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BIOCHEMICAL AND MOLECULAR EVALUATION OF NEUTROPHIL NOS IN SPONTANEOUSLY HYPERTENSIVE RATS

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Abstract - Resting neutrophils generate NO, while activation leads to the production of reactive oxygen and nitrogen species. Nowadays cardiovascular pathological conditions such as hypertension, cardiac ischemia, reperfusion and heart failure are associated with inflammation. This project explores the respiratory burst potential and NO generation status in the neutrophils, plasma, aorta, and kidneys from normotensive Wistar and spontaneously hypertensive rats (SHR). Total and protein associated nitrite content was quantitated using Griess reagent following cadmium reduction and mercuric chloride treatment respectively. NO and superoxide generation evaluated by Flowcytometry and peroxynitrite by spectrofluorimetric method. Expression of NOS isoforms was analyzed by RT-PCR. NO generation from SHR neutrophils was significantly augmented in comparison to normotensive counterparts. Neutrophils activated in response to arachidonic acid, PMA, fMLP or E. coli generated more superoxide radicals among SHR, and consequentially peroxynitrite. Expression of iNOS was significantly more in the SHR neutrophils, while that of nNOS remained unaffected. Results suggest that NO generated in SHR is utilized in scavenging superoxide radicals thereby limiting its bioavailability. Thus induction of NOS in neutrophils combined with augmented oxidative stress might influence its association with endothelium and contribute to inflammatory responses under hypertensive condition.

Keywords: Spontaneously hypertensive rats (SHR), neutrophils, NOS, NO, peroxynitrite ($ONOO^-$) an superoxide ($O_2^{\cdot-}$) radicals

INTRODUCTION

Much of the investigations in hypertensive disorders in past decades have been devoted to exploring the endothelial dysfunctions in terms of decline in the production of vasodilators such as prostacyclins and nitric oxide (NO) (10). Decrease in NO mediated vasodialatory consequences have been widely documented and is attributed to its altered production or bioavailability (28, 8, 18, 19). Endothelium endowed with eNOS has been the centre of interest among cardiovascular researchers, which led to the characterization and exploration of explicit signaling and regulatory parameters governing expression and functionality of eNOS.

Neutrophils, the forerunners in defense against pathogenic intrusion are able to synthesise NO at a rate comparable to endothelium (10-100nmoles/5min/10^6 cells) (37), thus significantly contribute to the circulating levels of NO and thereby encompass a profound influence on cardiovascular physiology, which have however, not been investigated so far. NO acts in an autocrine manner to modulate several neutrophil functions such as chemotaxis (3,11), adhesion (24,25), phagocytosis (32), oxidative burst (39,41,42,43), degranulation and apoptosis (23,27) for effective and timely resolution of the inflammatory process. NO also prevents interaction of neutrophils with the endothelium by reducing CD11b/CD18b expression (1), and platelet aggregation or intravascular vascular thrombosis (9). In fact relaxation of endothelium denuded aortic rings and inhibition of platelet aggregation in presence of neutrophils (34, 37) led to the characterization of NO release from the PMNs, subsequent to EDRF. Moreover, recent advocation for the presence of eNOS in these granulocytes along with previous characterization of nNOS and iNOS makes them a readily available and unique cellular system to explore NOS biology under diverse pathophysiological conditions.

Abbreviations: Nitric oxide synthase (NOS), Nitric oxide (NO), Endothelium derived relaxation facor (EDRF), Reactive oxygen species (ROs), Reactive nitrogen species (RNS), Polymorphonuclear leukocyte (PMN), Arachidonic acid (AA), Phorbol myristic acid (PMA), Bacterial peptide fMLP.

