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Review Article

Biochemistry Section

Nitric Oxide: It's Role in Immunity

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ABSTRACT

Nitric Oxide (NO), a lipophilic gas synthesised by the enzyme Nitric Oxide Synthase (NOS) from the substrate arginine, is an important biomolecule that mediates cellular signaling. It has a wide spectrum of biological functions including immunomodulation, inflammation, microbial and tumour eradication. Cells of the immune system such as macrophages on activation by cytokines and microbial antigens eradicate a number of microbes or parasites as well as tumour cells by releasing a number of effector molecules which also includes NO. However, this versatile molecule is shown to have dual roles. In lower concentration, this is beneficial and regulates the physiological processes in the body whereas at higher concentrations it is harmful, not only to the microbes or tumor cells but can produce undesirable effects on the host cells too. This suggests that NO has both protective and toxic roles that occur parallel in the body depending upon cellular microenvironment. Therefore, it is very essential to have the knowledge of the physiological processes involved in signaling cascades of NO since it might have novel clinical applications when therapeutic potential of NOS inhibitors and NO donors are to be considered.

Keywords: Macrophages, Microbes, Nitric oxide synthase, Tumour cells

INTRODUCTION

NO was initially discovered as the Endothelium Derived Relaxing Factor (EDRF) by Furchgott and Zawadki [1]. Joseph Priestly in 1772 was the first person to term it NO, however, till 1987, the biological functions of NO were not known, and therefore it was merely regarded as an air pollutant or toxic gas [2]. Only when the presence of nitrates and nitrites were detected in healthy rats and human volunteers, studies on the biological roles of NO came into light which was further followed by the discovery of the tumouricidal and antimicrobial activity of NO [3]. Later, the regulatory and protective roles of NO in cardiovascular system were described, that included blood pressure and vascular tone regulation, inhibition of leucocyte adhesion or platelet adhesion and prevention of proliferation of smooth muscles. This initiated extensive research on NO for its role and use as therapeutic agents in various diseases [4].

NO especially from iNOS (inducible Nitric Oxide Synthase) has important roles in immune regulation, inflammation and microbial invasion [3]. NO is also implicated in the pathophysiology of various cancers viz., breast, larynx, cervix, head and neck [5]. Tumour promoting role of NO is dependent upon the tumour type, NO concentration and its interaction with proteins, metals or free radicals and the genetic makeup of the host cells [6]. Lower concentration of NO is important for immune function, while NO at higher levels are shown to be immunosuppressive suggesting the dual role of this biomolecule. However, some contradictory reports also present a debating role of NO in immune response. Some state NO to be a causative agent of several diseases while others argue on its protective roles. This contradiction has led a conflict over whether NO serves as a protector from various infections or functions as a destroyer resulting in different disorders. Therefore, in this review, we emphasise on NO and its immunoregulatory, antimicrobial and tumouricidal role along with the involvement of phagocytes.

Synthesis

NO is synthesised from L-arginine by the enzyme NOS in the presence of two cofactors viz. NADPH and oxygen [7]. NOS has three isoforms represented as neuronal NOS (nNOS) or NOS 1, inducible NOS (iNOS) or NOS 2 and endothelial NOS (eNOS) or NOS 3 [7]. The genes for these isoforms 1, 2 and 3 are located on the chromosomes 12, 17 and 7 respectively [8]. All the isoforms of NOS are flavoproteins containing Tetrahydrobiopterin (THB) and haeme. THB is an important cofactor for NOS, since in its absence, NOS produces superoxide instead of NO [9].

