Involvement of Nitric Oxide and Its Up/Down Stream Molecules in the Immunity Against Parasitic Infections

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Nitric oxide (NO) is a potent mediator with diverse roles in regulating cellular functions and signaling pathways. The NO synthase (NOS) enzyme family consists of three major isoforms, which convey variety of messages between cells, including signals for vasorelaxation, neurotransmission and cytotoxicity. This family of enzymes are generally classified as neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). Increased levels of NO are induced from iNOS during infection; while eNOS and nNOS may be produced at the baseline in normal conditions. An association of some key cytokines appears to be essential for NOS gene regulation in the immunity of infections. Accumulating evidence indicates that parasitic diseases are commonly associated with elevated production of NO. NO plays a role in the immunoregulation and it is implicated in the host non-specific defence in a variety of infections. Nevertheless, the functional role of NO and NOS isoforms in the immune responses of host against the majority of parasites is still highly controversial. In the present review, the role of parasitic infections will be discussed in the controversy related to the NO production and iNOS gene expression in different parasites and a variety of experimental models.
Key-Words: iNOS, nitric oxide, NO, immunity, helminth, parasite, Protozoa.

The nitric oxide (NO) molecule consists of oxygen and nitrogen atoms, bound by a double bond [1]. NO is a product of L-arginine conversion to L-citrulline by nitric oxide synthase (NOS) enzyme in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) as a co-factor (Figure 1.). NO is a reactive free radical, and in the presence of oxygen, is oxidised to a variety of nitrogen oxides [2]. NO is known to react rapidly with oxyhemoglobin (*Oxy*-Hb) to give nitrate and *met*-Hb [3]. It has recently been found to be a potent immuno-modulator, which has alternative roles during inflammation, infection and transplant rejection [4]. Both oxygen and NO are vital for life processes, but too much of either can damage cells. It is suggested the attachment of NO to proteins enable them to activate gene(s) directly, but the body needs to keep NO in equilibrium by turning on and off expression of NO gene(s). NO also has an extraordinary physiological role with an ability to diffuse freely through cell membranes offering a new perspective on cell-cell communication [5].

Potential effector mechanisms of immunity against parasitic infections include antibodies, macrophages, T-cells, cytokines and a variety of other soluble mediators. Activated phagocytic cells generate large amounts of highly toxic molecules, reactive nitrogen and oxygen intermediate (RNI, ROI), H_2O_2, NO and many cytokines and enzymes [6-8]. NO reacts to form biologically active oxides, which react in several ways. In addition to numerous cytokines, the role of NO as a mediator in clinical parasitic diseases remains controversial.

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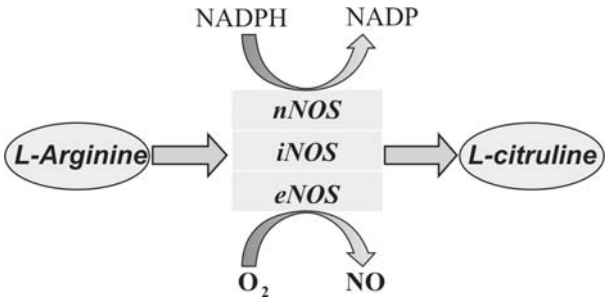
Figure 1. Nitric oxide production by three nitric oxide synthase isoforms. NADPH, nicotinamide adenine dinucleotide phosphate hydrogenase; NADP, nicotinamide adenine dinucleotide phosphate.



In previous reports, NO, RNI and NOS involvement in parasitic infections were investigated; however, variation in NO levels and NOS activities is under debate [9-11].