On the other hand, neutrophils, which are capable of vigorous respiratory burst, might cause significant oxidative damage to the surrounding milieu under pro-inflammatory conditions. Recent studies have documented that both genetic and acquired forms of hypertensive subjects are prone to oxidative stress (35). PMNs mediated oxidative stress might thus contribute to the complexity of hypertensive pathologies. ROS and /RNS cause damage through multifactorial aspects- indicating structural and functional damage to the vascular endothelium to limit the bio-availability of NO and generate ONOO- which, affect endothelial eicosanoids and oxidative modifications of LDL. Sela et al, (26,38) have reported contribution of primed PMNs in the oxidative stress and the augmented inflammatory status in hypertensive subjects. Moreover, intracellular ionized calcium in these granulocytes correlated with the mean arterial blood pressure in the hypertensives. It was therefore considered worthwhile to investigate the NO/NOS status and superoxide radical generation from neutrophils in SHR to assess the role of neutrophils and oxidative stress in hypertension.

MATERIALS AND METHODS

Animals

Genetically pre-disposed inbred strains of SHR were used in this study. Age and sex matched Wistar rats were used as controls. All the animals were procured from the National animal house facility of the Institute. Rats were housed in polypropylene cages, and provided with chow pellets and water *ad libitium*. All animal experimentations were approved and performed as per ethical guidelines of the Institute.

Chemicals

Dextran, Histopaque 1083,1119, cadmium pellets, sulphanilamide, phosphoric acid, ammonium chloride, hydrochloric acid, N-(1-naphthyl) ethylene diamine, diaminofluorescein diacetate (DAF-2DA), dihydroethidium (DHE), arachidonic acid, Mercuric chloride ($HgCl_2$), phorbol myristic acid (PMA), fMLP, scopoletin, Tri reagent were purchased from Sigma-Aldrich. Quickprep Micro mRNA Purification kit was procured from Amersham, and RETRO Script from Ambion.

Isolation of peripheral neutrophils

Rat blood was collected by cardiac puncture under ether anesthesia in sodium citrate (9:1 v/v). Neutrophils were isolated as reported earlier (45), briefly through dextran sedimentation followed by differential centrifugation on Histopaque gradients 1083, 1119 and suspended in HBSS. Cell viability was estimated by Trypan blue (2 mg/ml) exclusion assay, and always found to be >98%.

Estimation of total nitrite content

Total nitrite content in neutrophils ($2x10^7$), plasma (500 µl), kidney and aortic homogenates was estimated following reduction with cadmium pellets for 2hrs with constant shaking and subsequent treatment with Griess reagent (41). Optical density readings were recorded at 548 nm and 630 nm.

Estimation of protein associated nitrite

Plasma (500µl), PMNs ($2x10^7$), kidney and aortic homogenates were treated with 1mM $HgCl_2$, for 30mins and were subsequently treated with Griess reagent. Optical density was read at 548 nm along with of standards. Protein content in the aortic and kidney homogenate samples was estimated by the method of Lowry et al., (29).

Free radical generation from neutrophils

Superoxide radical and NO generation in neutrophils was measured by using cell permeable dyes like DHE ($O_2^{-\cdot}$, 5 µM), DAF-2DA (NO, 5 µM). The cells were incubated for (5-10) minutes with the dye at 37°C and further incubated in presence of neutrophil stimulants like arachidonic acid (2 µM), Phorbol myristic acid (PMA) (30 nM), fMLP (3 µM), and *E. coli* bacteria for 40 minutes. Samples were acquired on a flow cytometer (BD, FACS Caliber with argon laser) and mean fluorescence intensity was recorded for at least 10,000 neutrophils (42, 43). Data was analyzed using Cell-Quest pro software.

Peroxynitrite generation

$ONOO^-$ generation in resting and activated neutrophils following stimulation with arachidonic acid, PMA, fMLP and *E.coli* bacteria was estimated with scopoletin (20µM) as substrate by spectrofluorimetry at Exλ-305, Emλ-460. Decrease in fluorescence intensity of

scopoletin indicated oxidation of scopoletin due to the formation of $ONOO^-$ (31).