Broadly, the isozymes of NOS are categorised as constitutive NOS (cNOS) and iNOS. cNOS, which is constitutively present in the cell, is calcium dependent and comprises nNOS and eNOS while iNOS is calcium independent and expressed only after the stimulus is provided by cytokines [10]. The synthesis of NO by nNOS and eNOS is dependent on intracellular calcium ions and binding of these enzymes to calmodulin [11]. Increase in calcium level causes increased production of calmodulin that binds with eNOS and nNOS causing enhanced synthesis of NO by the respective enzymes [12]. For the activation, nNOS in central nervous system, the glutamate first binds with NMDA (N-methyl-D-aspartate) receptors causing opening of the voltage gated calcium channels and rise in calcium levels. In case of eNOS, it is activated when stimulus like blood shear stress or factors like substance P, kinins or thrombin receptors etc., cause release of calcium ions from the endoplasmic reticulum [1]. In the cells such as macrophages and monocytes, induction of iNOS by inflammatory cytokines (INF-γ, TNF-α and IL-2) and presence of L-arginine in sufficient amount leads to generation of NO [13].

Physiological Roles of No (Constitutive vs Inducible NOS)

Activation of iNOS leads to the sustained production of large amount of NO while that of constitutive forms (eNOS and nNOS) cause production of low levels of NO within seconds. NO from constitutive isoforms have direct and short lasting activities [14]. They interact with cytochrome p450, guanylate cyclase and NO itself. This leads to activation of guanylate cyclase that converts Guanosine Triphosphate (GTP) into cyclic Guanosine Monophosphate (cGMP) which in turn activates cGMP dependent protein kinase that further mediates the functions of NO such as increase in vascular permeability, vasorelaxation, antioxidant roles and antiplatelet activities [1]. In Central Nervous System (CNS) and Peripheral Nervous System (PNS), NO acts as a neurotransmitter

and is involved in neuronal apoptosis [15]. NO from eNOS is essential for maintaining tissue perfusion, protection against toxic lipids derived from Lipopolysaccharides (LPS) and preservation of RBC in septicaemia [16].

The functions of NO produced by iNOS is rather different. Such NO is produced by activated macrophages and is involved in microbial killing and immune regulation. NO combines with superoxide to form peroxynitrite that further mediates toxicity of NO which includes LDL oxidation, DNA damage, inhibition of TCA and mitochondrial oxidative enzymes, nitrosation [1].

NO and Immune Regulation

The exact function of NO in immune regulation is unclear. It is thought to be involved in the inhibition of genes responsible for cell proliferation or it may have anti-apoptotic roles [1]. NO inhibits Th1 and promotes Th2 cytokine response that leads to humoral immunity and allergic responses [2]. NO acts as the mediator of inflammation by increasing the activity of cycloxygenase enzyme and production of proinflammatory eicosanoids [17]. NO also inhibits the expression of the number of cytokines like IL-1β, TNF-α, IL-6, INF-γ in various immune cells such as lymphocytes, eosinophils and monocytes. This effect is mediated by nitrosylation of various transcription factors including JAK/STAT (Janus Kinase/ Signal Transducer and Activator of Transcription) and NF-κβ (Nuclear Factor kappa beta). NO from activated macrophages are involved in destruction of cellular targets, tumours or bacteria [1].

NO and proinflammatory cytokines

Cells contain various mechanisms to regulate the signaling pathways and transcription factors. Depending upon such regulation, NO is reported to either depress or stimulate the proinflammatory cytokine expression [18]. When the immune system is activated, there is induction of proinflammatory phase of innate immunity, a process which is termed as classical activation. Macrophages that are classically activated, also termed as M1 macrophage, results in production and release of proinflammatory cytokines [19].

Turpaev K et al., used DNA microarrays of macrophage cell lines in humans to identify the genes regulated by NO such as cFos, cJun and cell cycle regulator genes [20]. However, in some cases Reactive Nitrogen Species (RNS) act as regulatory molecules rather than NO.

Most of the immune pathways regulated by NO/RNS require NF-kβ for coordinating the outcomes of innate immunity. When the cells are at resting state, NF-kβ forms complex with the inhibitory proteins and are scattered in cytoplasm. Once the cells are activated by various stimuli such as bacterial LPS or TNF-α, there is proteosomal degradation of inhibitory proteins mediated by a cascade of phosphorylation reactions, thereby facilitating entry of NF-kβ to the nucleus where it modulates the respective genes of immune activity [21]. Studies have shown that NO/RNS at lower concentration augments NF-kβ mediated transcription of immune genes while higher levels inhibit the process.

