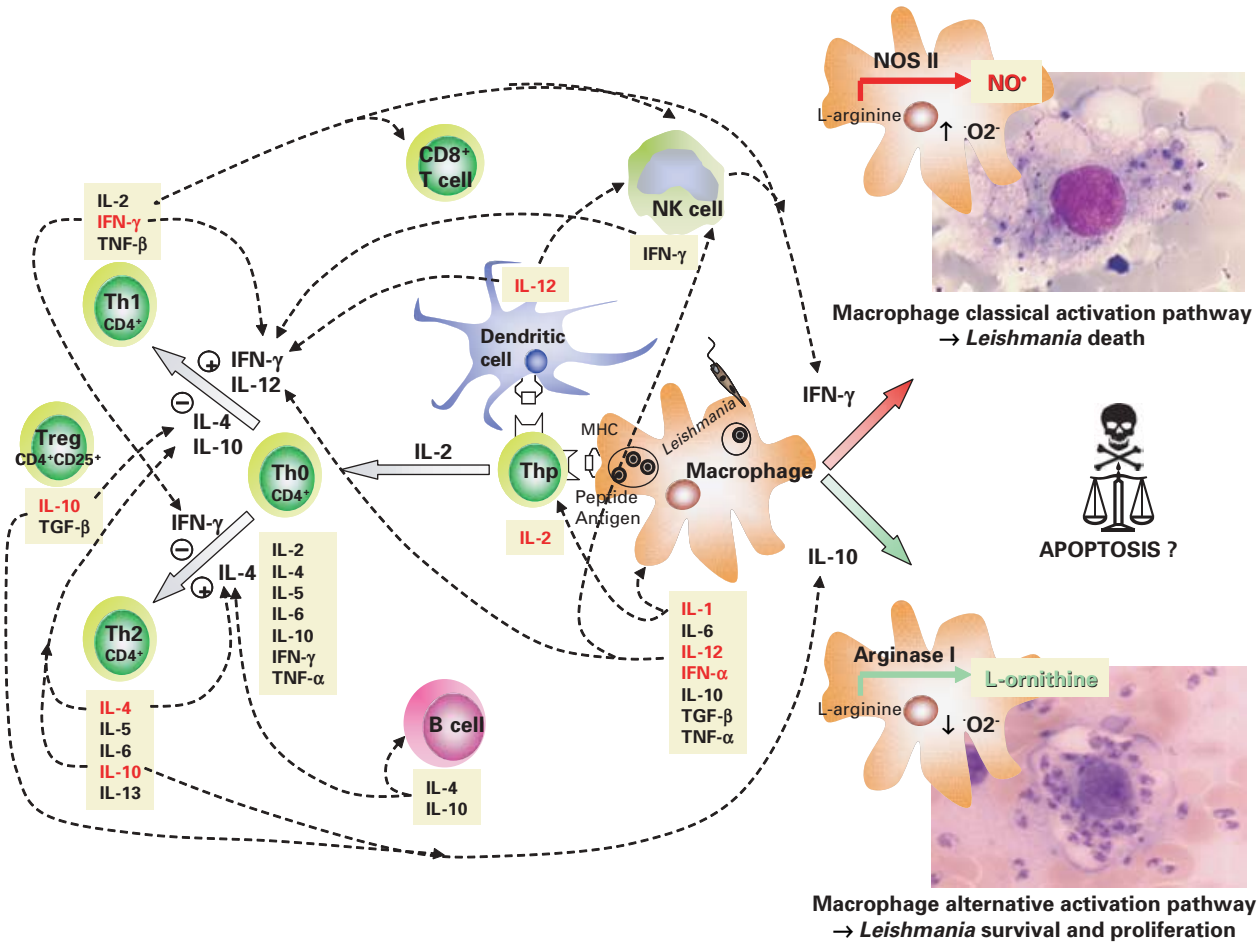

Fig. 1. Immunological determinants influencing *Leishmania* infection. Cytokines expressed by macrophages, dendritic cells and T- and B-lymphocytes determine the outcome of *Leishmania* parasites: from survival and proliferation to death. What role plays parasite apoptosis in this balance? Th: T helper, Thp: precursor T helper, Treg: T regulator, NK: natural killer, CD: cluster of differentiation, IL: interleukine, IFN-α: interferon α, IFN-γ: interferon γ, TNF-α: tumour necrosis factor α, TGF-β: transforming growth factor β, MHC: major histocompatibility complex, NOS: nitric oxide synthase, NO: nitric oxide.

Th1 response, leading to macrophage classical pathway activation and elimination of intracellular amastigotes through the induction of type II nitric oxide synthase (NOS II) and nitric oxide (NO) synthesis from L-arginine (Fig. 1). This prototypical model has been clearly evidenced in the murine experimental model for leishmaniasis but is now enlarged to natural hosts, such as humans and dogs (Green *et al.* 1990; Panaro *et al.* 1999; Sisto *et al.* 2001).

NO is a small molecule being a gas and a powerful intra- and extracellular messenger and that stirs up biological concepts of cellular communication. In the past, radicals had been associated with pathophysiology; now it is being appreciated that NO is a molecule with important signalling qualities (reviewed in Brune, 2003). Biological actions can often be attributed to 'reactive nitrogen species' (RNS) rather than NO itself. NO redox species effects can be propagated via addition or substitution reactions with thiol groups on cysteines and glutathione resulting in S-nitrosothiol (–S–NO) formation, or protein haeme groups that may account for protein nitrosylation (Eu *et al.* 2000; Jaffrey *et al.* 2001; Daiber *et al.* 2002; Espey *et al.* 2002; Thomas *et al.* 2002). S-nitrosothiol formation is reversible and is considered as the prototypic redox-based NO-signalling mechanism, predominantly implicated in cytostatic, cytotoxic or protective NO effects (Stamler, Lamas and Fang, 2001). NO has long been recognized as an important molecule involved simultaneously in the regulation of apoptotic death and cell viability (Bosca and Hortelano, 1999; Nicholson and Thornberry, 2003). First reports on NO-mediated apoptosis were proposed in 1993 (Albina *et al.* 1993; Sarih, Souvannavong and Adam, 1993). Nowadays, NO is described as an inducer of apoptosis in many different cell types (reviewed in Brune, von Knethen and Sandau, 1999). The mitochondrion represents a selective target for NO and there is accumulating evidence that inhibition of respiration may contribute to the pro-apoptotic effect of NO by membrane potential reduction, transition pore opening and release of

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cytochrome c (Boyd and Cadenas, 2002; Moncada and Erusalimsky, 2002). In fact, RNS and reactive oxygen species (ROS) interact for targetting substrate binding sites in several enzyme components of the bioenergetic pathways, thus inhibiting catalytic activity by forming complexes with haeme and iron-sulphur clusters present in many mitochondrial proteins (Hortelano *et al*. 1997, 1999; Brookes *et al*. 2002; Brown and Borutaite, 2002; Radi, Cassina and Hodara, 2002; Costa *et al*. 2003).

Cell death is now well defined in higher eukaryote cells. In fact, many studies have subdivided programmed cell death (PCD) into the three categories of apoptosis (type I), autophagy (type II) and necrosis (type III) based on criteria such as morphological alterations, initiating death signal, or the implication of a family of aspartate-directed cysteine proteases, the caspases (reviewed in Green *et al*. 2004 and in Bras, Queenan and Susin, 2005). Macrophages themselves are a notable and important exception, resisting apoptotic death upon activation. In the case of infectious diseases, this could help to prevent the development of parasitic strategies by phagocytosed pathogens. Nevertheless, an apoptotic-like death of phagocytosed pathogens induced by oxidative species such as NO could represent an escape mechanism at the parasitic population level. When infecting a mammalian host, *Leishmania* parasites are confronted by RNS and ROS and they exhibit a cell death that shares at least morphological features with apoptosis (Das, Mukherjee and Shaha, 2001; Holzmuller *et al*. 2002, 2005*a*, *b*, 2006; Mukherjee *et al*. 2002; Zangger, Mottram and Fasel, 2002; Mehta and Shaha, 2004; Gallego *et al*. 2005; Sousa-Franco *et al*. 2005). Interestingly, apoptotic-like programmed cell death seems to be the preferred way of dying for *Leishmania* parasites exposed to several stimuli, such as heat shock (Moreira *et al*. 1996), chemotherapeutic drugs (Sereno *et al*. 2001; Lee *et al*. 2002; Sudhandiran and Shaha, 2003; Jayanarayan and Dey, 2004, 2005; Paris *et al*. 2004; Verma and Dey, 2004), inhibitors of DNA topoisomerases (Mittra *et al*. 2000; Chowdhury *et al*. 2003; Sen *et al*. 2004*a*, *b*; Marquis, Hardy and Olivier, 2005; Singh, Jayanarayan and Dey, 2005), inhibitor of protein kinase (Arnoult *et al*. 2002), inhibitor of NAD-dependent deacetylases (Vergnes *et al*. 2005), water soluble cationic trans-platinum complexes (Nguewa *et al*. 2005), mutations (Selvapandiyan *et al*. 2004), growth factors (Tavares *et al*. 2005), or more naturally in culture or even *in vivo* (Lee *et al*. 2002; Vergnes *et al*. 2002; Lindoso Cotrim and Goto, 2004). Nevertheless, it is difficult to compare all these works since the type of programmed cell death is hard to define in *Leishmania* and there are many differences between the inducers used, the *Leishmania* species studied, the parasitic stage considered (i.e. promastigote or amastigote), and the state of maturation of the parasitic stage (i.e. dividing or non-dividing). This highlights the interest of studying 'natural' apoptosis-like inducers that are involved in anti-leishmanial strategies developed either by the host (vector or mammal) or by the clinician.