Expression of NOS isoforms in neutrophils

Total RNA was isolated from neutrophils of Wistar and SHR rats by using Tri reagent (Sigma).mRNA was prepared from total RNA by using Quickprep Micro mRNA Purification kit (Amersham). mRNA (100ng) was reverse-transcribed with RETRO Script (Ambion) using oligo(dT) primers, as per the manufacturer's instructions. The cDNA was amplified for different NOS isoforms. The PCR products were analyzed by electrophoresis on a 1.2% agarose gel and visualized with ethidium bromide staining. The primers were selected as reported earlier [44]. The sequence for nNOS (5' TTG ACC CCA CGA TGA AAA GC; 5' GGA TGC TCA GCA CAG GTT CTA TCT 3') amplified a 93 base pair (bp) fragment , iNOS (5' TGG TGA AAG CGG TGT TCT TTG ; 5' CTT ATA CTG TTC CAT GCA GAC AAC CTT 3') amplified a 176-bp fragment while alpha tubulin (5' TCT TGG ACA GAA TTC GCA AGC T 3' ; 5' GGA CTT CTT TCC GTA GTC GAC AGA) give 143 bp amplified product . The amplification reactions for 35 cycles were denaturation- 94°C, 30 s; annealing and extension- 60°C, 1 min for nNOS, iNOS and alpha tubulin.

Statistical analysis

Data are represented as Mean ± S.E.M, and were analyzed by using Prism 3 graph pad by one way ANOVA test followed by Newman-Keuls post analysis and Student's t-test analysis. Data were considered significant at $p< 0.05$.

RESULTS

Potential of NO generation from neutrophils

Total as well as protein bound nitrite content in the neutrophils from SHR were significantly more in comparison to the normotensive Wistar rats (Fig.1). In control rat neutrophils total nitrite and protein bound nitrite content were almost same. While total nitrite content and protein bound nitrite content in the SHR neutrophils was 14.36±3.7 µM/2x 10^7 cells and 4.57 ±0.64µM/2x 10^7 cells respectively.

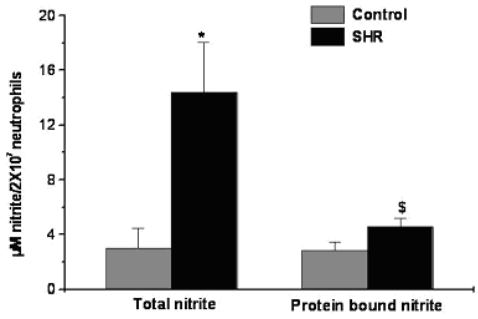
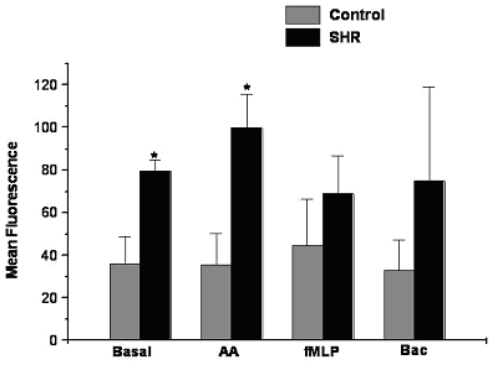
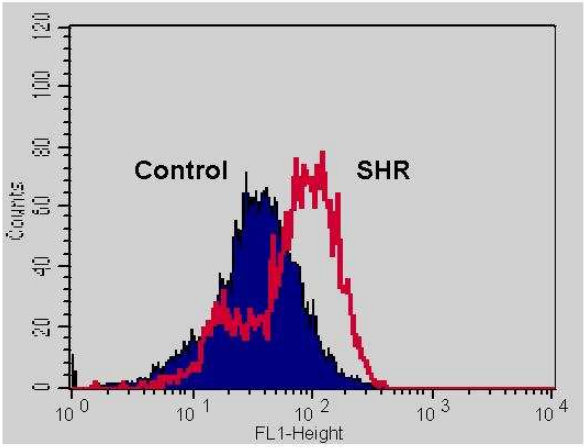


Figure 1. Histogram showing total (Cadmium treated) and protein associated (Mercuric chloride treated) nitrite content in neutrophils (µM/2x10^7 neutrophils)
Values are Mean ± SE for at least 4 experiments with $p<0.05$ (*), $p< 0.01$ (**) in comparison to the respective controls and $p<0.05$ ($) in comparison to the total nitrite content.