NO and anti-inflammatory signaling: Anti-inflammatory cytokines such as TGF-β, IL-13, IL-10 and IL-4 down regulate pro-inflammatory cytokines and facilitate the tissue repair [22]. The action of NO/RNS can be compared to that of a traffic officer as they critically regulate and redirect the signaling pathways by inducing one pathway and diminishing the other. Most of the immune receptors activated by anti-inflammatory cytokines are linked with synergy between JAK that causes activation of signaling pathway via stimulation of Signal Transducer and Activator of Transcription 6 (STAT6) through a cascade of phosphorylation reaction involving tyrosine residues. This finally leads to the expression of genes involved in tissue repair and restoration [23].

Immunosuppressive roles of NO: NO is also implicated to be involved in immunosuppression. Various mechanisms have been postulated as follows:

- Myeloid Suppressor Cell (MSC) mediated T cell suppression: NO produced by MSCs causes T cell immunosuppression by orchestrating the synergistic action of iNOS and arginase since activation of both the enzymes lead to depletion of L arginine, which inturn stimulates production of superoxide by iNOS in small amount. The superoxide then reacts with NO to form ONOO- and others forms of RNS that causes T cell apoptosis [24].
- Impairment in signaling cascade induced by IL-2R: NO at higher concentration blocks IL-2R mediated activation of JAKs (JAK 1and 2) and STAT 5 [25].
- Activation of mitochondrial intrinsic pathways that induces apoptosis: When NO is present in higher concentration in the cell, competition occurs between NO and oxygen to bind with the enzyme cytochrome oxidase C of inner membrane of mitochondria. This decreases the membrane potential of mitochondria causing production of Reactive Nitrogen Intermediates (RNI) that nitrosylate haeme iron of cytochrome oxidase causing its release to the cytosol where it assembles with adaptor protease activating factor 1 (Apaf 1) to form apoptosome that activates and mobilizes caspases 2,3,7 and 9 which induces apoptosis [26].
- Upregulation of p53 expression that may generate proapoptotic responses. Studies on mice thymocytes and human lymphocytes that were made p53 null and mutated at p53 gene respectively showed that these modified cells were resistant to NO induced apoptosis [27].
- S-nitrosylation of glucose-6-phosphate dehydrogenase (G6PD) results in its nuclear translocation and degradation [28].

Macrophages and Role of NO in Microbial Killing

Immune cells such as phagocytes have unique and non specific roles in host defence systems. They are also known as the janitors of innate immunity since they clear up the whole cellular debris. The phagocytes in human immune system may be microphages (neutrophils and eosinophils) or macrophages (phagocytes). Macrophages are larger than microphages and represent the first line barrier to the pathogens. Macrophages engulf pathogens even before they are detected by the lymphocytes [29].

Macrophages develop from haematopoietic stem cells and mature or proliferate into monocytes in presence of Macrophage Colony Stimulating Factor (MCSF) and Granulocyte Macrophage Colony Stimulating Factor (GMCSF). Monocytes migrate to various tissues and differentiate into macrophages. During this process, cells enlarge with the increase in mitochondrial and lysosomal number, along with the lysosomal enzymes such as cathepsin, β-glucuronidase, acid phosphatase and sulphatase [30].

Activation of macrophages and effect of NO: When macrophages encounter foreign molecules they are activated. Activated macrophages act on the pathogens via one of three mechanisms:

- Engulf and destroy the foreign molecules via lysosomal enzymes [30].
- Remove the invading substances from the interstitial fluid in assistance with the lymphocytes [31].
- Release a variety of chemical mediators such as ROS, NO or RNS, bioactive lipids and hydrolytic enzymes [30].

Production of these mediators facilitates cytotoxic as well as bactericidal action of macrophages. However, over production of, especially Reactive Oxygen Species (ROS) and RNS (NO, ONOO-) may lead to injury and toxicity to the host cells [30]. Fortunately, host cells are equipped with some protective mechanisms also known as antioxidant defence system that protect macrophage as well as other non infected cells from the toxic effects of RNS [32].