In this review, we have focused on cell death of the intracellular stage of the pathogen *Leishmania* induced by nitric oxide and redox derivatives: do we consider this cell suicide as apoptosis-like? Is it a way to maximise the *Leishmania* biological fitness? Could PCD represent a potential target for protozoan parasites control?

NO-MEDIATED APOPTOSIS-LIKE CELL DEATH IN *LEISHMANIA* AMASTIGOTES

Apoptosis or type I PCD is marked by morphological characteristics occurring in the dying cell including cell shrinkage, oligonucleosomal DNA fragmentation, chromatin condensation leading to the appearance of pyknotic nuclei, and controlled disintegration of the cell into so-called apoptotic bodies (Kerr, Wyllie and Currie, 1972; Clarke, 1990). NO-mediated cell death of *Leishmania* axenically-grown amastigotes was first assessed by Lemesre *et al*. in 1997. NO-mediated DNA fragmentation exhibiting features of apoptosis was further demonstrated in axenic amastigotes incubated with several NO donors (acidified sodium nitrite, nitrosylated albumin, SNAP, DETA/NONOate) by monitoring the genomic DNA status of treated versus untreated parasites. Nuclear DNA fragmentation into oligonucleosomal-sized fragments (720, 360 and 180 bp), a typical phenotypical characteristic of apoptotic cells, was readily visible in agarose gel in the case of NO-treated amastigotes and was confirmed by the use of the *in situ* TUNEL technique (Fig. 2A and B; Holzmuller *et al*. 2002). This characteristic of the DNA status during the course of cell death by apoptosis was also found in *Leishmania* undergoing programmed cell death induced by other stimuli (Moreira *et al*. 1996; Das *et al*. 2001; Sereno *et al*. 2001; Arnoult *et al*. 2002; Lee *et al*. 2002; Zangger *et al*. 2002; Sudhandiran and Shaha, 2003; Lindoso *et al*. 2004; Paris *et al*. 2004; Sen *et al*. 2004*a*, *b*; Verma and Dey, 2004; Gallego *et al*. 2005; Jayanarayan and Dey, 2005; Marquis *et al*. 2005; Singh *et al*. 2005; Tavares *et al*. 2005). The use of the molecular DNA intercalatant YOPRO-1, which penetrates specifically apoptotic cells exhibiting the phospholipid phosphatidylserine on their surface, which is normally hidden within the plasma membrane, was a new criterion defining the cell death induced by NO as apoptosis-like (Fig. 2C; Holzmuller *et al*. 2002). The Annexin V apoptotic detection method was used by other investigators to demonstrate the plasma membrane phospholipids reorganisation in *Leishmania* undergoing cell death (Mittra *et al*. 2000; Arnoult *et al*. 2002; Sudhandiran

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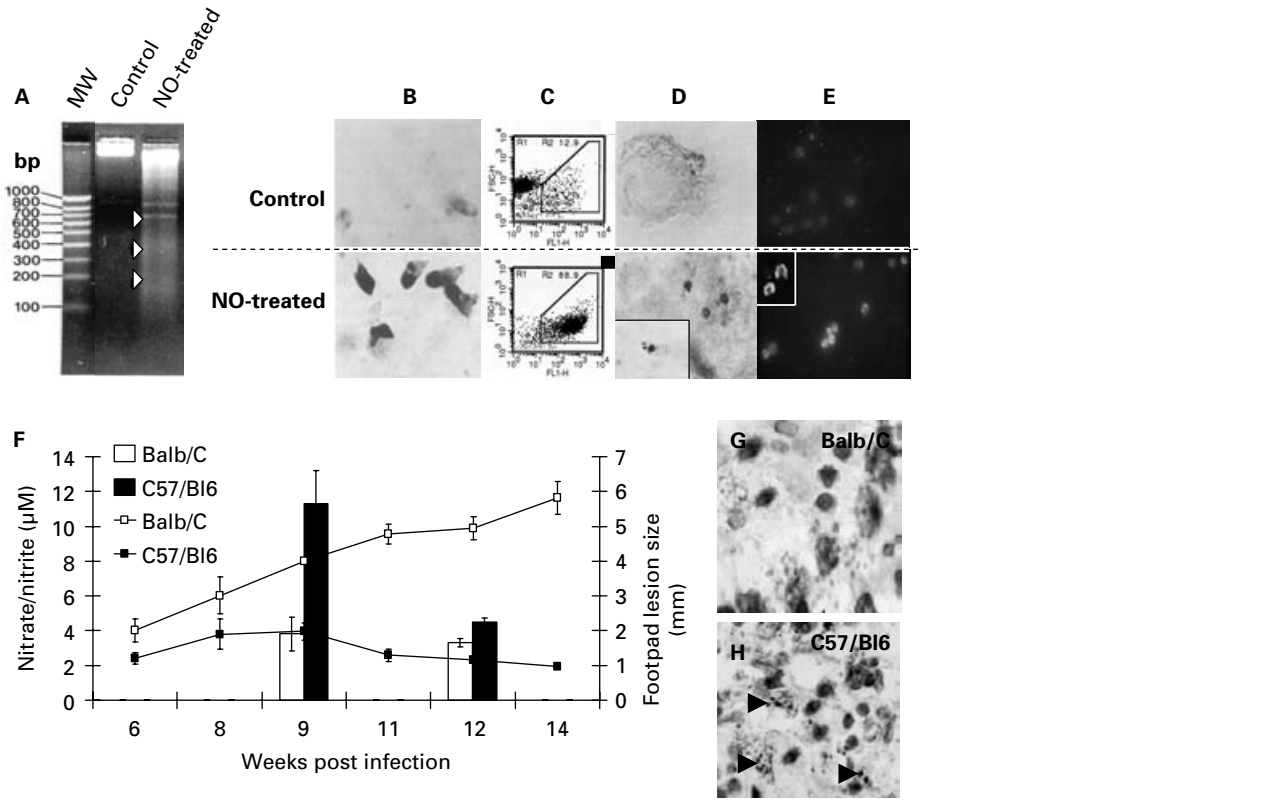

Fig. 2. NO-mediated programmed cell death exhibiting morphological features of apoptosis in *Leishmania* amastigotes. Control versus NO donor-treated axenic amastigotes DNA banding pattern in ethidium bromide-stained agarose gel (A), recognition of apoptotic DNA strand breaks by the *in situ* Terminal deoxynucleotidyl Transferase-mediated dUTP Nick-End Labeling (TUNEL assay kit, Alexis Biochemicals) in control versus NO donor-treated axenic amastigotes (B) and in intracellular amastigotes within control versus NO producing mouse macrophages (D), apoptotic fluorescent YOPRO-1 probe staining in flow cytofluorometry analysis of control versus NO donor-treated (6 hours with 5 mM acidified sodium nitrite) axenic amastigotes (C) and *in situ* in intracellullar amastigotes within control versus NO producing dog macrophages (E). Evolution of footpad lesion size and nitrate/nitrite levels in sera of Balb/C and C57/Black6 mice infected with *Leishmania amazonensis* (F) and *in situ* TUNEL in histological thin cryosections counterstained with Giemsa of footpad lesions of Balb/C (G) and C57/Black6 (H) mice.

and Shaha, 2003; Paris *et al*. 2004; Singh *et al*. 2005; Tavares *et al*. 2005). Nevertheless, we must also take into account that living amastigotes can exhibit phosphatidylserine (PS) on their surface. Exposed PS participates in amastigote internalization and induction of the macrophage alternative activation pathway increasing intracellular *Leishmania* amastigotes growth (de Freitas Balanco *et al*. 2001).

Otherwise, by using our *in vitro* culture system for axenically-grown amastigotes (Lemesre, 1994; Sereno and Lemesre, 1997), we showed that trivalent antimony induced *Leishmania* amastigotes cell death (Sereno *et al*. 2001). Changes upstream of DNA fragmentation included generation of oxidative molecules among which was nitric oxide that was primarily concentrated in the parasitophorous vacuole (Sudhandiran and Shaha, 2003). As an indirect proof of potential coordination of natural and chemotherapeutic anti-leishmanial molecules, we demonstrated recently that antimonial-resistant amastigotes were less susceptible to NO-mediated PCD (Holzmuller *et al*. 2005*b*). These data suggest that trivalent antimony could act both as an anti-leishmanial molecule and as a macrophage activating compound (Carter *et al*. 1989). Activation of mouse macrophages by the classical pathway leads to *L. amazonensis* intracellular amastigotes apoptosis-like death mediated by L-arginine-derived nitrogen oxides (Fig. 2D; Holzmuller *et al*. 2002). Very recently, Sousa-Franco *et al*. (2006) found that Balb/C peritoneal macrophages, which are unable to eliminate *L. amazonensis* without previous activation with cytokines or lipopolysaccharide (LPS), can kill *L. guyanensis* amastigotes through an apoptotic process that is independent of NO and is mediated by reactive oxygen species. As a whole, the oxidative machinery of macrophages leads to induction of *Leishmania* amastigotes' programmed cell death. Nevertheless, depending on the *Leishmania* species considered, the innate microbicidal mechanisms may be sufficient or supplemented by mechanisms triggered through the classical activation pathway of the macrophage by either acquired immunity or chemotherapeutic molecules. Finally, as previously

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suggested in cured *Leishmania*-infected dogs (Vouldoukis *et al*. 1996), integration of the above data strengthens the idea that usual chemotherapy and protective immunity are linked as they both lead to generation of reactive oxygen and nitrogen species (ROS an RNS) within the host cell. Under these conditions *Leishmania* parasites undergo a programmed cell death exhibiting morphological features of metazoan apoptosis and that can be considered as their preferred mode of death inside their mammalian hosts.