Immune Cell Types in NO Involvement During Infections

NO is produced by many cell types to induce many functions [12,13]. As part of the cytotoxic function, a variety of cells is involved in NO production including macrophages, neutrophils, Kupffer cells, lymphocytes and hepatocytes that are stimulated to produce RNI and ROI by some key cytokines such as TNF-α and IFN-γ [14,15]. NOS, NO or its stable metabolites have been identified as major effector molecules. An important protective role for RNI has been established in macrophage killing of intracellular protozoa [16-19], bacteria [20,21], fungus [22] and viruses [23]. A similar role has been demonstrated in neutrophil killing of *Candida albicans* [24] and *Staphilococcus aureus* [25]. In addition, there are data showing that eosinophils are able to kill intracellular protozoan parasites [26-28].

NOS Isoforms

The NOS family is generally classified as constitutive, calcium dependent as neuronal NOS (nNOS), endothelial NOS (eNOS) and calcium independent inducible NOS (iNOS) [29]. The molecular masses for the three NOS enzymes are reported to be 160 kDa (nNOS), 130 kDa (iNOS) and 135 kDa (eNOS) [30] (Table 1). The nNOS is constitutive with low output and mainly expressed in the central and peripheral nervous systems [29]. It normally generates low levels of NO for intracellular signalling and modulation of synaptic plasticity in the nervous system [31]. However, the overstimulated nNOS is implicated in ischemia, pain and several neurodegenerative disorders [32]. The iNOS was originally described as an enzyme that is expressed in activated macrophages, generates NO from the amino acid L-arginine and thereby contributes to the control of replication or killing of intracellular microbial pathogens [33]. It is expressed in response to endotoxins and inflammatory cytokines [32]. Inducible NOS is an inducible immune inflammatory factor with high output originally found in macrophages, hepatocytes, but later in glial cells [34]. Small quantities of iNOS-derived NO are critical for signal transduction events during infection [33]. The eNOS is also constitutive with low output [31] and mainly found in brain, neuronal tissue, neuroblastomas, skeletal muscle, vascular endothelial cells and b-cells of pancreatic islets [4]. The nNOS-derived NO, relaxes the vasculature, inhibits adhesion and platelet aggregation and maintains normal blood pressure [32].

Molecular Biology of NOS Isoforms

The cDNA for the three human NOS isoforms have been cloned and characterised. The human NOS genes are located on chromosomes: 12 (nNOS), 17 (iNOS) and 7 (eNOS). In mouse they are located in different chromosomes; 5 (nNOS), 11 (iNOS) and 5 (eNOS). The human genome contains at least two loci for the iNOS gene [35], however in mouse genome one locus has been recognised for the iNOS gene [30] (Table 2).

NOS Deficiency and Infections

Experiments conducted to confirm or deny the involvement of NO and RNI in infections, using experimental NOS knockout animals have been contradictory or inconclusive [36-38]. However, it is possible that iNOS deficient mice develop alternative pathways to overcome in-born deficiencies [36,39]. The role of NO as a single molecule in biological processes may ultimately require a triple-knockout mouse for iNOS, eNOS and nNOS to examine this question [39].

NO and Related Molecules During Infections

A role for antiparasitic effects of NO, RNI and NOS *in vivo* and *in vitro* have been demonstrated against a number of parasites including *Plasmodium sp.*, *Leishmania sp.*, *Toxoplasma gondeii*, *Schistosoma sp*. and *Trypanosoma brucei* [15, 40, 41]. There are several experimental evidences about NO involvement in the microbicidal activity of macrophages against a number of intracellular parasitic pathogens [28] including *L. major* [16], *T. gondii* [17], *T. cruzi* [19], *P. berghei* [42], or pathogenic bacteria e.g. *Mycobacterium leprae* [20], fungal infections e.g. *Candida albicans* [22] and viral pathogens e.*g. Herpes simplex* [23].