Microbial killing by NO occurs in a concentration dependent manner which in turn depends on NOS activity and availability of arginine in phagosomes. Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase and superoxide act synergistically in phagosomes to scavenge NO thereby causing increased nitrite levels which in acidic environment contribute to Cauldron effect. Like NO, nitrites can also generate NO_2/NO and N_2O_3 under favourable conditions. This mechanism is important during phagocytosis as acidic environment of phagosomes render suitable conditions for the production of RNS that lead to killing of pathogens [19].

Macrophages on encountering with foreign molecules are activated causing upregulation of NF-kβ which acts synergistically with inflammatory cytokines through JAK-STAT dependent pathway to increase the expression of iNOS and production of NO [21]. NO exerts immunity against various bacteria, viruses, fungi and parasites. The role of NO as a potent antimicrobial agent is due to formation of products also called as RNS such as peroxynitrite, NONOates, S-nitrosothiols and nitrous acid [3]. NO produced by skin cells provides the protective barrier against micro-organism. It also regulates melanogenesis and erythema formation on exposure to Ultra Violet (UV) light [33].

NO mediated immunity against bacteria: NO and RNS eradicate bacteria by nitrosylation and oxidation of bacterial macromolecules [34]. Three times more bactericidal activity can be observed when NO and H_2O_2 act synergistically, in comparison to killing by NO alone. Bacteria especially *E. coli* utilises iron sulphur proteins of the respiratory chain causing release of iron in the interior of bacteria where it binds with DNA. NO cannot easily enter the interior of bacterial cell; therefore peroxide through Fenton reaction interacts with these released iron leading to oxidative cleavage of bacterial DNA. On the other hand NO which can diffuse to mammalian cells easily, serves as an antioxidant and protects the cells from ROS mediated toxicity [35]. Destruction of bacteria like *Staphylococcus* involves the sequential exposure of bacteria to respiratory burst followed by NO exposure [19]. *Mycobacterium tuberculosis* is another bacterial example killed by NO produced from alveolar macrophages. The survival of this organism is guarded by methionine sulfoxide reductase but the acidic environment of alveolar phagosomes favours the generation of RNS from NO that causes oxidation of methionine group of the enzyme. NO also protects from *Mycobacterium* mediated T cell apoptosis and also helps in eradication of disease from adaptive immune system [36]. Not all the bacteria are susceptible to killing by NO, however NO reduces the infectivity of such bacteria. High levels of NO cause cytostasis and bacterial imprisonment in the infected cells via nitrosation of thiol groups of proteins responsible for pore formation in cell membrane thereby, preventing the bacterial exit from the infected cell and spread of infection to the other cells. At the same time, it also allows other components of the immune system to eradicate the bacteria [37].

Leggett R et al., detected intracellular NO from phagolysosome using conjugate of fluorescently tagged metalloprotein-gold. They developed NO specific biosensor by conjugating cytochrome c with alexa fluor dye fixed on a gold nanoparticles. These biosensors detected increasing concentrations of NO in macrophages during bacterial phagocytosis [38]. Similarly, Yang Z et al., investigated the gender based resistance against pneumonia among female and oestrogen treated male mice. They observed increased bacterial clearance with low inflammation and improved survival rate in those mice. They showed that oestrogen activates NOS-3 in macrophages causing increased production of NO that is responsible for bacterial eradication [39].

NO mediated immunity against viruses: NO also acts as an important element in the control of some viruses like rhino-virus, cytomegalo virus (CMV), herpes virus, vaccinia virus etc [40]. Most of these viruses cause induction of iNOS via Toll like receptor-3 (TLR-3) activation [41]. NO combats viral infection by causing nitrosation of cysteine residues of proteins essential for viral replication and infection. Some of the examples of such proteins include integrases and nucleocapsid proteins which on nitrosation are unable to bind DNA and hence do not function as topoisomerases thereby preventing the integration of viral DNA to the host cell DNA. However, during clearance of virus, high levels of NO can produce some undesirable effects on the host, like haemorrhagic fever [42].

