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Nitric oxide and the immune system: a literature review

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Abstract

Nitric oxide (NO) was discovered as a biologically active molecule over two decades ago and it has since been recognised as one of the most versatile components of the immune system, with involvement in both cytotoxic and regulatory functions. It is a readily diffusible gas that has been established as a universal messenger, capable of mediating cell-cell communication throughout the body. It is involved in the pathogenesis and also the control of infectious diseases, autoimmunity, chronic degenerative diseases and tumours. This review will collate, contrast and compare recently published literature, to provide an up-to-date and contemporary overview of the substantial role that NO plays within the immune system.

Introduction

Nitric oxide is formed following enzyme activation of one of three NO synthase isoforms, converting the amino acid substrate L-arginine to citrulline. Two of the synthases are constitutively expressed within the body; endothelial NO synthase (eNOS) and neuronal NO synthase (nNOS) (Guzik *et al* 2003). Contrary to their given names, expression is not restricted to neurons or endothelial cells. nNOS is found in high amounts in the CNS, testes and skeletal muscle where it is involved (among other physiological functions) with endocrine regulation, platelet aggregation, neuromodulation and skeletal muscle contraction (Bredt 1999). eNOS is bound to the Golgi apparatus of endothelial cells, where it regulates vascular reactivity in the

periphery and brain (Deckel 2001). Activation is primarily brought about by Ca^{2+} fluxes and subsequent binding of the calcium regulatory protein – calmodulin (Bogdan 2001). The third isoform of NOS is referred to as inducible NOS (iNOS). It is not normally expressed under physiological conditions but, stimulation by pro-inflammatory mediators, such as cytokines or endotoxin, cause its rapid formation (Bredt 1999). One of the distinguishing features of this isoform is that it does not require an increase in cellular Ca^{2+} levels to become activated (Deckel 2001). Activation of iNOS leads to the

production of micromolar levels of NO, much higher than the nanomolar levels generated by the constitutive eNOS and nNOS (Achike and Kwan 2003). Prolonged exposure to a large amount of NO can inhibit the activity of several enzymes, such as aconitase and cytochrome c oxidase, resulting in potential cytotoxicity and the development of pathogenesis (Nathan and Hibbs 1991). It is these pathogenic responses to NO and the pathways underlying them, which shall form the basis of this review. The possible functions of the different NOS isoforms within immune cells will be examined and the mechanisms behind the regulation of NOS activity shall be explored.

NO production in the immune system and mechanisms of its regulation

The generation of NO is a key feature of many immune cells, including dendritic cells, NK cells, mast cells, macrophages and other phagocytic cells. Either iNOS or eNOS has been found in all of these cell types but the evidence remains uncertain as to whether primary T or B lymphocytes express the NOS isoforms (Bogdan 2001). This is an area to which further research could be targeted, to clarify the contradicting results gained through other studies. Previous work carried out by Relling (1996) relied solely on PCR methods to detect NOS mRNA within T cells, increasing the possibility of false positives, through the contamination of other cells. Much focus within previous research has been placed upon the classical phagocytic cells, such as macrophages, examining the role they play in NO production. There seems to be much less information regarding T and B lymphocytes and the position that they hold with respect to the expression of NOS.

All three isoforms of NOS are regulated at various levels during an immune response. They have similar regulation processes however, because eNOS and nNOS are preformed within the cell, their activity can be switched on by Ca^{2+} level increases and calmodulin, as previously explained. In contrast, iNOS mRNA has to be synthesised under the appropriate stimuli and the gene promoter for this provides a mode of regulation under the influence of cytokines (Pellacani *et al.* 2001). Some of the participating transcription factors include NF-κB, AP-1 and STAT-1α. Depending on the microbial or cytokine stimulus, different upstream signalling pathways are involved which either promote or inhibit iNOS expression. The NF-κB is present in an inactive form in the cytosol as a complex with IkB. When an inducer, such as LPS, stimulates the cell, the IkB- NF-κB complex is phosphorylated, allowing the NF-κB to translocate to the nucleus and induce iNOS gene expression (Aktan 2004). Supportive studies carried out by Musial and Eissa (2001) found that some compounds were also able to suppress iNOS induction by blocking IkB degradation. NO production itself can regulate its own production, as was described by Connelly *et al.* (2001). He showed that upon initial macrophage stimulation by cytokines, when the NO concentrations were low, NFκB was upregulated and further iNOS was produced (positive feedback). Under high NO conditions, the opposite phenomena was observed. This negative feedback could be a mechanism to help prevent the overproduction of NO and the associated pathologies. In a similar study conducted by Perez-Sala *et al.* (2001), the biphasic effect of NO synthesis was shown to be mediated through the inhibition of NFκB. This regulatory site

within the signalling pathway could be investigated further as a potential intervention point for therapies associated with inflammatory diseases.