This raises the question of the occurrence of *Leishmania* apoptotic-like cell death *in vivo*. Fig. 2F shows the outcome of the infection by *L. amazonensis* promastigotes in both a susceptible and a resistant mouse model. In Balb/C mice (i.e. *Leishmania*-susceptible mice) the footpad lesion grows continuously during the time-course of the experiment without development of a Th1-type cellular immune response, as indicated by the absence of increasing levels of NO-end products in the serum (Fig. 2F). By contrast, regression of the footpad lesion size in C57/Black6 mice (i.e. *Leishmania*-resistant mice) correlates with increased detection of nitrites in the serum indicative of NO production, due to an efficient Th1 cellular immune response (Fig. 2F). The *in situ* TUNEL technique reveals amastigotes dying by apoptosis in histological thin sections of the regressive lesion (Fig. 2H). Interestingly, apoptotic amastigotes were also observed in the spleen and the liver of hamsters infected with *L. chagasi*, but the potential stimuli involved are only suggested and discussed (Lindoso *et al*. 2004). Nevertheless, according to the available data, we can reasonably postulate that phenotypically-defined apoptosis is the natural type of cell death induced *in vivo* by nitric oxide, or more generally by both ROS and RNS, as a consequence of a Th1-type polarised cellular immune response involved in *Leishmania* resistance.

Furthermore, we recently described the capacity of naturally secreted antigens easily purified from culture supernatant of *L. infantum* promastigotes (*Li*ESAp), successfully cultivated in completely defined medium (Lemesre, 1994; Merlen *et al*. 1999), to protect dogs, a natural host for visceral leishmaniasis, against experimental *L. infantum* infections (Lemesre *et al*. 2005). We show that vaccine-induced protection correlates with an early establishment of a strong and long-lasting predominantly Th1-type cellular immune response specifically directed against *Li*ESAp as demonstrated by anti-*Li*ESAp IgG2 reactivity, *Li*ESAp-specific lymphocyte proliferation assays and enhanced NO-mediated anti-leishmanial activity of canine monocyte-derived macrophages (CM-DM) in response to higher IFNγ production by T cells. The use of both *in situ* TUNEL and *in situ* YOPRO-1 techniques (Fig. 2E) indicates *Leishmania* amastigote death by apoptosis inside T lymphocytes-activated CM-DM derived from dogs immunised with *Li*ESAp (Holzmuller *et al*. 2005*a*). These studies confirm that the NO-mediated apoptosis of intracellular *Leishmania* amastigotes that we previously demonstrated in a murine laboratory experimental model, also occurs in a canine model, a natural reservoir for *L. infantum*/*L. chagasi*, the etiologic agents of visceral leishmaniasis, in response to cell-mediated protective immunity.

BIOCHEMICAL PATHWAYS INVOLVED IN NO-MEDIATED PROGRAMMED CELL DEATH IN *LEISHMANIA*

Apoptosis defined in higher eukaryote cells (i.e. mammalian cells) is regulated by two well-characterized executive pathways (reviewed in Green, 2000). The first involves the proteolytic activation of caspases (reviewed in Hengartner, 2000), and could be considered as an evolutionary step acquired by metazoa. The second one is more complex and involves the mitochondrion, with outer membrane permeabilisation leading to the release into the cytosol of mitochondrial intermembrane space proteins that either induce caspase activation, e.g. cytochrome c, or promote the induction of caspase-independent pathways, e.g. apoptosis inducing factor (AIF) (reviewed in Zamzami and Kroemer, 2001). This second pathway can be considered as inherited during the nucleated cell/bacteria-derived mitochondrion symbiosis and is characterized by apoptosis regulators belonging to the Bcl-2 protein family (Reed, 1994; Henkart and Grinstein, 1996; Gross, McDonnell and Korsmeyer, 1999; Martinou and Green, 2001). Caspase activities seem to be essential for the induction of the typical nuclear features of apoptosis, such as chromatin condensation and oligonucleosomal DNA fragmentation, whereas they are not required, in several circumstances, for the induction and execution of PCD (Sperandio, de Belle and Bredesen, 2000). Moreover, evidence is now accumulating that non-caspase proteases including cathepsins, calpains, granzymes and the proteasome complex, also have roles in mediating and promoting cell death (Orlowski, 1999; Johnson, 2000).

Caspase-like activities have been described in *Leishmania* through the cleavage of specific substrates or the use of specific inhibitors. In 2001, Das *et al*. made the first report of a caspase-like-regulated cell death in *L. donovani* promastigotes exposed to ROS. They demonstrated a significant increase in the ability of parasite lysates to cleave a substrate for the CED-3/CPP32 group of proteases. Pre-treatment of cells with a specific inhibitor of this group of proteases reduces the number of cells showing apoptosis-like features and inhibits the cleavage of a poly(ADP)ribose-polymerase (PARP)-like

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protein (Das *et al*. 2001). Camptothecin, an inhibitor of topoisomerase I, induces formation of ROS in *L. donovani*, which increases cytosolic calcium levels and decreases both intracellular pH and potassium levels inducing the apoptotic process through activation of caspase 3-like proteases CED-3/CPP32 (Sen *et al*. 2004*a*). Endogenous ROS formation causes subsequent elevation in the level of lipid peroxidation that are potentially involved in the loss of mitochondrial membrane potential and cytochrome c release (Sen *et al*. 2004*b*). Activation of CED-3/CPP32 and ICE group of proteases occurs downstream of mitochondrial injuries (Sen *et al*. 2004*b*). Interestingly, novobiocin, an inhibitor of topoisomerase II, also induces a CED-3/CPP32-regulated apoptosis but without inducing ROS (Singh *et al*. 2005). This suggests that different biochemical pathways could be exploited by *Leishmania* parasites to die by apoptosis. This hypothesis is strengthened by the partial effect of cell permeable caspase inhibitors on amphotericin B-induced apoptosis in *Leishmania* promastigotes and axenic amastigotes (Lee *et al*. 2002). Moreover, drug resistance may influence the biochemical pathway involved in PCD since PARP cleavage was evident in the wild type strain but not in the arsenite resistant strain of *L. donovani* undergoing apoptosis (Jayanarayan and Dey, 2004).

Furthermore, in our experiments with nitric oxide, there was no evidence of CED-3/CPP32 proteases family activation in the *L. amazonensis* amastigote NO-induced apoptosis as indicated by the lack of effect of specific caspase inhibitors Z-VAD-fluoromethylketone and Z-DEVD-fluoromethylketone, or a general cysteine-protease inhibitor [(2*S*,3*S*)-*trans*-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester E-64d (Holzmuller *et al*. 2002). No inhibitory effect was observed with the caspase inhibitors in *L. mexicana* axenic amastigotes undergoing apoptosis whereas they were active in amastigotes isolated from mouse macrophages (Zangger *et al*. 2002). The authors hypothesised either uptake of macrophage caspases by the parasite or the involvement of *Leishmania* cathepsins in the DEVDase activity measurement used to provide evidence of caspases 3 and 7 (Zangger *et al*. 2002). This shows the limits of the specificity of the substrates used to characterize caspase-like activities in PCD in *Leishmania*. Moreover, to date no caspase homologues have been demonstrated in any unicellular eukaryotes (Aravind, Dixit and Koonin, 2001). However, genes encoding for metacaspases, which belong to an ancestral metacaspase, paracaspase and caspase super-family have been identified in protozoa (Uren *et al*. 2000; Szallies, Kubata and Duszenko, 2002; Mottram *et al*. 2003). The functions of these metacaspases, in particular in promoting cell death, and sensitivity to classical caspase inhibitors have yet to be elucidated. In fact, to our present knowledge, cathepsin or calpain-like cysteine protease activities strengthen the evolutionary hypothesis of a less complex ancestral biochemical pathway mediating PCD in *Leishmania*. In contrast to the lack of effect of caspase inhibitors, we observed a significant inhibitory effect on NO-induced apoptosis in *L. amazonensis* amastigotes with specific proteasome inhibitors, in particular lactacystin and calpain inhibitor I (Holzmuller *et al*. 2002). The delay in NO-mediated amastigote apoptosis-like death in the presence of reversible proteasome inhibitors supports the view that protease activities of the proteasome complex are involved in promoting apoptosis-like changes in NO-exposed amastigotes (Holzmuller *et al*. 2002). Recent studies have pointed out in two *Leishmania* species the existence of an active proteasome, one similar to the proteasomes of other eukaryotes (Robertson, 1999; Christensen *et al*. 2000; Paugam *et al*. 2003). Protease activities of this proteasome, in particular calpain, could participate in the cleavage of PARP-like proteins inhibiting DNA repair and favouring nuclear events of apoptosis, as demonstrated in human neuroblastoma cells (McGinnis *et al*. 1999). More recently, calpain inhibitor I was shown to interfere with apoptotic DNA fragmentation in *L. donovani* promastigote death induced by miltefosine (Paris *et al*. 2004). Moreover, Arnoult *et al*. (2002) have identified a calpain-like sequence in the kinetoplastid database and suggested that *L. major* cysteine proteinases, inhibitable by both broad caspase and cysteine protease inhibitors, are calpain-like proteases.

