Introduction

“Start by doing what is necessary, then what is possible and suddenly you are doing the impossible”

-St. Francis of Assisi
Neutrophils/polymorphonuclear leukocytes (PMNs) are the primary guards of the innate immune system, which comprises 60-70% of the total white blood cells (WBCs) population and recruited earliest to the sites of infection, injury or inflammation. PMNs play an important function in first line of defense against invading bacteria, fungi and protozoa by internalization and destroy them by the sequential formation of phagosomes (Phagocytosis). This process involves recruitment of lysosomes and various types of granules to release proteolytic enzymes, anti-microbial peptides and free radical formation (Klebanoff, 2005; Nathan, 2006; Segal, 2005). The neutrophil-mediated inflammatory response can be regarded a multi step process involving the initial adhesion of circulating neutrophils to activate endothelium, subsequent extravasation and migration towards invaders and elimination through phagocytosis, generation of reactive species (ROS and RNI) and release of microbicidal substances (Faurschou and Borregaard, 2003).

Neutrophils are produced in the bone marrow, released into blood, circulate briefly, and migrate into tissue spaces. PMN development in the bone marrow has classically been divided into six stages myeloblasts (MBs), promyelocytes (PMs), myelocytes (MCs), metamyelocytes (MMs), band cells (BCs) and segmented neutrophil on the basis of cell size, nuclear morphology and granule content (Bainton et al., 1971; Borregaard and Cowland, 1997). In addition to the appearance of cytoplasmic granules, during maturation process there is decrease in cell size, cytoplasmic basophilia and number of mitochondria along with nuclear lobulation. Mitosis occur only during the first three stages; most take place during the myelocyte stage (Bainton et al., 1971; Theilgaard-Monch et al., 2006). Transit time for generation of neutrophils in marrow is approximately 10-14 days and the marrow maintains a five-day supply of mature neutrophils (Bainton et al., 1971). Neutrophil retention in the bone marrow mainly depends on the interaction of SDF1 (stromal derived factor-1; CXCL12) with the chemokine receptor CXCR4. Moreover CXCR4 deficiency results in decrease in bone marrow but increase in peripheral neutrophils as identified by the marker Gr-1 (von Vietinghoff and Ley, 2008). The daily turnover of neutrophils production is $10^{10}$-$10^{11}$ per human body. Neutrophils have a life span of only 4 -10 hours in circulation and one or two days in the tissue.

Blood cells such as neutrophils, monocytes, eosinophils, platelets, and red blood cells synthesize nitric oxide (NO). Among these, polymorphonuclear leukocytes (PMNs) constitute an important proportion and the major participant in a
number of pathological conditions with suggestive involvement of NO. NO is synthesized by a class of nicotinamide adenine dinucleotide phosphate (NADPH)-dependent NO synthases (NOS) by the conversion of L-arginine to L-citrulline along with NO (Bredt and Snyder, 1994; Moncada et al., 1989). NOS exists in three isoforms, neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). Constitutive NOS (cNOS) including endothelial (eNOS) and neuronal NOS (nNOS) are calcium dependent and produce low level of NO; however iNOS is augmented by inflammatory cytokines and is calcium independent to produce large amount of NO for prolonged period of time (Alderton et al., 2001). NO helps to repair the cells, move out of the bone marrow, dilates blood vessels and improves the oxygen uptake (Jablonska et al., 2005).

Neutrophils are predicted to generate NO at a rate of 10-100 nmoles/5min/10^6 cells, comparable to the endothelial cells (Salvemini et al., 1989; Wright et al., 1989) implicating a potential impact on vascular homeostasis. Neutrophils capability to synthesize NO was first discovered by its ability to relax aortic rings (Dikshit et al., 1993; Rimele et al., 1988) and by the inhibition of platelet aggregation, which was abolished by NOS inhibitors (Dikshit et al., 1993; Faint et al., 1991). Nitrite (NO$_2^-$) content (Miles et al., 1995; Rodenas et al., 1995) and NOS activity as measured by the conversion of L-arginine to L-citrulline (Chen and Mehta, 1996) have been correlated with NO production in rat and human PMNs.

Michurina et al., (2004) demonstrated NO as a modulator of haematopoietic activity. Bone marrow stromal-cell-derived eNOS has been found essential for the mobilization of stem and progenitor cells (Aicher et al., 2003). NO donors in vitro have however been shown to differentially regulate the generation of myeloid and erythroid colonies by CD34$^+$ cells (Shami and Weinberg, 1996). Induction of iNOS in bone marrow and CD34$^+$ cells after stimulation with IFN$\gamma$ or TNF-$\alpha$ suggested negative regulation of haematopoiesis (Maciejewski et al., 1995). Asthma exacerbates the number of CD34$^+$ circulating progenitors expressing high levels of iNOS implicating role of NO to prevent cell growth and colony formation in a paracrine and autocrine manner but it was not sufficient to prevent their proliferation in the circulation (Wang et al., 1999). Bone marrow derived neutrophils produce NO as assessed by flow cytometry after IFN$\gamma$, GM-CSF, LPS and L-arginine treatment (Punjabi et al., 1992). Bone marrow derived neutrophils possess nNOS (Wallerath et
Introduction