NO generation from neutrophils was also evaluated by flow cytometry using DAF-2DA. The N-nitrosation of DAF, a NO detection dye yielding a highly green fluorescent triazole form (DAF-2T), offers the advantages of specificity, sensitivity, and effectivity for direct NO detection. NO generation in neutrophils from SHR was significantly enhanced in comparison to those from control Wistar rats (Fig 2), which was in accordance with the trend as observed with nitrite content in neutrophils. Following stimulation with arachidonic acid, fMLP or bacteria the NO generation was increased but it was statistically significant only after AA induced stimulation (Fig 2a,b).



a



b

Figure 2. (a) Flow cytometric evaluation of NO generation in neutrophils from SHR and normotensive Wistar rats using DAF-2DA

Values are Mean fluorescence of DAF-2T ± SE for at least 4 experiments with p<0.05 (*) in comparison to the respective controls.(b) Histogram presentation of NO generation in terms of FL-1Height response for DAF-2T in control and SHR.

Expression of NOS isoforms in neutrophils

The expression of nNOS and tubulin remained unaltered (Fig.3) in the SHR neutrophils. The expression of iNOS was however found to be upregulated in SHR neutrophils (Fig.3). This could attribute to the enhanced NO generation status from neutrophils of the SHR group in comparison to the normotensives.

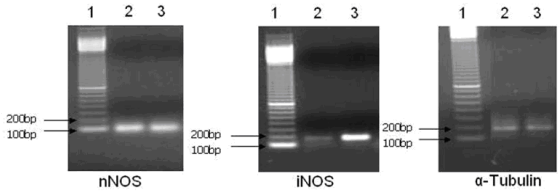


Figure 3. RT-PCR products of NOS isozymes and α-tubulin in rat PMNs

Lane 1, Molecular weight marker; lane 2, 3 PCR products of nNOS, iNOS and α-tubulin as indicated of Wistar and SHR respectively.

Superoxide generation in neutrophils

The basal DHE fluorescence in resting neutrophils was higher in SHR than those of normotensive rats, which was further enhanced to a significant extent among the activated neutrophils stimulated with arachidonic acid, PMA, a bacterial peptide fMLP or E.coli bacterial challenge (Fig.4).Presumably the stimulants trigger superoxide generation more over NO generation from activated neutrophils since the production of NO stimulated by arachidonic acid, fMLP or bacterial challenge did not enhance the levels of basal NO to a significant extent amongst SHR.

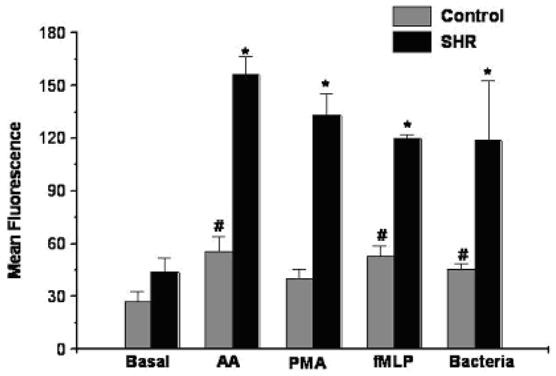


Figure 4. Flow cytometric evaluation of superoxide generation from neutrophils of SHR and control matched Wistar rats using DHE

Values are Mean fluorescence values for DHE ± SE for at least 4 experiments with p<0.05 (*) in comparison to the respective controls, p<0.05 (#) basal vs stimulated control PMNs.