Finally, as caspase activities have only been indicated by the use of inhibitors or substrates that are also effective on other cysteine proteases, oxidative stress-mediated PCD in *Leishmania* could be executed by cysteine proteases belonging to the cathepsin or the calpain families, or to a new cysteine protease family generated during the evolution of protozoans. In fact, *Leishmania* parasites contain different cysteine proteases, among which cathepsin B- and L-like proteases play an important role in the proliferation and differentiation processes (Frame, Mottram and Coombs, 2000) and could participate in the regulation of parasite death or survival in the host (Selzer *et al*. 1999). After the activation of the death programme, cysteine proteases related to the calpain family could promote the induction of nuclear apoptosis-like features in *Leishmania* parasites. Sequential involvement of both cathepsin and calpain families could represent a prototype of the caspase cascade occurring in metazoan apoptosis. Further investigations that must consider both the *Leishmania* species studied and the apoptotic stimuli used, are needed to determine either the precise role of cysteine proteases in executing PCD or the existence of other biochemical pathways controlling PCD.

NO MOLECULAR TARGETS ASSOCIATED WITH *LEISHMANIA* PROGRAMMED CELL DEATH

S-nitrosylation reactions with thiol groups on cysteines represent the prototypic molecular NO redox-based mechanism of interaction with proteins (reviewed in Stamler, Lamas and Fang, 2001). In this regard, there is unquestionable evidence that the active site of caspases can be S-nitrosylated, which results in loss of enzyme function (Dimmeler *et al*. 1997; Liu and Stamler, 1999). Although caspase homologues have, to date, not been formally demonstrated in any unicellular eukaryotes (Aravind *et al*. 2001), we could hypothesise that if caspase-like or rather metacaspase activities (Uren *et al*. 2000; Szallies *et al*. 2002; Mottram *et al*. 2003) exist in *Leishmania*, they represent high affinity NO molecular targets and that NO-mediated PCD is under control of other molecules. Moreover, recent studies demonstrate inactivation of *Leishmania* cysteine protease by NO (Fig. 3, A and B; Salvati *et al*. 2001; Ascenzi *et al*. 2004). Although the exact role of cysteine proteases in *Leishmania* killing is unclear, it has been demonstrated that *Leishmania* can not grow within macrophages in the presence of cysteine protease inhibitors (Mottram *et al*. 1996). Cysteine proteases are involved in several biological functions of the parasite (Mottram, Brooks and Coombs 1998). For example, cathepsin-B and -L-like proteases are essential for *Leishmania* virulence (Denise *et al*. 2003). Furthermore, calpain-like cysteine proteases are present in *Leishmania* (Arnoult *et al*. 2002; Mottram, Coombs and Alexander, 2004) and they are described as playing a crucial role in NO-mediated cell injuries (Volbracht *et al*. 2005). In fact, considering the definition of apoptosis characterized in metazoans, almost all potential effector molecules belong to the cysteine protease super-family and represent targets inhibitable by NO. Nevertheless, kinetics of inactivation of protozoan cysteine proteases by NO exhibit second-order to pseudo-first-order reaction kinetics depending on NO concentration (Bocedi *et al*. 2004). Based on the observation of differential inhibition of *L. amazonensis* cysteine proteases of amastigotes incubated with NO donors (Holzmuller *et al*. unpublished data), we could also suggest differential sensitivity to NO depending on the catalytic site conformation. In this view, further research is needed to analyse the potential role of cysteine proteases in promoting NO-mediated *Leishmania* PCD.

Other molecular targets, involved in both glycolysis and citric acid cycle, have been shown to be inhibited by NO in *Leishmania* parasites, in particular *cis*-aconitase (Fig. 3C; Lemesre *et al*. 1997) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Mauel and Ransijn, 1997). Interestingly, NO seems to disrupt crucial steps of the energetic metabolism to induce cell death. Up-stream impairment of mitochondrial respiration (Szabo and Salzman, 1995; Lemesre *et al*. 1997), NO inhibition of glycolysis through ADP ribosylation of GAPDH (Zhang and Snyder, 1993; Mauel and Ransijn, 1997), concomitant with depletion of cellular NAD+ pools (Radons *et al*. 1994), could represent the death signal initiating PCD. In fact, apoptosis is a form of cell death that requires energy. By blocking the metabolic pathway involved in the conversion of the source of energy (i.e. glucose), we can postulate that NO action forces *Leishmania* parasites to use the stocks of energy to ensure a silent cell death. It makes sense if we consider the NO disruption of the citric acid cycle as the effector signal promoting PCD since inhibition of respiration may contribute to the NO pro-apoptotic effect (Boyd and Cadenas, 2002; Moncada and Erusalimsky, 2002). A biologically indirect argument supporting our hypothesis is that amastigotes appeared to be less sensitive than corresponding promastigotes to NO action (Lemesre *et al*. 1997). This difference was consistent with the relatively weak development of the mitochondria in amastigotes compared to promastigotes (Mukkada *et al*. 1985). In terms of co-evolution of *Leishmania* inside the host cell, decreased mitochondrial development could represent an adaptative strategy to manage NO toxicity and related cell death induction. Furthermore, NO-mediated PCD in *Leishmania* amastigotes is abolished by supplementation with the products of these two enzymes: either *cis*-aconitate for *cis*-aconitase or 1,3-bisphosphoglycerate for GAPDH (Fig. 3D). This suggests that these two enzymes are key targets involved in NO-mediated apoptosis. We recently demonstrated that *L. infantum* amastigotes selected *in vitro* for their NO resistance over-express both *cis*-aconitase and GAPDH (Fig. 3E; Holzmuller *et al*. 2006). In fact, parasitic over-expression of NO molecular targets may protect the amastigotes both directly and indirectly. Directly, enzymes act as NO scavengers and consequently detoxify the cell. Indirectly, increased GAPDH could prevent ATP depletion and consequently cell death, by engaging anaerobic glycolysis as observed in NO-treated glucose-fed human epithelial cells (Le Goffe *et al*. 2002). Moreover, over-expression of *cis*-aconitase, which is considered as a two-faced protein, i.e. acting firstly as an enzyme and secondly as an iron regulatory protein (Beinert and Kennedy, 1993), would increase regulation of iron homeostasis, which plays a crucial role in tumour cell protection from the pro-apoptotic effect of NO (Feger *et al*. 2001). In addition to *cis*-aconitase and GAPDH, we evidenced over-expression of 6-phosphogluconate dehydrogenase (6PGDH) (Fig. 3E; Holzmuller *et al*. 2006). The 6PGDH is the third enzyme of the pentose phosphate pathway (PPP), which generates NADPH and ribulose-5-phosphate (Barrett, 1997).

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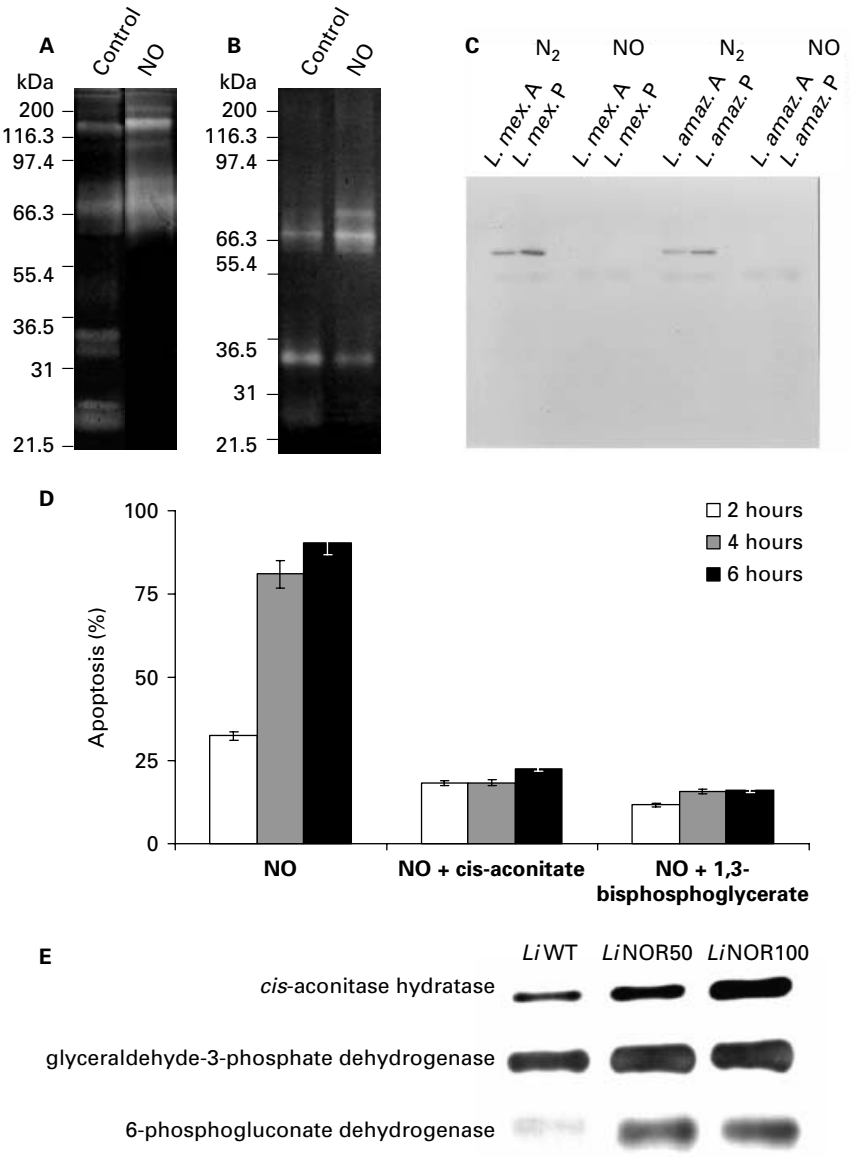