Many of the studies investigated for this review used a combination of LPS and IFNγ to stimulate immune cells, such as macrophages, to produce NO. This is because certain combinations of cytokines or LPS with IFNγ are able to induce iNOS expression synergistically (Chong *et al.* 2002). They signal through the Jak-STAT pathway, where STAT1 becomes activated and translocates to the nucleus, increasing IRF-1 levels and ultimately inducing iNOS (Aktan 2004).

NO, leukocyte adhesion and chemotaxis

Various studies have shown that NO inhibits the adhesion of platelets and leukocytes to endothelium and also significantly impeding their rolling and transmigration (Grisham *et al.* 1998). Both Grisham *et al* (1998) and Bogdan (2001) used monocytes and granulocytes with a monolayer of endothelial cells. They performed the experiments using flow chambers and endogenously produced NO in addition to NO from donors. Both independent studies came to the same conclusions; that the NO was indeed acting to inhibit the adhesion of the leukocytes. The methods behind this inhibition were not explored within these studies and there also seems to be a lack of published literature to explain these mechanisms. The possible effects that NO could exert on the adhesion of other immune cells, such as T and B lymphocytes also proved difficult to find within any of the papers researched. This is an area to which further investigations and studies could be targeted to gain a deeper insight into this feature of NO. The chemotaxis of leukocytes has however been demonstrated, within several studies, to be influenced by NO. Bogdan (2000) showed that it can modulate chemokine production, such as IP-10 and monocyte chemoattractant protein-1. In addition to this, Cheria and Garu (2001) showed that NO was able to inhibit the activity of chemokines, such as IL8, and to function as an intracellular messenger in chemokine signalling pathways.

NO and the thymus

The thymus has a central role in immunology, as it is the place where T cells diversify and differentiate by positive and negative selection processes (Goldsby *et al.* 2003). Those cells which respond too strongly to self-MHC are induced to die through apoptosis, thereby preventing autoimmunity. Because of NO's capacity to induce apoptosis, many scientists have investigated its possible role within the thymus, acting to assist in correct T cell development. In a study by Allello *et al* (2000), human thymocytes were found to be lacking the iNOS protein. However, epithelial and dendritic cells within the medulla and corticomedullary junction were shown to constitutively express iNOS and this was further upregulated upon contact with self antigens. Developing on the ideas presented by Allello *et al* (2000), Moullan (2001) and his team discovered that TCR-activated double-positive thymocytes were particularly sensitive to killing by NO. These combined data strongly suggest that NO, released by iNOS expressing cells within the thymus, contributes to the deletion of double-positive thymocytes.

NO in infection and inflammation

NO plays a crucial role at all stages of infection and it has a diverse and somewhat contradictory spectrum of activities. Rollinghoff *et al.* (2000) used $iNOS^{-/-}$ mice in their informative study, concluding that a vast array of immunological effects are indeed mediated by NO. The functions of NO which they described were; antiviral, antimicrobial, immunostimulatory, immunosuppressive, cytotoxic and cytoprotective.

The antimicrobial powers of NO are thought to occur through several mechanisms including, mutation of DNA, inhibition of DNA repair and synthesis, alteration of proteins and inactivation of enzymes (Bogdan 2001). Further iNOS-dependent protective effects during infection include the inhibition of tissue fibrosis and the termination of the immune response by apoptosis of activated CD4+ T cells (Dalton *et al.* 2000). The *in vitro* killing via NO of *Mycobacterium avium-intracellulare* and *Leishmania major* by activated macrophages was shown in the studies by Kronck *et al* (1998). This notion was developed further by Iniesta *et al* (2001). Their study suggested that Nω-hydroxy-L-arginine, an intermediate of the L-argine-iNOS-NO pathway, contributes to the killing of intracellular *Leischmania* by blocking arginase activity within the parasite and the macrophage. This compares similarly to the outcome of an additional study, where *Leischmania major*-infected mice were treated with the NOS inhibitor NMMA, resulting in substantially increased parasite loads and the development of larger skin legions, validating the importance of this mechanism in regulating parasite growth *in vivo* (Gobert *et al.* 2000).