A strong correlation has been established between expression of nNOS in bone marrow and the ability of these cells to support hematopoietic stem cells, furthermore, NO donor SNAP can further increase this ability (Krasnov et al., 2008). Exposure of irradiated mice to systemic NOS inhibition increased the number of stem cells in the bone marrow followed by a transient increase in the number of neutrophils in the peripheral blood (Michurina et al., 2004) by an un-identified mechanism. Recently, circulating neutrophils have been shown to maintain blood pressure by suppressing bacteria and IFNγ-dependent iNOS expression in the vasculature of healthy mice (Morton et al., 2008) as neutrophil depletion led to decrease in blood pressure, suggesting requirement of PMNs to maintain the optimal vascular tone. Neutrophils maturation is adversely affected in diseases like chronic myeloid leukemia (CML), acute myeloid leukemia (AML) and neutropenia but NO/NOS status is not defined in these diseases. Since NO plays a role in neutrophil generation and is also constitutively present in peripheral PMNs, it is pertinent to identify the biochemical and molecular characteristics of NOS in the precursor cells of neutrophils obtained from bone marrow.

NOS in PMNs have been topic of debate at functional and molecular level including mRNA and protein expression. iNOS mRNA, protein and enzymatic activity was reported in circulating rat PMNs after culture (Miles et al., 1995) and in human PMNs after cytokine-treatment (Evans et al., 1996) and bacterial infection (Wheeler et al., 1997). nNOS in neutrophils was though demonstrated at mRNA level (Greenberg et al., 1998), but failed to observe at the protein level (Wallerath et al., 1997). Moreover, Gatto et al., (2000) reported over expression of neutrophil nNOS mRNA and protein in Parkinson's disease. Constitutive expression of iNOS in human neutrophils has been documented (Cedergren et al., 2003), while presence of eNOS has been observed only by de et al., (2001) in human neutrophils, warranting further investigations. A detailed study from our lab by using RT-PCR, Western blotting and immuno electron microscopy demonstrated the presence and intracellular distribution of nNOS and iNOS in rat PMNs (Saini et al., 2006). Neutrophil iNOS was augmented in SHR rat neutrophils (Chatterjee et al., 2007), while NOS activity and expression was found to be regulated by ascorbate (Chatterjee et al., 2008).

Studies from this lab as well as from others have identified NO as an important modulator of free radical generation in PMNs (Lee et al., 2000; Patel et al.,
2009; Pieper et al., 1994; Sethi et al., 1999; Sharma et al., 2004). NO and superoxide (O$_2^-$) anion react to generate peroxynitrite (ONOO$^-$) anion, which is more cytotoxic. Superoxide dismutates O$_2^-$ to form hydrogen peroxide, and MPO halogenates the hydrogen peroxide to HOCl and its reaction to NO can form NO$_2$Cl, a very potent oxidative moiety. The observations from our lab convincingly indicate towards NO mediated augmentation of free radical generation from PMNs (Seth et al., 1994; Sethi et al., 2001; Sethi et al., 1999). Intracellular and extracellular calcium also have a modulatory impact on NOS activity and free radical generation (Dikshit and Sharma, 2002). Recently, effect of NO donors on neutrophil respiratory burst suggests involvement of K$^+$ channels and kinases in NO mediated augmentation of respiratory burst (Patel et al., 2009).

A novel mechanism, formation of neutrophil extracellular traps (NETs), to eliminate invading pathogens has been recently reported (Brinkmann et al., 2004). NETs have been considered as beneficial suicide (Brinkmann and Zychlinsky, 2007) of neutrophils that binds microorganisms, prevents them from spreading, and ensures a high local concentration of antimicrobial agents. NETs contents are expectedly abundant at the site of infection and acute inflammation (Beiter et al., 2006; Brinkmann et al., 2004; Buchanan et al., 2006; Clark et al., 2007; Gupta et al., 2005). Since chronic granulomatous disease (CGD) patients did not form NETs, it was delineated that NADPH oxidase dependent generation of reactive oxygen species (ROS) mediate NETs release (Fuchs et al., 2007). Recently, a novel innate immune deficiency of impaired neutrophil extracellular traps (NETs) formation in human neonates have demonstrated with glucose oxidase (Yost et al., 2009), it is therefore desirable to identify new mediators of NETs formation.

NO modulates cell proliferation and apoptosis depending on cell type and redox status (Griscavage et al., 1995; Liu et al., 2003; Villalobo, 2006). Takagi et al., (1994) have shown NO to block the cell cycle of mouse macrophage-like cells in the early G$_2$/M phase. An earlier study using NO-donating agents, SNP showed that NO could suppress the growth and induce the monocytic differentiation of a human leukemia cell line, HL-60 (Magrinat et al., 1992). Recently, Wang et al reported that NO inhibits the proliferation of HL-60 cells by inducing G$_0$/G$_1$ arrest and apoptosis in a dose- and time-dependent manner through AKT pathway (Wang et al., 2007). The human leukemic cell line HL60 has been widely used as model cell line for neutrophil...
differentiation. Since our studies found differential expression of NOS isoforms and NO level in bone marrow cells during neutrophil maturation, we considered it logical to assess effect of NO donors and nitrite, a NO metabolite on the HL60 cell cycle to assess the proliferative and apoptotic effects of NO.

The present study was undertaken to achieve following objectives

- Molecular and biochemical characterization of NOS isoforms in the rat and human neutrophil precursor cells and circulating neutrophils
- NO mediated regulation of neutrophil free radical generation and NETs release from human neutrophils
- Effect of NO donors and nitrite on HL-60 cell proliferation and apoptosis