Fig. 3. NO molecular targets in *Leishmania*. Proteinase activities in gelatin-copolymerised SDS-PAGE stained by Coomassie blue of *L. amazonensis* amastigotes protein lysates either incubated in the presence or absence of 5 mM acidified sodium nitrite during gel revelation steps (A) or prepared from axenic parasites cultured for 4 hours in acidified PBS in the absence or presence of 5 mM sodium nitrite (B). *Cis*-aconitase activity of N2- versus NO-treated promastigotes (P) and axenic amastigotes (A) of *L. amazonensis* (*L. amaz.*) and *L. mexicana* (*L. mex.*) by multilocus enzyme electrophoresis (MLEE) (C). Apoptosis of *L. amazonensis* axenic amastigotes cultured in the presence of NO donor in a medium supplemented by either *cis*-aconitate (product of *cis*-aconitase) or 1,3-bisphosphoglycerate (product of glyceraldehyde-3-phosphate dehydrogenase) (D). MLEE profiles revealed with the *cis*-aconitase hydratase (E.C.4.2.1.3), glyceraldehyde-3-phosphate dehydrogenase (E.C.1.2.1.12), 6-phosphogluconate dehydrogenase (EC1.1.1.44) of wild-type amastigotes (LiWT) and amastigotes resistant to 50 mM (LiNOR50) and to 100 mM (LiNOR100) DETA/NONOate (E).

One hypothesis is that the principal function of PPP in *Leishmania* amastigotes is the production of NADPH, which is known to protect the parasite *Trypanosoma brucei* against oxidative stress (Dardonville *et al*. 2003).

As a whole, data on NO molecular targets open the way for further investigations on the molecular characterization of NO-mediated PCD in *Leishmania*. In particular, it would be of interest to elucidate the NO targeting of mitochondrial enzymes, correlated with subsequent release of calcium from the de-energized organelle to the cytosol (Richter *et al*. 1994), leading to activation of calpain-related enzymes for the execution of the nuclear features of cell death. This could therefore define a PCD specific to protozoan parasites and represent the first evolutionary step to apoptosis defined in metazoa.

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WHY DO *LEISHMANIA* PARASITES DIE BY APOPTOSIS?

The important question still remaining is what could be the role of PCD in trypanosomatid parasites? It is fair to assume that, if unicellular organisms have retained such a very complex PCD pathway during evolution, it is because this pathway must be beneficial or essential for survival of the species or population. Two main hypotheses have been proposed to answer the fascinating question concerning the benefits of apoptosis for unicellular organisms (Debrabant *et al*. 2003; Debrabant and Nakhasi, 2003; Nguewa *et al*. 2004; Wanderley *et al*. 2005). First, cell death can be important for population size control in response to limited resources (Welburn, Barcinski and Williams, 1997; Al-Olayan, Williams and Hurd, 2002; Lee *et al*. 2002) or to avoid host death (parasitic organisms) (Heussler, Kuenzi and Rottenberg, 2001). In this case unicellular apoptotic cells show altruistic behaviour, dying for the benefit of others. The second explanation is that apoptotic cells, which will not necessary die (apoptotic mimicry), could provide signals that enhance the survival of the entire population (Lee *et al*. 2002; Zangger *et al*. 2002). For example, the evidence that phagocytosis of apoptotic cells reduces the secretion of mammalian-derived pro-inflammatory cytokines or signals as growth factors, offers the interesting speculation that the ability of intracellular *Leishmania* to undergo apoptosis may reduce the host immune response and favour overall parasite survival (de Freitas Balanco *et al*. 2001; DosReis and Barcinski, 2001).

Apoptosis in *Leishmania* promastigotes and axenically-grown amastigotes has been well demonstrated *in vitro* (Das *et al*. 2001; Sereno *et al*. 2001; Arnoult *et al*. 2002; Holzmuller *et al*. 2002; Lee *et al*. 2002) and then *ex vivo* in interacellular amastigotes (Sereno *et al*. 2001; Holzmuller, 2002, 2005*a*). For the moment, only one published study reports the occurrence of apoptosis in *Leishmania* amastigotes *in vivo* (Lindoso *et al*. 2004), suggesting that PCD could constitute a mechanism that regulates growth of the parasite population during *Leishmania* infection. Moreover, we show in this paper that apoptotic amastigotes were detected in regressive lesions of experimentally infected resistant mice, allowing us to suggest that PCD could occur *in vivo* as a consequence of a Th1-type polarised cellular immune response involved in *Leishmania* resistance. Furthermore, even if apoptotic PCD also seems to be important in vector/parasite interactions in both malaria (Hurd, Carter and Nacer, 2005) and African trypanosomiasis (Welburn and Murphy, 1998), apoptosis during *Leishmania* metacyclogenesis within the sandfly vector still needs to be demonstrated.

In order to demonstrate successfully the real purpose of the PCD, whether altruistic or otherwise, in *Leishmania* or other trypanosomatid parasites, it is essential to establish definitively that PCD really occurs *in vivo* in the insect vectors and/or in the mammalian hosts (inside or outside the macrophage for *Leishmania*). A better and more detailed understanding of the *in vivo* role of PCD in *Leishmania*, as in other unicellular parasites, is needed because it could be exploited to identify new targets for therapeutic intervention. The clarification of its potential relevance in silencing the host immune response to favour parasite survival or infection (alternative activation of macrophages, limitation of antigen presentation to the immune system, *Leishmania* antigen-specific suppression of the T-cell response) and in increasing the host survival time allowing the pathogen to live in pseudo-symbiosis with the host (regulation of cell population in tissues and organs, control of the growth and/or selection of parasitic population by parasite-derived signals, control of the virulence, mechanisms to promote and maintain clonality within the population) will help to define precisely the role played by PCD in the establishment and the maintenance of the *Leishmania*/host relationship.

Finally, the most important benefit of PCD knowledge in kinetoplastids would be the design of more active and less toxic drugs directed towards specific molecular targets of the parasites.

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REFERENCES

Albina, J. E., Cui, S., Mateo, R. B. and Reichner, J. S. (1993). Nitric oxide-mediated apoptosis in murine peritoneal macrophages. *Journal of Immunology* **150**, 5080–5085.

Alexander, J. and Russell, D. G. (1992). The interaction of *Leishmania* species with macrophages. *Advances in Parasitology* **31**, 175–254.

Al-Olayan, E. M., Williams, G. T. and Hurd, H. (2002). Apoptosis in the malaria protozoan, *Plasmodium berghei*: a possible mechanism for limiting intensity of infection in the mosquito. *International Journal for Parasitology* **32**, 1133–1143.

Aravind, L., Dixit, V. M. and Koonin, E. V. (2001). Apoptotic molecular machinery: vastly increased complexity in vertebrates revealed by genome comparisons. *Science* **291**, 1279–1284.

Arnoult, D., Akarid, K., Grodet, A., Petit, P. X., Estaquier, J. and Ameisen, J. C. (2002). On the evolution of programmed cell death: apoptosis of the unicellular eukaryote *Leishmania major* involves cysteine proteinase activation and mitochondrion

https://doi.org/10.1017/S0031182006000837 Published online by Cambridge University Press

permeabilization. *Cell Death and Differentiation* **9**, 65–81.

Ascenzi, P., Bocedi, A., Gentile, M., Visca, P. and Gradoni, L. (2004). Inactivation of parasite cysteine proteinases by the NO-donor 4-(phenylsulfonyl)-3-((2-(dimethylamino)ethyl)thio)-furoxan oxalate. *Biochimica et Biophysica Acta* **1703**, 69–77.

Barrett, M. P. (1997). The pentose phosphate pathway and parasitic protozoa. *Parasitology Today* **13**, 11–16.

Beinert, H. and Kennedy, M. C. (1993). Aconitase, a two-faced protein: enzyme and iron regulatory factor. *FASEB Journal* **7**, 1442–1449.

Bocedi, A., Gradoni, L., Menegatti, E. and Ascenzi, P. (2004). Kinetics of parasite cysteine proteinase inactivation by NO-donors. *Biochemical and Biophysical Research Communications* **315**, 710–718.

Bogdan, C. and Rollinghoff, M. (1998). The immune response to *Leishmania*: mechanisms of parasite control and evasion. *International Journal for Parasitology* **28**, 121–134.

Bosca, L. and Hortelano, S. (1999). Mechanisms of nitric oxide-dependent apoptosis: involvement of mitochondrial mediators. *Cellular Signaling* **11**, 239–244.

Boyd, C. and Cadenas, E. (2002). Nitric oxide and cell signaling pathways in mitochondrial-dependent apoptosis. *Biological Chemistry* **383**, 411–423.

Bras, M., Queenan, B. and Susin, S. A. (2005). Programmed cell death via mitochondria: different modes of dying. *Biochemistry* (*Moscow*) **70**, 231–239.