Generation of peroxynitrite (ONOO-)

$ONOO^-$ generation in resting and stimulated neutrophils following treatment with arachidonic acid, PMA, or fMLP was significantly more in SHR in comparison to matched normotensive group (Fig.5).

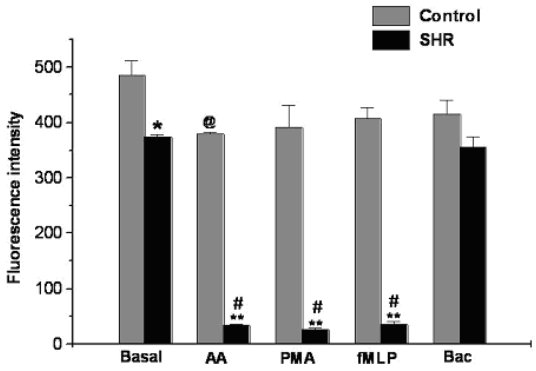


Figure 5. Peroxynitrite generation from neutrophils of SHR and normotensive Wistar rats in resting state and following stimulation with arachidonic acid, PMA, fMLP and E.coli

Values are Mean Scopoletin Fluorescence intensity ± SE for at least 3 experiments with p< 0.05 (*), p<0.01 (**) in comparison to the respective controls p< 0.05 (@) in comparison to basal levels in control and p< 0.05 (#) in comparison to basal levels in SHR .

Most of these agents did not affect the NO generation potential in activated neutrophils to a great extent in comparison to the resting cells.

This rise in ONOO- generation could be attributed to the enhanced superoxide generation under the influence of these stimulants. NO having a natural affinity towards superoxide, scavenges this radical to generate ONOO-. However the increased generation of ONOO- at the basal levels among SHR could be attributed to the difference in NO generation potential rather than superoxide generation from resting neutrophils from both groups which did not exhibit a significant difference <Fig. 4>. Seemingly the SHR neutrophils are more efficient in generation of NO at the basal or unstimulated state but when activated trigger the switch towards superoxide generation in copious amounts that is subsequently scavenged at the expense of the NO produced in excess producing ONOO-.

Status of nitrite content in aortic tissue, plasma and kidney

Total nitrite content or protein associated nitrite in the plasma showed no significant difference between the two groups (Fig.6A). Nitrite content however, exhibited a decrease in the aortic tissue (Fig.6B) from SHR compared to the normotensive Wistar rats. The NO in kidneys was mainly trapped as protein bound form and was found to be more in the SHR group (Fig.6C).