NO and Related Molecules During Parasitic Infections

NOS, NO or its stable metabolites have been identified as major effector molecules during the majority of parasitic infections. It seems that NO is not only necessary, but is also sufficient to account for the entire antiparasitic activity [43]. There is evidence that the activated macrophages are able to kill intracellular *L. major, T. gondeii* and extracellular *Schistosoma sp*. parasites by the release of NO and RNI [41,42]. The formation of NO, RNI and $ONOO^-$ has been reported in majority of parasitic infections including *Giardia lamblia, Entamoeba coli* [44], *L. amazonensis* [45], *T. gondii* [46], *L. mexicana* [47], *Schistosoma mansoni* [28], *Opisthorchis viverrini* [48] and *Clonorchis sinensis* [15].

NO and Related Molecules in Protozoal Infections

Inducible NO, synthesized by the iNOS, is an anti-pathogen and tumoricidal agent. However, its production requires a tight control because of cytotoxic and immune-modulation activity [49]. Although there are several immunological mechanisms to eliminate the intracellular pathogens, they have elaborated a variety of strategies to escape immune response and to make possible survival and replication in the host. Several parasites are highly sensitive to NO and their derivatives, however some parasites modulate the production of toxic molecules [50]. Interestingly, hemoglobin, myoglobin, and neuroglobin may protect intracellular protozoa from the antiparasitic effects of NO [51]. Despite the wide evidence about anti-protozoal effects of NO, little efforts have been made to develop NO-based drugs in human medicine. This is mainly due to the difficulty in designing chemical carriers able to release the right amount of NO, in the right place and in the right time, to avoid toxic effects against non-target host cells [49].

Leishmania sp.

NO produced by human and canine macrophages has been involved in the intracellular killing of leishmania. Mechanisms of parasite survival and persistence in the host have been thoroughly investigated, and include suppression of iNOS and the parasite entry into iNOS negative cells [49]. The iNOS expression by macrophages plays an important role during the control of leishmania infection in dogs [52], thisNO may be involved in the long-term protection of dogs against natural Leishmania infection and in the clinical presentation of canine leishmaniasis [53]. Application of potential prodrugs to cultures of infected mouse macrophages that were deficient in iNOS caused rapid death of the intracellular protozoan parasite *Leishmania major* with no host cell toxicity [54]. Therefore, some antileishmanial drugs act via NO modulation [55-57].

Table 1. Comparison of three nitric oxide synthase isoforms.

Isoform	Protein weight (KDa)	Ca^{2+} dependency	Expression	Tissue distribution
nNOS	160	Ca^{2+} dependent	constitutive	Central and peripheral nervous system
iNOS	130	Ca^{2+} independent	inducible	Macrophage
eNOS	135	Ca^{2+} dependent	constitutive	Endothelial cells

Table 2. Chromosomal localization of nNOS, iNOS and eNOS sequences. (Mayer, 1998; Xu & Liu, 1998).

Name	Other names	Type	Regulated by	Present in	Human chromosome	Mouse chromosome
nNOS	Neuronal NOS (NOS1)	Constitutive	Ca^{2+}/ calmodulin	Brain and other tissues	12 12q24.1-12q24.31	5 (site 56.0)
iNOS A	Inducible NOS (NOS2A)	Inducible	Endotoxin, cytokines	Macrophages Neutrophiles Chondrocytes Hepatocytes	17 17q11.2-17q12	11 (site 45.6)
iNOS B *iNOS C*	Inducible NOS (NOS2B) (NOS2C)	Unknown	Unknown	Unknown	17 17p11.2-17q11.2	11 (site 45.6)
eNOS	Endothelial NOS (NOS3)	Constitutive	Ca^{2+}/ calmodulin	Endothelial cells	7 7q35-7q36	5 (site 9.0)

Plasmodium sp.