Brookes, P. S., Levonen, A. L., Shiva, S., Sarti, P. and Darley-Usmar, V. M. (2002). Mitochondria: regulators of signal transduction by reactive oxygen and nitrogen species. *Free Radical Biology and Medicine* **33**, 755–764.

Brown, G. C. and Borutaite, V. (2002). Nitric oxide inhibition of mitochondrial respiration and its role in cell death. *Free Radical Biology and Medicine* **33**, 1440–1450.

Brune, B. (2003). Nitric oxide: NO apoptosis or turning it ON? *Cell Death and Differentiation* **10**, 864–869.

Brune, B., von Knethen, A. and Sandau, K. B. (1999). Nitric oxide (NO): an effector of apoptosis. *Cell Death and Differentiation* **6**, 969–975.

Carter, K. C., Alexander, J., Baillie, A. J. and Dolan, T. F. (1989). Visceral leishmaniasis: resistance to reinfection in the liver following chemotherapy in the BALB/c mouse. *Experimental Parasitology* **68**, 375–381.

Chowdhury, A. R., Mandal, S., Goswami, A., Ghosh, M., Mandal, L., Chakraborty, D., Ganguly, A., Tripathi, G., Mukhopadhyay, S., Bandyopadhyay, S. and Majumder, H. K. (2003). Dihydrobetulinic acid induces apoptosis in *Leishmania donovani* by targeting DNA topoisomerase I and II: implications in antileishmanial therapy. *Molecular Medicine* **9**, 26–36.

Christensen, C. B., Jorgensen, L., Jensen, A. T., Gasim, S., Chen, M., Kharazmi, A., Theander, T. G. and Andresen, K. (2000). Molecular characterization of a *Leishmania donovani* cDNA clone with similarity to human 20S proteasome a-type subunit. *Biochimica et Biophysica Acta* **1500**, 77–87.

Clarke, P. G. (1990). Developmental cell death: morphological diversity and multiple mechanisms. *Anatomy and Embryology* **181**, 195–213.

Costa, N. J., Dahm, C. C., Hurrell, F., Taylor, E. R. and Murphy, M. P. (2003). Interactions of mitochondrial thiols with nitric oxide. *Antioxidants and Redox Signaling* **5**, 291–305.

Daiber, A., Frein, D., Namgaladze, D. and Ullrich, V. (2002). Oxidation and nitrosation in the nitrogen monoxide/superoxide system. *Journal of Biological Chemistry* **277**, 11882–11888.

Dardonville, C., Rinaldi, E., Hanau, S., Barrett, M. P., Brun, R. and Gilbert, I. H. (2003). Synthesis and biological evaluation of substrate-based inhibitors of 6-phosphogluconate dehydrogenase as potential drugs against African trypanosomiasis. *Bioorganic and Medicinal Chemistry* **11**, 3205–3214.

Das, M., Mukherjee, S. B. and Shaha, C. (2001). Hydrogen peroxide induces apoptosis-like death in *Leishmania donovani* promastigotes. *Journal of Cell Science* **114**, 2461–2469.

Debrabant, A., Lee, N., Bertholet, S., Duncan, R. and Nakhasi, H. L. (2003). Programmed cell death in trypanosomatids and other unicellular organisms. *International Journal for Parasitology* **33**, 257–267.

Debrabant, A. and Nakhasi, H. (2003). Programmed cell death in trypanosomatids: is it an altruistic mechanism for survival of the fittest? *Kinetoplastid Biology and Disease* **25**, 7.

de Freitas Balanco, J. M., Moreira, M. E., Bonomo, A., Bozza, P. T., Amarante-Mendes, G., Pirmez, C. and Barcinski, M. A. (2001). Apoptotic mimicry by an obligate intracellular parasite downregulates macrophage microbicidal activity. *Current Biology* **11**, 1870–1873.

Denise, H., McNeil, K., Brooks, D. R., Alexander, J., Coombs, G. H. and Mottram, J. C. (2003). Expression of multiple CPB genes encoding cysteine proteases is required for *Leishmania mexicana* virulence *in vivo*. *Infection and Immunity* **71**, 3190–3195.

Dimmeler, S., Haendeler, J., Nehls, M. and Zeiher, A. M. (1997). Suppression of apoptosis by nitric oxide via inhibition of interleukin-1beta-converting enzyme (ICE)-like and cysteine protease protein (CPP)-32-like proteases. *Journal of Experimental Medicine* **185**, 601–607.

DosReis, G. A. and Barcinski, M. A. (2001). Apoptosis and parasitism: from the parasite to the host immune response. *Advances in Parasitology* **49**, 133–161.

Espey, M. G., Thomas, D. D., Miranda, K. M. and Wink, D. A. (2002). Focusing of nitric oxide mediated nitrosation and oxidative nitrosylation as a consequence of reaction with superoxide. *Proceedings of the National Academy of Sciences, USA* **99**, 11127–11132.

Eu, J. P., Liu, L., Zeng, M. and Stamler, J. S. (2000). An apoptotic model for nitrosative stress. *Biochemistry* **39**, 1040–1047.

Feger, F., Ferry-Dumazet, H., Mamani Matsuda, M., Bordenave, J., Dupouy, M., Nussler, A. K., Arock, M., Devevey, L., Nafziger, J., Guillosson, J. J., Reiffers, J. and Mossalayi, M. D. (2001). Role of iron in tumor cell protection from the pro-apoptotic effect of nitric oxide. *Cancer Research* **61**, 5289–5294.

Frame, M. J., Mottram, J. C. and Coombs, G. H. (2000). Analysis of the roles of cysteine proteinases of *Leishmania mexicana* in the host-parasite interaction. *Parasitology* **121**, 367–377.

Gallego, C., Estevez, A. M., Farez, E., Ruiz-Perez, L. M. and Gonzalez-Pacanowska, D. (2005). Overexpression of AP endonuclease protects *Leishmania major* cells against methotrexate induced DNA fragmentation and hydrogen peroxide. *Molecular and Biochemical Parasitology* **141**, 191–197.

Green, D. R. (2000). Apoptotic pathways: paper wraps stone blunts scissors. *Cell* **102**, 1–4.

Green, D. R., Knight, R. A., Melino, G., Finazzi-Agro', A. and Orrenius, S. (2004). Ten years of publication in cell death. *Cell Death and Differentiation* **11**, 2–3.

Green, S. J., Crawford, R. M., Hockmeyer, J. T., Meltzer, M. S. and Nacy, C. A. (1990). *Leishmania major* amastigotes initiate the L-arginine-dependent killing mechanism in IFN-gamma-stimulated macrophages by induction of tumor necrosis factor-alpha. *Journal of Immunology* **145**, 4290–4297.

Gross, A., McDonnell, J. M. and Korsmeyer, S. J. (1999). BCL-2 family members and the mitochondria in apoptosis. *Genes and Development* **13**, 1899–1911.

Hengartner, M. O. (2000). The biochemistry of apoptosis. *Nature* **407**, 770–776.

Henkart, P. A. and Grinstein, S. (1996). Apoptosis: mitochondria resurrected? *Journal of Experimental Medicine* **183**, 1293–1295.

Heussler, V. T., Kuenzi, P. and Rottenberg, S. (2001). Inhibition of apoptosis by intracellular protozoan parasites. *International Journal for Parasitology* **31**, 1166–1176.

Holzmuller, P., Cavaleyra, M., Moreaux, J., Kovacic, R., Vincendeau, P., Papierok, G. and Lemesre, J. L. (2005*a*). Lymphocytes of dogs immunised with purified excreted-secreted antigens of *Leishmania infantum* co-incubated with *Leishmania* infected macrophages produce IFN gamma resulting in nitric oxide-mediated amastigote apoptosis. *Veterinary Immunology and Immunopathology* **106**, 247–257.

Holzmuller, P., Hide, M., Sereno, D. and Lemesre, J. L. (2006). *Leishmania infantum* amastigotes resistant to nitric oxide cytotoxicity: Impact on *in vitro* parasite developmental cycle and metabolic enzyme activities. *Infection, Genetics and Evolution* **6**, 187–197.

Holzmuller, P., Sereno, D., Cavaleyra, M., Mangot, I., Daulouede, S., Vincendeau, P. and Lemesre, J. L. (2002). Nitric oxide-mediated proteasome-dependent oligonucleosomal DNA fragmentation in *Leishmania amazonensis* amastigotes. *Infection and Immunity* **70**, 3727–3735.

Holzmuller, P., Sereno, D. and Lemesre, J. L. (2005*b*). Lower nitric oxide susceptibility of trivalent antimony-resistant amastigotes of *Leishmania infantum*. *Antimicrobial Agents and Chemotherapy* **49**, 4406–4409.

Hortelano, S., Alvarez, A. M. and Bosca, L. (1999). Nitric oxide induces tyrosine nitration and release of cytochrome c preceding an increase of mitochondrial transmembrane potential in macrophages. *FASEB Journal* **13**, 2311–2317.

Hortelano, S., Dallaporta, B., Zamzami, N., Hirsch, T., Susin, S. A., Marzo, I., Bosca, L. and Kroemer, G. (1997). Nitric oxide induces apoptosis via triggering mitochondrial permeability transition. *FEBS Letters* **410**, 373–377.

Hurd, H., Carter, V. and Nacer, A. (2005). Interactions between malaria and mosquitoes: the role of apoptosis in parasite establishment and vector response to infection. *Current Topics Microbiology and Immunology* **289**, 185–217.