NO mediated immunity against parasites: The human parasites subjected to expulsion via NO/RNS mediated mechanisms include Plasmodium, Leishmania and Toxoplasma, the most common one being Plasmodium infection, also known as malaria [19]. According to Kun and Weinberg etal, increased production of NO and protection against malaria are associated with a single nucleotide polymorphism of iNOS gene promoter sequence called NOS_2 lambarene (G954C) mutation. Leishmania is also successfully destroyed by NO [43]. Increased expression of iNOS also inhibits the infectivity of Toxoplasma and prevents the disease progression to other systems especially central nervous system (CNS) while inactivity of iNOS allows uncontrolled replication of the organism [40]. NO is also involved in provided immunity against Giardia intestinalis. In study of Zarebavani M et al., there was a sharp increase in NO and its derivatives among the patients infected with Giardiasis. NO eliminates the parasites by cytostatic or cytotoxic effects. It can also inhibit parasitic replication and differentiation [44].

Hence, the overall effect of NO on microbial killing is that, it targets the microorganisms by inducing DNA damage, impairment in DNA replication and repair, impairing mitochondrial respiration and inducing apoptosis or disabling the pathways that are used by that organism to escape the other immune responses.

Besides humans, insects such as lepdopterans, hemipterans, dipterans etc depend on NO to mediate immune response. NO in insects acts both as signalling molecule and a cytotoxic component to combat parasites [45]. Negri P et al., conducted study on Apis mellifera hematocytes and reported that not only NO is produced in large amount in hematocytes when larvae are injured by mite called Varroa destructor (that feeds on larvae of Apis mellifera), but is there also increase in subsets of hematocytes that can produce NO after injury [46].

Tumour Cells and NO

Tumour develops due to genetic alterations that cause uncontrolled growth and proliferation of the cells. Initially the immune system act to eliminate such abnormal cells via the process known as immune-surveillance [47]. During the first stage of immune-surveillance, the immune cells succeed to destroy the developing transformed cells and prevent the formation of tumour mass. However, if the surveillance process fails, then the emerging transformed cells transit to the second phase, also known as equilibrium phase in which immune system can control but not clear up the tumour cells. During this phase the immune system constantly pressures the tumour cells in order to remove many original variants but additional mutation may occur causing generation of the new variants, some of which may escape the immune-surveillance and enter the third phase known as escape phase, in which the transformed cells grow in an unrestricted manner [48].

Macrophages among the immune cells are the most prominent ones that infiltrate deep into the hypoxic area of the tumour mass to combat and eliminate the pathogens and tumour cells. In some cases macrophages comprise about 50% of the tumour mass [49].

Tumour inhibition by NO: NO/RNS when present in higher concentration cause cellular death by stimulating modification of death related target protein receptors or affecting the respiratory chain via changes in the permeability of mitochondrial outer membrane causing cytochrome C release and cellular apoptosis

[50]. Besides these mechanisms, another mechanism of tumour inhibition by NO/RNS includes phosphorylation of Ser15 of wild type P53 that leads to its activation and initiates the process of apoptosis. It also causes S-nitrosylation of Cys 62 of NF-κβ (an-antiapoptotic factor) leading to its inhibition [51].

Tumour promoting action of NO: Though high levels of NO/RNS are used as a killing mechanism by phagocytes, it has also been shown that NO/RNS can cause carcinogenesis as well as support the progression of pre-existing tumour which is at escape phase from the immune system.

Carcinogenic Role

NO/RNS being lipophilic can easily diffuse through the cell membrane and cause oxidation or deamination of nitrogen bases, produces DNA breaks and cross links, all of which induce mutations. NO can also activate the oncogenes or cause deactivation of tumour suppressor genes. S-Nitrosylation or nitration of proteins involved in DNA repair by NO/RNS affects the cellular repair mechanism and genomic stability [52].