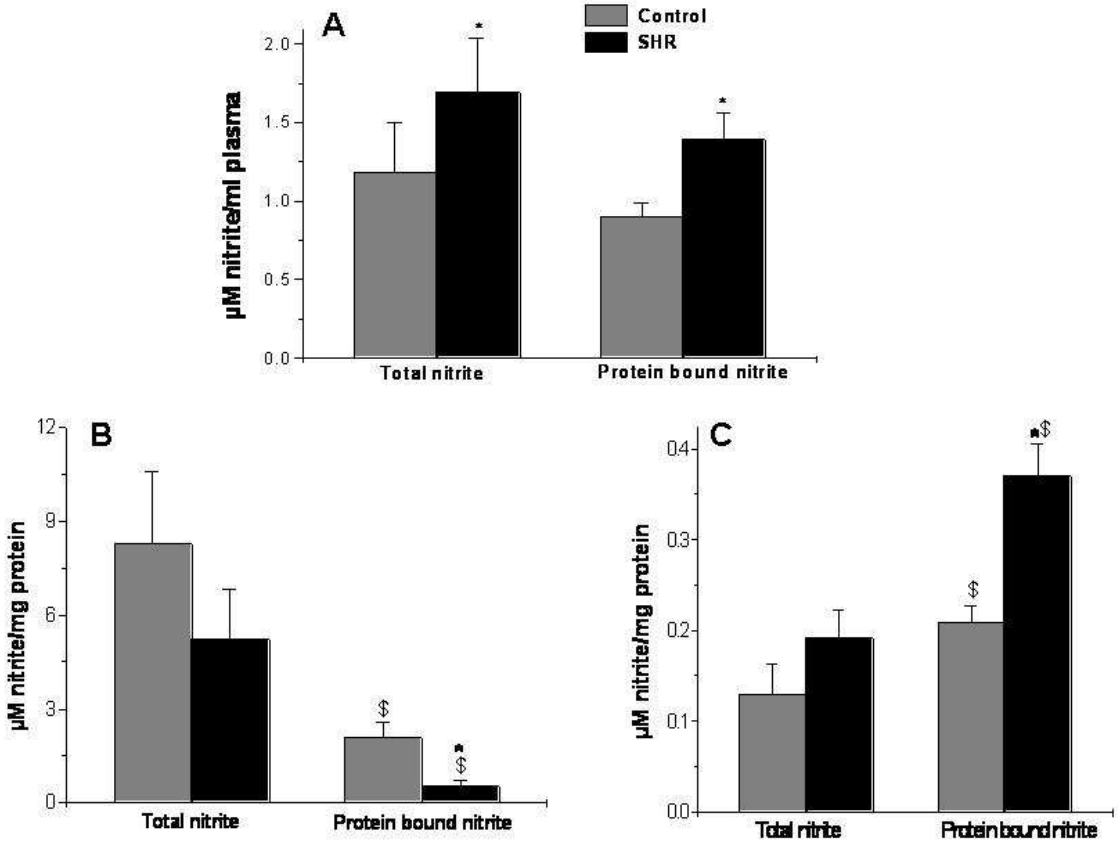


Figure 6. Histogram representing total (Cadmium treated) and protein associated (Mercuric chloride treated) nitrite content in (A) plasma (µM/ml of plasma), (B) aortic homogenate (µM/mg of protein), (C) and kidney (µM/mg of protein) homogenate from SHR in comparison to the matched normotensive Wistar rats

Values are Mean ± SE for at least 4 experiments with $p<0.05$ (*) in comparison to the controls and $p<0.05$ ($) in comparison to total nitrite content.

Interestingly the amount of total nitrite content was found to be higher than that of protein associated nitrite in plasma and aortic tissue as seen in neutrophils with the exception of kidney where most of the nitrite was found to be in a protein associated form. Moreover there existed a significant difference between total and protein associated nitrite content in aortic tissue from SHR as seen in neutrophils although following the opposite trend with respect to the normotensives. A significant enhancement in protein associated nitrite in kidneys among the hypertensive group suggests protein nitrosylation.

DISCUSSION

Neutrophils generate NO almost at a rate comparable to the endothelial cells, which could contribute significantly to the circulating NO levels. NO generated from neutrophils modulate leukocyte-endothelial and platelet-neutrophil interactions. It might also have some influence on the vascular tone. It is however, surprising that the status of neutrophil NOS has not been subjected so far to detailed investigations. Presence of both nNOS and iNOS has been accepted unequivocally (4, 12, 14), while occurrence of eNOS (7), is currently being advocated. The extent and kinetics of NO generation varies between isoforms (46), depending on the microenvironment of the intracellular compartments; and differential amounts of NO thus generated at effector sites modulates the functional features of the cell. Therefore presumed alteration at the transcriptional regulation of different NOS isoforms under pathological conditions as hypertension could serve as peripheral marker and moreover offer a critical look into the molecular regulatory device operating to modulate NO generation by these cells during such pathologies. The present study was therefore undertaken to explore the status of NO generation and NOS isoform expression in the neutrophils obtained from SHR and normotensive Wistar rats. NO generation as assessed by DAF-2T as well as total nitrite and protein associated nitrite content, were found to be significantly augmented in the SHR group (Fig.1, 2a,b). Interestingly expression of iNOS was also significantly augmented, while nNOS expression remained unaltered (Fig 3). We have however not observed presence of eNOS in the rat neutrophils at the expression level (36), presence of eNOS protein has been documented in the human PMNs (7), and can not be completely ruled out in rat PMNs also. Increase in iNOS expression in the PMNs could presumably be correlated to the alteration in the profile of pro-inflammatory cytokines during hypertension as evidenced earlier (2, 15, 39). Expression of iNOS has recently been shown to be enhanced by eNOS (13). Therefore the underlying reasons for the enhanced expression of iNOS in SHR neutrophils remain to be explored. Since neutrophils possess both nNOS, eNOS (calcium dependent) and iNOS (calcium independent), augmented NO level could be due to the elevated intracellular calcium levels as evidenced by Sela et al. (38) as well as expressional up regulation on the part of iNOS thus assuring a two pronged stimulatory effect on NO synthesis; or it could be triggered by the endothelium or neutrophil in an autocrine manner to prevent adverse neutrophil-endothelial interactions.