Jaffrey, S. R., Erdjument-Bromage, H., Ferris, C. D., Tempst, P. and Snyder, S. H. (2001). Protein S-nitrosylation: a physiological signal for neuronal nitric oxide. *Nature Cell Biology* **3**, 193–197.

Jayanarayan, K. G. and Dey, C. S. (2004). Altered expression, polymerisation and cellular distribution of alpha-/beta-tubulins and apoptosis-like cell death in arsenite resistant *Leishmania donovani* promastigotes. *International Journal for Parasitology* **34**, 915–925.

Jayanarayan, K. G. and Dey, C. S. (2005). Altered tubulin dynamics, localization and post-translational modifications in sodium arsenite resistant *Leishmania donovani* in response to paclitaxel, trifluralin and a combination of both and induction of apoptosis-like cell death. *Parasitology* **131**, 215–230.

Johnson, D. E. (2000). Noncaspase proteases in apoptosis. *Leukemia* **14**, 1695–1703.

Kerr, J. F., Wyllie, A. H. and Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer* **26**, 239–257.

Lee, N., Bertholet, S., Debrabant, A., Muller, J., Duncan, R. and Nakhasi, H. L. (2002). Programmed cell death in the unicellular protozoan parasite *Leishmania*. *Cell Death and Differentiation* **9**, 53–64.

Le Goffe, C., Vallette, G., Charrier, L., Candelon, T., Bou-Hanna, C., Bouhours, J. F. and Laboisse, C. L. (2002). Metabolic control of resistance of human epithelial cells to H2O2 and NO stresses. *Biochemical Journal* **364**, 349–359.

Lemesre, J. L. (1994). Methods for the culture *in vitro* of different stages of tissue parasites, *International Publication* WO 94/26899.

Lemesre, J. L., Holzmuller, P., Cavaleyra, M., Goncalves, R. B., Hottin, G. and Papierok, G. (2005). Protection against experimental visceral leishmaniasis infection in dogs immunized with purified excreted secreted antigens of *Leishmania infantum* promastigotes. *Vaccine* **23**, 2825–2840.

Lemesre, J. L., Sereno, D., Daulouede, S., Veyret, B., Brajon, N. and Vincendeau, P. (1997). *Leishmania* spp.: nitric oxide-mediated metabolic inhibition of promastigote and axenically grown amastigote forms. *Experimental Parasitology* **86**, 58–68.

Lindoso, J. A., Cotrim, P. C. and Goto, H. (2004). Apoptosis of *Leishmania* (*Leishmania*) *chagasi* amastigotes in hamsters infected with visceral leishmaniasis. *International Journal for Parasitology* **34**, 1–4.

Liu, L. and Stamler, J. S. (1999). NO: an inhibitor of cell death. *Cell Death and Differentiation* **6**, 937–942.

Mallinson, D. J. and Coombs, G. H. (1989). Interaction of *Leishmania* metacyclics with macrophages. *International Journal for Parasitology* **19**, 647–656.

Marquis, J. F., Hardy, I. and Olivier, M. (2005). Resistance mechanism development to the

topoisomerase-I inhibitor Hoechst 33342 by *Leishmania donovani*. *Parasitology* **131**, 197–206.

Martinou, J. C. and Green, D. R. (2001). Breaking the mitochondrial barrier. *Nature Reviews – Molecular Cell Biology* **2**, 63–67.

Mauel, J. and Ransijn, A. (1997). *Leishmania* spp.: mechanisms of toxicity of nitrogen oxidation products. *Experimental Parasitology* **87**, 98–111.

McGinnis, K. M., Gnegy, M. E., Park, Y. H., Mukerjee, N. and Wang, K. K. (1999). Procaspase-3 and poly(ADP)ribose polymerase (PARP) are calpain substrates. *Biochemical and Biophysical Research Communications* **263**, 94–99.

Mehta, A. and Shaha, C. (2004). Apoptotic death in *Leishmania donovani* promastigotes in response to respiratory chain inhibition: complex II inhibition results in increased pentamidine cytotoxicity. *Journal of Biological Chemistry* **279**, 11798–11813.

Merlen, T., Sereno, D., Brajon, N., Rostand, F. and Lemesre, J. L. (1999). *Leishmania* spp: completely defined medium without serum and macromolecules (CDM/LP) for the continuous *in vitro* cultivation of infective promastigote forms. *American Journal of Tropical Medicine and Hygiene* **60**, 41–50.

Mittra, B., Saha, A., Chowdhury, A. R., Pal, C., Mandal, S., Mukhopadhyay, S., Bandyopadhyay, S. and Majumder, H. K. (2000). Luteolin, an abundant dietary component is a potent anti-leishmanial agent that acts by inducing topoisomerase II-mediated kinetoplast DNA cleavage leading to apoptosis. *Molecular Medicine* **6**, 527–541.

Moncada, S. and Erusalimsky, J. D. (2002). Does nitric oxide modulate mitochondrial energy generation and apoptosis? *Nature Reviews – Molecular Cell Biology* **3**, 214–220.

Moreira, M. E., Del Portillo, H. A., Milder, R. V., Balanco, J. M. and Barcinski, M. A. (1996). Heat shock induction of apoptosis in promastigotes of the unicellular organism *Leishmania* (*Leishmania*) *amazonensis*. *Journal of Cellular Physiology* **167**, 305–313.

Mottram, J. C., Brooks, D. R. and Coombs, G. H. (1998). Roles of cysteine proteinases of trypanosomes and *Leishmania* in host-parasite interactions. *Current Opinion in Microbiology* **1**, 455–460.

Mottram, J. C., Coombs, G. H. and Alexander, J. (2004). Cysteine peptidases as virulence factors of Leishmania. *Current Opinion in Microbiology* **7**, 375–381.

Mottram, J. C., Helms, M. J., Coombs, G. H. and Sajid, M. (2003). Clan CD cysteine peptidases of parasitic protozoa. *Trends in Parasitology* **19**, 182–187.

Mottram, J. C., Souza, A. E., Hutchison, J. E., Carter, R., Frame, M. J. and Coombs, G. H. (1996). Evidence from disruption of the lmcpb gene array of *Leishmania mexicana* that cysteine proteinases are virulence factors. *Proceedings of the National Academy of Sciences, USA* **93**, 6008–6013.

Mukherjee, S. B., Das, M., Sudhandiran, G. and Shaha, C. (2002). Increase in cytosolic Ca2+ levels through the activation of non-selective cation channels induced by oxidative stress causes mitochondrial depolarization leading to apoptosis-like death in *Leishmania donovani* promastigotes. *Journal of Biological Chemistry* **277**, 24717–24727.

Mukkada, A. J., Meade, J. C., Glaser, T. A. and Bonventre, P. F. (1985). Enhanced metabolism of *Leishmania donovani* amastigotes at acid pH: an adaptation for intracellular growth. *Science* **229**, 1099–1101.

Nguewa, P. A., Fuertes, M. A., Iborra, S., Najajreh, Y., Gibson, D., Martinez, E., Alonso, C. and Perez, J. M. (2005). Water soluble cationic trans-platinum complexes which induce programmed cell death in the protozoan parasite *Leishmania infantum*. *Journal of Inorganic Biochemistry* **99**, 727–736.

Nguewa, P. A., Fuertes, M. A., Valladares, B., Alonso, C. and Perez, J. M. (2004). Programmed cell death in trypanosomatids: a way to maximize their biological fitness? *Trends in Parasitology* **20**, 375–380.

Nicholson, D. W. and Thornberry, N. A. (2003). Apoptosis. Life and death decisions. *Science* **299**, 214–215.

Noel, W., Raes, G., Hassanzadeh Ghassabeh, G., De Baetselier, P. and Beschin, A. (2004). Alternatively activated macrophages during parasite infections. *Trends in Parasitology* **20**, 126–133.

Orlowski, R. Z. (1999). The role of the ubiquitin-proteasome pathway in apoptosis. *Cell Death and Differentiation* **6**, 303–313.

Panaro, M. A., Acquafredda, A., Lisi, S., Lofrumento, D. D., Trotta, T., Satalino, R., Saccia, M., Mitolo, V. and Brandonisio, O. (1999). Inducible nitric oxide synthase and nitric oxide production in *Leishmania infantum*-infected human macrophages stimulated with interferon-gamma and bacterial lipopolysaccharide. *International Journal of Clinical and Laboratory Research* **29**, 122–127.

Paris, C., Loiseau, P. M., Bories, C. and Breard, J. (2004). Miltefosine induces apoptosis-like death in *Leishmania donovani* promastigotes. *Antimicrobial Agents and Chemotherapy* **48**, 852–859.

Paugam, A., Bulteau, A. L., Dupouy-Camet, J., Creuzet, C. and Friguet, B. (2003). Characterization and role of protozoan parasite proteasomes. *Trends in Parasitology* **19**, 55–59.

Radi, R., Cassina, A. and Hodara, R. (2002). Nitric oxide and peroxynitrite interactions with mitochondria. *Biological Chemistry* **383**, 401–409.

Radons, J., Heller, B., Burkle, A., Hartmann, B., Rodriguez, M. L., Kroncke, K. D., Burkart, V. and Kolb, H. (1994). Nitric oxide toxicity in islet cells involves poly(ADP-ribose) polymerase activation and concomitant NAD+ depletion. *Biochemical and Biophysical Research Communications* **199**, 1270–1277.