Role in Escape Phase of Tumour Cells

Tumour cells at escape phase deploy a mechanism that enables them to maintain NO/RNS at lower levels which supports the progression of tumour. Anti-inflammatory mediators like TGF-β decreases production of NO by suppressing iNOS mRNA transcription. Also, the enzyme arginase, expressed by Tumour Associated Macrophages (TAMs) and Myeloid Derived Suppressor Cells (MDSCs) degrades L-arginine thereby creating scarcity of this substrate for iNOS [53].

Lower levels of NO/RNS act as anti-apoptotic factor. The S-nitrosylation of caspases blocks the process of apoptosis and prevents the degradation of anti apoptotic proteins, hence protects the cells from death. Lower NO/RNS levels also facilitate angiogenesis and promote tumour growth and metastasis [54]. Thus by all these mechanisms, tumor cells are able to reprogram macrophages ensuring their proangiogenic activation there by deviating the macrophages towards the fulfillment of tumour needs.

NO Production From Tumour Cells and Therapeutic Intervention

Since high levels of NO/RNS induces apoptosis it is plausible to manipulate the tumour cells to maintain their concentrations at the higher levels, so that they can be used as a means of therapeutic intervention. In a study using mice model, the tumour cells transfected with iNOS gene using viral vectors showed the increased ability to produce NO, pro-angiogenic proteins and prevented the tumour progression [55].

Another therapeutic approach for the treatment of tumour could be induction of M1 macrophages. Macrophages may be isolated from the patient and activated as M1 macrophages ex vivo, then introduced back to the patients.

However macrophages in tumour therapy can only be used until they are skewed towards M1 activation. Tumour mass reduces after surgery or chemotherapy. At this stage, MDSCs are decreased along with the tumour micro-environment being less hypotoxic and immunosuppressive. This period is regarded as window period that allows the opportunity for a successful antitumourtherapy using macrophages. Recently combined therapies using macrophages and activation of innate immune system are being explored [56].

Inhibitors of programmed cell death proteins-1 (PD-1) are novel drugs developed for treatment of various cancers. However, most of the patients either donot or partially respond to PD-1 inhibitors which is attributed to cancer associated immunosuppression. Therefore, a combination therapy has been proposed which involved coupling of PD-1 inhibitors with NOS inhibitors. Such combination modulates the immunosuppressive microenvironment of tumour cells and aids the patients who are unresponsive to anti PD-1 monotherapy. Moreover, further experimental analysis is still in progress to rule out the responders and nonresponders [57].

CONCLUSION

The effects of NO in humans are an area of interest for most of the researchers both at basic experimental levels or clinical studies. Remarkable studies have been conducted in the past decades to understand the role of NO in immunity. It not only acts as a potent antimicrobial agent but also has a protective function against tumours. However, despite of these beneficial effects, NO has the potential to switch from the protector to destroyer, i.e., it either protects from or leads to diseases. Over production by excess stimulation of NOS can lead to neurodegenerative disorders, cancer or inflammation. It is therefore essential to understand the roles of NO in immune system which requires the discrimination between the dual natures of this biomolecule. Observations from animal models regarding the interaction between macrophages and pathogens must be correlated carefully to human studies so that the importance of NO in providing protection against emerging pathogens can be stressed out. Also NO can be used as a novel therapeutic regimen in the treatment of refractory tumours which can be achieved by sensitising the tumour cells to immunotherapy. But validation of such strategies requires further clinical trials, so that NO mediated therapies can be developed in the prevention and treatment of cancer. Though most of the studies are focused on the immunological role of NO from iNOS; nNOS and eNOS can also be up-regulated to produce large amounts of NO. But the contribution of these variants of NO in immunoregulation are yet to be clarified. Therefore, further in-depth studies, on a much larger scale, for the identification of NO signaling cascade, molecular mechanisms of NO action and their targets should be carried out so that justification on how, when, where and why the cells are targeted by NO can be provided, thereby aiding in further exploration of therapeutic and clinical applications of such a versatile molecule

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