It has also been found that the antimicrobial activity of NO may be mediated by indirect effects. One type of infectious pathogen, African trypanosomes, along with many others, are dependent on exogenous arginine for the production of polyamines and cell proliferation. Bryk *et al* (2000) used these pathogens in their experiments to show that when arginine levels become depleted by the induction of iNOS, it can result in the growth inhibition, or death of the parasite.

A good indication for the defence role of NO at the interface between the body and the external environment, has been demonstrated on the surface of the tongue, in a dated but interesting study by Benjamin *et al* (1994). They demonstrated the fact that in the mouth, facultative anaerobic bacteria reduce nitrate within the saliva rapidly to nitrite. When this is swallowed, large amounts of NO are generated in the stomach, partly due to the acidic conditions. Similar studies have also been carried out, which show that NO is continuously released from the skin's surface (Weller *et al* 1996). Within this research by Weller *et al* (1996), commensal bacteria was shown to reduce the nitrate present within sweat to nitrite where it was then reduced further, forming NO -again due to the acidic conditions on the surface of the skin. Although the studies mentioned here are fairly dated, they provide a valuable insight into a further defensive role that NO plays within the immune system, outside of the more obvious roles and features of this multi-functional gas molecule.

In addition to the beneficial effects that NO exerts when the body is challenged with infection, it is also known to display pathogenic properties when sustained at high concentrations (Aktan 2004). NO can, under certain circumstances, mediate cytotoxicity and tissue damage, inhibit T cell proliferation, induce T cell apoptosis, generate viral escape mutants and also have direct positive effects on viral or microbial growth (Bogdan 2001). When NO is produced in large amounts by myeloid cells, it is generated also with equally large amounts of superoxide anion (O_2^-). These two compounds can then form peroxynitrite ($ONOO^-$), which is a mediator of the cytotoxic effects of NO (Guzik 2003). The production of $ONOO^-$, $NO^\cdot$ and other reactive oxidising compounds in the presence of superoxide radicals or peroxidises, can cause the reversal of NO effects from protective to deleterious (Aktan 2004). These products can target numerous proteins and enzymes which are critical for cell survival and signalling; nitrating cysteines within their structures, leading to their activation or inactivation (Coleman 2001). Such toxic effects of NO have been

studied and are shown to underlie the pathogenesis of septic shock. Thiemermann (1997) indicated that increased amounts of nitric oxide (as occurs during endotoxic or cytokine-induced shock) contribute to excessive vasodilation, resulting in a decrease in vascular tone and a fall in blood pressure. The plasma NO concentrations in patients with septic shock were shown to be hugely increased and the administration of a specific NOS inhibitor was able to increase the blood pressure but with side effects. A study carried out Parrett (2000) also found that the use of NOS inhibitors as a treatment for septic shock produced a detrimental side effect of decreased cardiac output.

Several research papers have been produced from the results of investigative work into the field of NO and its role in inflammatory pathologies. It can occasionally become part of a dysregulated immune response, resulting in chronic inflammatory disorders.

In recent years, NO has become associated with the initiation and maintenance of inflammatory bowel disease. In a study carried out by Boughton-Smith *et al* (1994) rectal biopsy sections were examined from patients with active ulcerative colitis (UC). The results showed that citrulline concentrations (the co-product of NO synthase) were much higher in the biopsies from UC sufferers than in those with normal histology. Incubating the biopsy samples with L-NMMA, an inhibitor of NOS, significantly reduced the citrulline concentrations, therefore indicating that the increase must be as a result of NOS activity (Boughton-Smith *et al* 1994). Kolios *et al* (2004) found similar results when studies were undertaken using patients with both UC and Crohn's disease (CD). The colonic mucosa of the patients' biopsies showed iNOS activity eightfold higher than in the control subject's mucosa.