The main control of malaria in human and animals, is achieved by NO-mediated mechanisms. Protection from severe malaria in African children has been found associated with polymorphisms of the iNOS promoter; however, a pathogenic role of endogenous NO has been documented in cerebral malaria [49]. There is conflicting evidence regarding the role of NO in the process of resistance against malaria parasites. Schizonts treated *in vitro* with NO donors caused a delayed infection to mice in a dose and time-dependent manner, which, suggest an inhibitory role for NO [58] with influence on parasitemia and survival of *Plasmodium berghei* in infected mice [59] or *P. berghei Anka* in rats [60]. Moreover, human severe malaria is associated with decreased NO production [61] and iNOS variants in regions of differing disease manifestation [62]. Low NO bioavailability might contribute to pathologic activation of the immune system [63] and to the experimental cerebral malaria [64]. Mechanism of NO action in malaria explain the presence of its molecules in food vacuole which is a critical parasitic compartment involved in hemoglobin degradation, heme detoxification and a target for antimalarial drug action [65]. The role of other haem enzymes including iNOS is indicated in malaria infection [66]. NO and peroxynitrite concentration is reported to be higher than hemoglobin concentration, and yet no parasite killing was detected, therefore hemoglobin protects Plasmodium parasites from oxygen radicals [67].

Toxoplasma sp.

In macrophages, *Toxoplasma gondii* inhibit NO production, which suggests that an iNOS suppression mechanism might be used for better survival in macrophages [68]. Treatment of mice with a NOS inhibitor partially inhibited the host-cell apoptosis induced by the parasite infection. Apoptosis in host cells is due to the secretion of NO and other soluble factors released by parasite infected cells [69]. *T. gondii* has a nitrite production and a putative NOS motif genomic sequence. Recombinant protein derived from the putative genomic sequence of *T. gondii*, is able to produce nitrites [70]. In addition, NO modulates IFN-γ production in *T. gondii*-infected mice, and that NO is involved in mediating a protective response in toxoplasmosis susceptible, but not resistant, mice strain during acute infection [71].

Trypanosoma sp.

Both intracellular and extracellular morphotypes of *Trypanosoma cruzi* are killed by NO *in vitro* and *in vivo*. The NO donation was shown to kill *T. cruzi* epimastigotes in culture. [49]. iNOS is a potent modulator of chemokine expression which is critical to triggering the generation of the inflammatory infiltrate during *T. cruzi* infection [72]. DNA damage and NOS activity was seen in infected mice with *T. cruzi* [73]. Moreover, cytokine and NO is produced by *T. brucei* infection in rats [74]. NO and iNOS was detected in myocardium and spleen of dogs in the acute stage of infection with metacyclic or blood trypomastigotes of *T. cruzi* [75].Immunisation with a major *T. cruzi* antigen promotes pro-inflammatory cytokines and NO production [76]. Therefore, NO may not be the sole contributor to intestinal dysfunction resulting from *T. cruzi* infection [77].

Cryptosporidium sp.

An *in vivo* role for peroxynitrite formation in acute mucosal defense against a noninvasive intestinal epithelial

Cryptosporidium parvum pathogen was defined [78]. *C. parvum* infection revealed the location, mechanism of induction, specificity, and consequence of iNOS expression in neonatal piglets [79]. These data suggest that NO may reduce the parasite load in experimental cryptosporidiosis [80] and it is indicated that number of *C. parvum* oocysts in feces, proportion of CD4+, CD3+ T cells in blood, serum IFN-ã, and NO content in intestinal tissue were all higher than those of infected control group [81]. NO serves as a proximal mediator of PGE_2 synthesis and barrier function in *C. parvum* infection [82]. Taken together these data suggest that both reactive nitrogen and reactive oxygen species play protective roles in experimental cryptosporidiosis [83].

Trichomonas sp.

NO is a macrophage-mediated cytotoxicity against *Trichomonas vaginalis* [84] and protozoa produce NO and display NOS activity [85]. Killing of extracellular protozoa such as *T. vaginalis* by activated macrophages is also mediated by NO [49] and protozoa degrades NO as a new pathogenic mechanism of action *in vitro* [86]. These results suggest that RNI radicals may have a role in limiting *T. vaginalis* infection in asymptomatic women [87].

Giardia sp.