Previous studies from the lab have shown that extracellular addition of NO donors augment the free radical generation from the activated neutrophils (41). It might thus be possible that increase in the NO availability in hypertensive rats prime these cells or promote pronounced free radical generation. Currently hypertensive disorders are being associated to proinflammatory predisposition and neutrophils though primarily meant to offer protection against invading pathogens; under inflammatory conditions these cells however inflict damage to the host tissue. Bestowed with the functional NADPH oxidase complex in the membranous sites of plasma membrane or phagolysosomal compartments activated neutrophils liberate compelling amounts of $O_2^{\cdot -}$ in the external surrounding and phagolysosomal vesicles. In the present study we have evaluated $O_2^{\cdot -}$ generation from resting and activated neutrophils. Neutrophils from SHR were found to be more activated and capable of generating profuse amount of $O_2^{\cdot -}$ when stimulated in comparison to the control normotensive rats, (Fig. 4) in accordance with the observations of previous workers showing increased SOD response in neutrophils from hypertensive subjects (26,38). The high basal fluorescence for DHE in the resting neutrophils from SHR denotes by far a more activated state than those from the

normotensives. We did not observe any significant difference in the phagocytic potential though suggesting both endocytose equivalent bacterial loads during phagocytic engulfment (data not shown). However, enhancement in surface expression of CD11b among SHR neutrophils also substantiated their active status in peripheral circulation (data not shown). It would be worthwhile exploring the execution of activated neutrophils thereby modulating the course of inflammatory response as part of future studies. Sela et al., (26) have documented increased necrosis in neutrophil population, further complicating the inflammatory adversities among hypertensive subjects. Rise in the repertoire of activated peripheral neutrophils has also been documented and the possibility of chemotactic migration to peripheral circulation in response to chemokines and their subsequent inflammatory reaction and demise would further enhance their importance in regulating cardiovascular hemostasis.

Now both generation of NO and $O_2^{\cdot-}$ being higher in neutrophils among SHR, it could affect the bioavailability of the NO as a vasodilatory agent meant to compensate for endothelial malfunctioning among SHR. Generation of peroxynitrite, a reaction product of NO and $O_2^{\cdot-}$ was far greater by a wide margin in resting as well as activated neutrophils from SHR when stimulated with arachidonic acid, PMA, fMLP and *E.coli* (Fig.5) . Nonetheless generation of $ONOO^-$ could bear cytotoxic effects not only upon the neutrophils generating it but also on the surrounding extracellular environment wielding pro-inflammatory results and as an effective nitrosylating agent could be involved in modulation of intracellular signaling offering an on and off switch regulating protein functionality.