Reed, J. C. (1994). Bcl-2 and the regulation of programmed cell death. *Journal of Cell Biology* **124**, 1–6.

Richter, C., Gogvadze, V., Schlapbach, R., Schweizer, M. and Schlegel, J. (1994). Nitric oxide kills hepatocytes by mobilizing mitochondrial calcium. *Biochemical and Biophysical Research Communications* **205**, 1143–1150.

Robertson, C. D. (1999). The *Leishmania mexicana* proteasome. *Molecular and Biochemical Parasitology* **103**, 49–60.

Sacks, D. and Noben-Trauth, N. (2002). The immunology of susceptibility and resistance to *Leishmania major* in mice. *Nature Reviews – Immunology* **2**, 845–858.

https://doi.org/10.1017/S0031182006000837 Published online by Cambridge University Press

Salvati, L., Mattu, M., Colasanti, M., Scalone, A., Venturini, G., Gradoni, L. and Ascenzi, P. (2001). NO donors inhibit *Leishmania infantum* cysteine proteinase activity. *Biochimica et Biophysica Acta* **1545**, 357–366.

Sarih, M., Souvannavong, V. and Adam, A. (1993). Nitric oxide synthase induces macrophage death by apoptosis. *Biochemical and Biophysical Research Communications* **191**, 503–508.

Scott, P. (1991). IFN-gamma modulates the early development of Th1 and Th2 responses in a murine model of cutaneous leishmaniasis. *Journal of Immunology* **147**, 3149–3155.

Selvapandiyan, A., Debrabant, A., Duncan, R., Muller, J., Salotra, P., Sreenivas, G., Salisbury, J. L. and Nakhasi, H. L. (2004). Centrin gene disruption impairs stage-specific basal body duplication and cell cycle progression in *Leishmania*. *Journal of Biological Chemistry* **279**, 25703–25710.

Selzer, P. M., Pingel, S., Hsieh, I., Ugele, B., Chan, V. J., Engel, J. C., Bogyo, M., Russell, D. G., Sakanari, J. A. and Mckerrow, J. H. (1999). Cysteine protease inhibitors as chemotherapy: lessons from a parasite target. *Proceedings of the National Academy of Sciences, USA* **96**, 11015–11022.

Sen, N., Das, B. B., Ganguly, A., Mukherjee, T., Bandyopadhyay, S. and Majumder, H. K. (2004*a*). Camptothecin-induced imbalance in intracellular cation homeostasis regulates programmed cell death in unicellular hemoflagellate *Leishmania donovani*. *Journal of Biological Chemistry* **279**, 52366–52375.

Sen, N., Das, B. B., Ganguly, A., Mukherjee, T., Tripathi, G., Bandyopadhyay, S., Rakshit, S., Sen, T. and Majumder, H. K. (2004*b*). Camptothecin induced mitochondrial dysfunction leading to programmed cell death in unicellular hemoflagellate *Leishmania donovani*. *Cell Death and Differentiation* **11**, 924–936.

Sereno, D., Holzmuller, P., Mangot, I., Cuny, G., Ouaissi, A. and Lemesre, J. L. (2001). Antimonial-mediated DNA fragmentation in *Leishmania infantum* amastigotes. *Antimicrobial Agents and Chemotherapy* **45**, 2064–2069.

Sereno, D. and Lemesre, J. L. (1997). Axenically cultured amastigote forms as an *in vitro* model for investigation of antileishmanial agents. *Antimicrobial Agents and Chemotherapy* **41**, 972–976.

Singh, G., Jayanarayan, K. G. and Dey, C. S. (2005). Novobiocin induces apoptosis-like cell death in topoisomerase II over-expressing arsenite resistant *Leishmania donovani*. *Molecular and Biochemical Parasitology* **141**, 57–69.

Sisto, M., Brandonisio, O., Panaro, M. A., Acquafredda, A., Leogrande, D., Fasanella, A., Trotta, T., Fumarola, L. and Mitolo, V. (2001). Inducible nitric oxide synthase expression in *Leishmania*-infected dog macrophages. *Comparative Immunology, Microbiology and Infectious Diseases* **24**, 247–254.

Sousa-Franco, J., Araujo-Mendes, E., Silva-Jardim, I., L-Santos, J., Faria, D. R., Dutra, W. O. and Horta, M. D. (2005). Infection-induced respiratory burst in BALB/c macrophages kills *Leishmania guyanensis* amastigotes through apoptosis: possible involvement in resistance to cutaneous leishmaniasis. *Microbes and Infection* **8**, 390–400.

Sperandio, S., De Belle, I. and Bredesen, D. E. (2000). An alternative, nonapoptotic form of programmed cell death. *Proceedings of the National Academy of Sciences, USA* **97**, 14376–14381.

Stamler, J. S., Lamas, S. and Fang, F. C. (2001). Nitrosylation. the prototypic redox-based signaling mechanism. *Cell* **106**, 675–683.

Sudhandiran, G. and Shaha, C. (2003). Antimonial-induced increase in intracellular Ca2+ through non-selective cation channels in the host and the parasite is responsible for apoptosis of intracellular *Leishmania donovani* amastigotes. *Journal of Biological Chemistry* **278**, 25120–25132.

Szabo, C. and Salzman, A. L. (1995). Endogenous peroxynitrite is involved in the inhibition of mitochondrial respiration in immuno-stimulated J774.2 macrophages. *Biochemical and Biophysical Research Communications* **209**, 739–743.

Szallies, A., Kubata, B. K. and Duszenko, M. (2002). A metacaspase of *Trypanosoma brucei* causes loss of respiration competence and clonal death in the yeast *Saccharomyces cerevisiae*. *FEBS Letters* **517**, 144–150.

Tavares, J., Ouaissi, A., Lin, P. K., Tomas, A. and Cordeiro-Da-Silva, A. (2005). Differential effects of polyamine derivative compounds against *Leishmania infantum* promastigotes and axenic amastigotes. *International Journal for Parasitology* **35**, 637–646.

Thomas, D. D., Miranda, K. M., Espey, M. G., Citrin, D., Jourd'heuil, D., Paolocci, N., Hewett, S. J., Colton, C. A., Grisham, M. B., Feelisch, M. and Wink, D. A. (2002). Guide for the use of nitric oxide (NO) donors as probes of the chemistry of NO and related redox species in biological systems. *Methods in Enzymology* **359**, 84–105.

Uren, A. G., O'Rourke, K., Aravind, L. A., Pisabarro, M. T., Seshagiri, S., Koonin, E. V. and Dixit, V. M. (2000). Identification of paracaspases and metacaspases: two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma. *Molecular Cell* **6**, 961–917.

Vergnes, B., Sereno, D., Madjidian-Sereno, N., Lemesre, J. L. and Ouaissi, A. (2002). Cytoplasmic SIR2 homologue overexpression promotes survival of *Leishmania* parasites by preventing programmed cell death. *Gene* **296**, 139–150.

Vergnes, B., Vanhille, L., Ouaissi, A. and Sereno, D. (2005). Stage-specific antileishmanial activity of an inhibitor of SIR2 histone deacetylase. *Acta Tropica* **94**, 107–115.

Verma, N. K. and Dey, C. S. (2004). Possible mechanism of miltefosine-mediated death of *Leishmania donovani*. *Antimicrobial Agents and Chemotherapy* **48**, 3010–3015.

Volbracht, C., Chua, B. T., Ng, C. P., Bahr, B. A., Hong, W. and Li, P. (2005). The critical role of calpain versus caspase activation in excitotoxic injury induced by nitric oxide. *Journal of Neurochemistry* **93**, 1280–1292.

Vouldoukis, I., Drapier, J. C., Nussler, A. K., Tselentis, Y., Da Silva, O. A., Gentilini, M., Mossalayi, D. M., Monjour, L. and Dugas, B. (1996).

Canine visceral leishmaniasis: successful chemotherapy induces macrophage antileishmanial activity via the L-arginine nitric oxide pathway. *Antimicrobial Agents and Chemotherapy* **40**, 253–256.

Wanderley, J. L., Benjamin, A., Real, F., Bonomo, A., Moreira, M. E. and Barcinski, M. A. (2005). Apoptotic mimicry: an altruistic behavior in host/*Leishmania* interplay. *Brazilian Journal of Medical and Biological Research* **38**, 807–812.

Welburn, S. C., Barcinski, M. A. and Williams, G. T. (1997). Programmed cell death in trypanosomatids. *Parasitology Today* **13**, 22–26.

Welburn, S. C. and Murphy, N. B. (1998). Prohibitin and RACK homologues are up-regulated in trypanosomes induced to undergo apoptosis and in naturally occurring terminally differentiated forms. *Cell Death and Differentiation* **5**, 615–622.

Zamzami, N. and Kroemer, G. (2001). The mitochondrion in apoptosis: how Pandora's box opens. *Nature Reviews – Molecular Cell Biology* **2**, 67–71.

Zangger, H., Mottram, J. C. and Fasel, N. (2002). Cell death in *Leishmania* induced by stress and differentiation: programmed cell death or necrosis? *Cell Death and Differentiation* **9**, 1126–1139.

Zhang, J. and Snyder, S. H. (1993). Purification of a nitric oxide-stimulated ADP-ribosylated protein using biotinylated beta-nicotinamide adenine dinucleotide. *Biochemistry* **32**, 2228–2233.

https://doi.org/10.1017/S0031182006000837 Published online by Cambridge University Press