Giardia intestinalis produce NO and display NOS activity [85]. NO and arginine was detected as central components in a novel cross-talk between a luminal pathogen *G. lamblia* and host intestinal epithelium [88]. Moreover, NO accounts for trophozoite killing and this effect is not mediated by peroxynitrite [44]. Neuronal NOS is necessary for elimination of *G. lamblia* infections in mice [89], however serum levels of NO increased only in some cases during Giardiasis [90].

Entamoeba sp.

Trophozoites of *Entamoeba histolytica* produce NO in culture by activated macrophages as a major cytotoxic molecule against *E. histolytica* trophozoites [91]. NO produced and that O_2- and H_2O_2 may be cofactors for the NO effector molecules [92-93]. NO production and iNOS mRNA expression were confirmed in experimental hepatic amoebiasis [94] and during development of liver abscess in hamster inoculated with *E. histolytica* [95]. Unlike in mice, amoebic liver abscess in hamsters [96] and in humans [97] is due to an excess in NO production. NO is involved in the neutrophil and macrophage killing of the *E. histolytica* [98]. Although, *E. histolytica* selectively induces macrophage by modulating iNOS and NO, allowing the parasites to survive within the host [99], *E. histolytica* inhibits NO-mediated amoebicidal activity of macrophages by consuming L-arginine [100]. The resistance of the mice probably lies in non-specific immune responses, among which the neutrophils activation and NO production may be important amoebicide factors [101]. TNF-α and TGF-β are demonstrated to be associated with NO-dependent macrophage cytotoxicity against *E. histolytica* [92].

NO and Related Molecules in Helminthic Infections

NO possesses antiparasitic effects on both protozoa and metazoa in definitive and intermediate hosts [51]. Several groups have previously presented evidence for NOS activity and immunoreactivity in several parasitic platyhelminthes, indicating that NO release may play an important role in helminth physiology [102]. Most helminthes induce inflammation in the host associated with NO production through somatic and excretory-secretory antigens of adult worm and larvae [103]. An association was hypothesized that helminthes may induce protection through immunoglobulin E (IgE) and the CD23/NO pathway [104]. Our data imply that NO production in host defense against the extracellular parasite is probably in response to an IFN-ã activating signal. Concomitant enhanced levels of IFN-ã and nitrite represent useful indicators of the clinical aggressiveness of hydatidosis [105].

Nematodes sp.

NO is produced in several nematodes including *Ascaris suum* [106], *Brugia malayi* and *Acanthocheilonema viteae* [107]. A potential role for NO as a neurotransmitter at the neuromuscular junction was observed in *A. suum* [108]. In addition, an inhibitory effect on nematode somatic muscle is mediated by NO [109]. Although, the biochemical presence of NOS activity is indicated in *A. suum* tissue [110] and in *B. malayi*, NOS may play a role in developmental signaling [111].

Toxocara sp.

Antigens of *T. canis* is reported to produces NO and PGE_2 *in vitro* [112]. *In vivo* production of NO during *Toxocara canis* infection causes direct host damages and it is strongly related to the oxidative stress and larval NO is effective in migration [113]. A potential therapeutic strategy is presented for experimental granulomatous hepatitis caused by *T. canis* in mice through manipulation of iNOS expression [114]. *In vivo* inhibition of iNOS decreases lung injury induced by this nematode in infected rats [112]. Cytokines and iNOS is involved in the cerebral pathology during infection with *T. canis* [115], whereas, iNOS inhibition can protect the brains of infected mice from damage [116].

Schistosoma sp.

NO and NOS was significantly involved in different stages of human schistosomiasis including *Schistosoma mansoni*, [117], *S. haematobium* [118] and *S. japonicum* infections [119]. Although, *S. japonicum* infection induce the expression of iNOS in a time-dependent manner in the liver of the host, the eggs may be the main factor in this induction [119]. There are controversial reports about NOS activity in murine schistosomiasis; NOS inhibits egg-induced granuloma formation in the mouse liver [120], however NOS activity increased during granulomatous inflammation in the colon and caecum of pigs infected with *S. japonicum* [121] and its inhibition reduces liver injury in mice [122]. An important

regulatory role for the iNOS biosynthetic pathway as a critical determinant in the pathogenesis of granuloma was observed [123].