A decline in the NO generation status from aortic tissue (Fig 6b) as evidenced in the present study is in accordance with previous documentations that recorded malfunctioning of aortic endothelium. During development of hypertension, SHR exhibits a decrease in the activity and protein expression of eNOS in the aorta associated with an elevation of blood pressure when compared with those from age-matched Wistar Kyoto rats (WKY) (6, 21). On the contrary attenuation of functional basal NO despite increased eNOS enzymatic activity was observed in other studies where the bioavailability of NO was drastically reduced. Since vascular endothelium is prone to oxidative stress among hypertensive subjects increased scavenging of NO by O_2^- would then limit NO availability despite increased synthesis (16). eNOS itself generates O_2^- (22) in absence of substrate or reduced tetrahydrobiopterin content when NOS becomes partially uncoupled. Impaired synthesis of tetrahydrobiopterin and enhanced expression of iNOS has been documented in SHR. It has also been suggested that tetrahydrobiopterin supplementation can reverse endothelial dysfunction caused during cardiovascular diseases, including atherosclerosis, coronary artery disease, and hypertension. Chronic treatment with biopterin (10 mg/kg per day IP) significantly improved the impaired vascular responses to acetylcholine, suppressed the development of hypertension in SHR. (20).

Total or protein associated nitrite content in the plasma showed significant difference among SHR and normotensive group as evident from (Fig 6a). This could be attributed to the neutrophil derived NO in peripheral circulation but since much of it is trapped in an oxidized or protein bound form it is not available for vasodilatory actions.

Essential hypertension is often associated with renal arteriolar thickening, fibrinoid deposition in glomeruli and proteinuria. The detrimental effects of systemic hypertension on renal vascular bed and also the renin-angiotensin system depend on the extent of vascular pressure exerted upon renal microcirculation (30). NO and its derivatives effectively modulate vascular tone in the microcirculation under pathophysiological conditions. Nava *et al.*, have also reported elevated calcium dependent NOS activity in renal medulla and hypothesised that impairment in medullary control of arterial pressure was not due to reduced production of NO (33). This instigated us to explore the total and protein associated nitrite content in the kidneys fron SHR and Wistar rats. Much of the nitrite is found to be associated with proteins either in the nitrosylated or nitrated form (Fig 6c) and more prominent in the SHR group in comparison to control. Since $ONOO^-$ is a potent nitrosylating agent, it indirectly signifies the generation of $ONOO^-$ and therefore more of $O_2^{\cdot-}$ subjecting kidneys to oxidative damage. It is well established that ONOO promotes generation of renin via the CoX-2–prostaglandin synthesis pathway. On the other hand Enalapril an ACE inhibitor effectively prevents vascular damage in NO deficient SHR. Moreover Angiotensin -1

(AT-1) receptor blockade (Losartan 30 mg/kg/day) initiated early in the course of the disease has been shown to prevent target organ damage and preserve renal and vascular NOS (47). Thus NOS and the renin-angiotensin system in kidneys correlate to modulate blood pressure electrolyte and fluid homeostasis. Considering the context of neutrophils infiltration of activated leukocytes has been reported earlier which contribute much to the generation of ROS/RNS in the nephrous tissues. Moreover in effect these events limit the levels of vasodilatory NO in the nephral microcirculation and consequentially might cause predisposition to end organ damage (5).

Considering hypertension as an inflammatory disorder (15) has opened a new area of research and neutrophils could be considered as one of the prime candidates to exert their influence on the phenomenon. They not only augment the adversities of hypertensive disorders by altered endothelial-neutrophil associations, but might also contribute to the secondary inflammatory response even in absence of primary inflammation. The target organ damage encountered in experimental models of hypertension illustrates the infiltration of activated macrophages to the target sites (17). Neutrophils bearing equivalent potentials as macrophages can possibly play a role in target organ damage during hypertension as they do in sepsis. As an outcome of this investigation it might be concluded that neutrophils being the largest population of circulating leukocytes profoundly affect the vascular hemostasis under hypertensive condition bearing pro-oxidant and proinflammatory attributions, predisposing the hypertensive rats to oxidative stress and further encompassing pathological complexities.

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