These results, along with those of countless other studies show a definite link between the up-regulation of iNOS and the clinicopathological features found in many chronic IBDs. The excessive concentrations of NO may exacerbate the features of these pathologies by direct cytotoxicity, vasodilation, activation of neutrophils and by the formation of the highly toxic peroxynitrite radical (Kolios *et al* 2004).

NO and tumours

NO's involvement with tumour development seems extremely complex and some what contradictory. It has both tumouricidal effects and, more recently discovered, deleterious tumour-promoting effects. Macrophage-derived NO can cause cytostasis or kill tumour cells *in vitro* (Bogdan 2001). Kroncke *et al* (1998) found that iNOS protein could be detected in the vasculature, infiltrating macrophages and the tumour cells of human brain, breast, lung and colon tumours. He revealed that the NO produced by the tumour itself could potentially inhibit proliferation or induce apoptosis of T cells, which would in turn suppress the host's immune system. In contrast to this, a study performed by Kwak *et al* (2000), found that tumour cell death could be achieved by the induction of iNOS within the tumour cells. This iNOS was stimulated in response to IFNγ and TNF, which was released by cytotoxic lymphocytes. This evidence seems to imply that the tumour cells can use nitric oxide for their own advantage but at the same time, it can be targeted against the tumour to assist in its destruction. Further research by Xu *et al* (2000) confirmed that the iNOS expressed by the tumour is able to promote its growth, neovascularisation and invasiveness, by the induction of p53 mutations and the upregulation of VEGF (vascular endothelial growth factor). He also found that by exposing the tumour cells to NO, there was an upregulation of the catalytic subunit of the DNA-dependent protein kinase - essential for repairing DNA breaks. This important discovery suggests that the tumour cells could be protected from the potential toxic effects exerted by NO but also protected from the therapeutic agents used to treat the tumour. As suggested by Bogdan (2001), NO-based strategies intended for use as a tumour treatment, must take into account the results gained within these and other such studies.

There is much research which suggests the involvement of NO in the carcinogenesis of some cases of breast carcinoma (Gatti *et al* 2003). A study by Umansky and Schirrmacher (2001) used a human tumour cell line transfected for constitutive expression of iNOS to investigate this theory. They found that the cells showed increased growth rate, vascular density and micro-invasiveness. Supporting these observations were the results of a further study, by Harmey *et al* (2002) where benign and malignant breast cancer tissues were compared for levels of NOS activity. The benign tissues had NOS levels below that of detection whereas the levels were continually elevated within the malignant tissues. Loibl *et al* (2002) also came to similar conclusions when they carried out assays and discovered that the detection of iNOS within benign breast lesions was extremely rare.

NO and neurodegenerative diseases

Parkinson's and Alzheimer's diseases are both neurodegenerative conditions that are associated with oxidative stress, resulting from mitochondrial dysfunction and the production of ROS (Achike and Kwan 2003). Neuronal damage in these diseases has been linked to an increase in nNOS and NO production (Hantraye *et al* 1996). iNOS is also believed to be involved in the pathogenesis, due to its activation within astrocytes,

by the β-amyloid peptide (characteristic of the amyloid plaques) (Achike and Kwan 2003). Supportive conclusions can be found within a study carried out by Achike and Kwan (2003). Post-mortem specimens of brain tissue were analysed and it was found that iNOS expression was high in the demyelinated regions of the brain.

Conclusions

In summary of this review of literature, nitric oxide has been shown to have diverse biological functions and effects within the immune system. It is an intra- and intercellular signalling molecule that can shape the immune response and sustain homeostasis. NO is a potent anti-microbial defender and has many host-protective effects that are most evident during invasion by infectious agents. It also has a possible role in thymic selection processes and the regulation of T cell differentiation. However, in contrast to these beneficial roles, NO has been shown to have the potential to switch from being an indispensable regulator to a harmful destroyer. The overstimulation of NOS can assist with disease generation, inflammatory pathologies, neurodegenerative diseases and also cancer progression.

Within the scope and limitations of this review, only a brief overview could be given of the relationship between nitric oxide and the immune system. It covers such a broad and detailed area that it would be beneficial to dissect the research down much further, allowing the in-depth analysis of just one specific factor, such as NO and inflammation or cancer. The field of immunology is dynamic and exciting with new discoveries and theories constantly being developed. Further informative research into the fascinating nitric oxide molecule shall no doubt be only a short time away.

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