Fasciola sp.

The levels of both superoxide and NO radicals were reported to be significantly higher in patients with *Fasciola hepatica* in compare with control group, indicating these radicals may have a role in the immunity against human [124] and rodent infections [125]. Some parasiticidal effects of peroxynitrite was reported on bovine liver flukes including *F. hepatica* and *Dicrocoelium dendriticum in vitro* [126]. The mechanism of cytotoxicity was dependent on the production of NO and required attachment of effector cells to the newly excysted juvenile liver *F. hepatica* tegument in infected hosts [127]. Trematoda immune suppression, decreases NO production by host peritoneal cells and it is one of the strategies of the parasite to avoid the potential killing effect of NO during peritoneal migration [128].

Echinoccocus sp.

The results of experimental infection with *Echinococcus granulosus, E. multilocularis* and *E. alveolaris* showed that serum NO level was significantly increased, which is needed for *in vitro* killing of protoscoleces [117, 129, 130]. The NO elevation on hepatic pathological lesions of disease showed a marked reduction of granuloma size with absence of concentric fibrosis [117]. Controversially, some researchers reported NO-mediated immunosuppression following murine *Echinococcus* infection [131] and the high level of NO production during chronic infection, which contribute more to immunosuppression than to limitation of parasite growth [132]. Results indicated that IFN-ã mediated iNOS is induced as one of host defense mechanism against human *E. granulosus* infection [133]. Collectively, the data indicated that NO concentration correlate with IFN-γ levels, and overall suggest that their production together play a role in the host defense mechanisms in human hydatidosis [134].

Taenia sp.

Macrophage activation and NO production are effector mechanisms that importantly contribute in host resistance to *Taenia crassiceps* infection [135]. NO contributes directly to a component of inhibitory transmission in guinea-pig *T. coli* [136], therefore a possible role of L-arginine-NO pathway is presented in the modulation of transmission in this guinea-pig cestoda [137]. In addition, the source and role of basal NO *in vitro* in proximal segments of another species of taenia (*T. caeci*) in guinea pig was also indicated [138].

Conclusive Remarks

Taken together, the data provided by researchers highlight the fact that NO and/or its related molecules are involved in many infectious diseases, but the involvement is not independent of other immune events. It is indicated that NO is an important, but possibly not essential contributor in the control of acute phase of parasitic infections. Although, the protective immune responses against microorganisms are multifactorial, the final effector molecules that mediate organism death are not known, NOS, NO, and RNI have been significantly implicated. It is concluded that NO is only part of an immunopathological chain against infection and the anti-microbial function did not relate only to NO action, therefore a combination of NO and other immune factors is required to resolve pathogens. In summary, it is highlighted the complexity and variation of NO-released by different NOS isoforms in parasitic infections and discussed that NOS activation could have both pro- and anti-inflammatory effects. Moreover, it is emphasised here that iNOS-derived NO may have an extra-functional qualification to overcome pathogenic parasites. It is possible that these effects could be critically dependent on the type and concentration of NO generated. Finally, it is still unclear whether NOS inhibition would be a good therapeutic target in parasitic infections. It is suggested that the detrimental effect of NOS is related to the L-arginine and NO concentrations, because NO at high concentration has a clear anti-inflammatory effect [139-142]. Thus, activation of NO could be a potential therapeutic strategy to suppress parasitic infections [143-145]. Nevertheless, the functional role of NO and NOS isoforms in the immune responses of host against the majority of parasites is still highly controversial. Therefore, the involvement of NO and its up / downstream molecules in parasitic infections is cuurently under debate and it is still required more investigations [146-147].

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