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Molecular and biochemical characterization of nitric oxide synthase isoforms and their intracellular distribution in human peripheral blood mononuclear cells

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ABSTRACT

Nitric oxide synthase (NOS) expression and catalytic status in human peripheral blood mononuclear cells (PBMCs) is debatable, while its sub-cellular distribution remains unascertained. The present study characterizes NOS transcripts by real time PCR, NOS protein by immunoprecipitation (IP)/Western blot (WB), nitric oxide (NO) generation by DAF-2DA and NOS sub-cellular distribution by immunogold electron microscopy in resting PBMCs, monocytes and lymphocytes obtained from healthy donors. We observed constitutive expression of full length NOS isoforms (nNOS, iNOS and eNOS) in PBMCs: with the highest expression of iNOS in comparison to nNOS and eNOS. Isolated monocytes expressed more eNOS transcript and protein as compared to nNOS and iNOS. Lymphocytes however had more iNOS transcripts and protein than nNOS and eNOS. NOS was catalytically active in PBMCs, monocytes as well as in lymphocytes as evident by NO generation in the presence of substrate and cofactors, which was significantly reduced in the presence of NOS inhibitor. Immunogold electron microscopy and morphometric analysis revealed the distinct pattern of NOS distribution in monocytes and lymphocytes and also exhibited differences in the nuclear–cytoplasmic ratio. nNOS localization was much more in the cytosol than in the nucleus among both monocytes and lymphocytes. Interestingly, iNOS distribution was comparable in both cytosol and nucleus among monocytes, but in lymphocytes iNOS was predominantly localized to the cytosol. The present study exhibits constitutive presence of all the NOS isoforms in PBMCs and reports the distinct pattern of NOS distribution among monocytes and lymphocytes.

1. Introduction

Nitric oxide (NO) is generated by the blood cells including PBMCs which regulates vascular hemostasis, peripheral immune response and hematopoiesis, by modulating signaling, gene expression and the balance between proliferation and differentiation [1–3]. Beneficial and cytotoxic actions of NO have been correlated with its concentration/availability [4–6]. There is a dearth of studies regarding NOS localization in PBMCs, while contradictions exist on the ability of PBMCs to generate NO, NOS expression and the presence [7–10] or absence [11–13] of NOS protein among PBMCs. It was demonstrated that as compared to murine macrophages, human macrophages failed to secrete NO (<0.1 μmol/10^6 cells/24 h), even after treatment with endotoxin, interferon-gamma, granulocyte–macrophage colony-stimulating factor, tumor necrosis factor-alpha, bacteria, or proliferating lymphocytes [13].

Expression of both cNOS and iNOS has been reported among PBMCs at transcript and/or protein level by using different experimental approaches. Constitutive expression of iNOS was detected by RT-PCR and Northern blot analysis in monocytes of control subjects but could not be confirmed by Western blot analysis [7]. The presence of eNOS transcript was also demonstrated in IgD+ or IgD− B cells, peripheral blood, tonsillar T cells and in circulating monocytes [8]. However, the quantitative expression of NOS isoforms at mRNA and protein levels in monocytes and lymphocytes has not been well explored. NOS activity could not be measured in sub-cellular fractions of human macrophages even after tetrahydrobiopterin (BH_4) supplementation [13]. Others have also dissociated NO generation from the microbicidal actions of monocytic phagocytes [11,12]. These reports are contrary to those advocating expression of NOS and NO production from the PBMCs [7–10]. Thus biochemical and molecular characterization of the NOS system in PBMCs needs to be explored in a systematic manner.

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The extent of NO generation from a particular cell in question might be modulated by post-translational regulations imposed on NOS even after genetic check points have been passed. The microenvironment inside sub-cellular organelles harboring NOS may influence catalysis through variations in pH, co-factor and substrate availability, or by interaction with regulatory protein partners to either promote or impede catalysis while in association with NOS. Distinct sub-cellular distribution of various NOS isoforms might affect their catalytic potential and modulate organelle specific as well as overall cellular functions. Analyzing the intracellular localization of NOS [14–20] thus may facilitate our understanding of cell physiology and be an informative tool for the studies related to NO generation, nitrosylation of signaling proteins and trafficking of certain cargo proteins [4,21,22]. Studies exploring NOS localization in neutrophils [23], endothelial cells [24], astrocytes [20], adipocytes [15], and eosinophils [25] have highlighted the importance of NOS distribution/trafficking to various intracellular compartments, which is unexplored in PBMCs. The present study was therefore undertaken to systematically identify and quantify the NOS isoforms at mRNA and protein level as well as to assess the NO generation in resting PBMCs, and in isolated monocytes and lymphocytes. Moreover, we have further extended this study to demonstrate the relative sub-cellular distribution pattern of NOS isoforms in PBMCs obtained from healthy human volunteers.

2. Materials and methods

2.1. Reagents and antibodies

RevertAid™ H Minus First Strand cDNA synthesis kit and 2× SYBR green PCR master mix were procured from Fermentas. L- [^{3}H] arginine, 10 nm and 15 nm gold conjugated goat anti-mouse and goat anti-rabbit antibodies were purchased from Amersham Biosciences (Sweden). Uranyl acetate was obtained from Polaron Equipment Ltd. (England). Antibodies against human nNOS (rabbit polyclonal), iNOS (mouse monoclonal) and goat anti-rabbit IgG HRP were from Santa Cruz (USA). Immunoprecipitation starter pack was purchased from GE Healthcare (USA). Monoclonal antibody against human eNOS (mouse monoclonal) and all other chemicals were procured from Sigma Aldrich Co. (USA).

2.2. Human subjects

Blood was collected in citrate–phosphate–dextrose (CPD) (1:7) from healthy volunteers. A detailed medical history and physical examination was carried out before phlebotomy. The donors were free from heart, lung and kidney diseases, cancer, epilepsy, diabetes, tuberculosis, abnormal bleeding tendency, allergic disease, sexually transmitted diseases, jaundice, malaria, typhoid and thyroid/or any other endocrine disorders. Donors were also free from any prior medication for the last 72 h before sample collection. The study was performed in accordance with guidelines set by the ethical committee of the institute.

2.3. Isolation of peripheral blood mononuclear cells (PBMCs), monocytes and lymphocytes

Peripheral blood mononuclear cells (PBMCs) were isolated by loading diluted blood (1:1 with saline) on Histopaque 1077 and centrifuged at 400 g for 30 min at 20 °C (3 K30 Sigma Centrifuge, Germany) [26]. PBMCs collected from the interface were washed twice with Hank's Balanced Salt Solution (HBSS) by centrifugation at 100 g for 10 min at 20 °C and re-suspended in RPMI 1640 and were cultured for 2 h[27]. All the steps were carried out under sterile conditions. Adhered monocytes and floated lymphocytes were collected for subsequent analysis. The purity of the isolated monocytes and lymphocytes was assessed by using CD14 and CD3 antibodies in a flow cytometer (FACS Calibur, Becton Dickinson, USA) and was found more than 94% and 95% respectively. Viability of the isolated blood cells was also more than 90% as assessed by Trypan blue exclusion test.

2.4. NOS transcripts by quantitative (real-time PCR)

Total RNA was isolated from human PBMCs lymphocytes and monocytes using Tri reagent (Sigma, USA). 5 μg of total RNA was reverse transcribed with RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas) using oligo (dT) primers, as per the manufacturer's instructions. The cDNA was amplified in PCRs by using primers for neuronal NOS (F-5′TCTAACAGGCTGGCAATGAAG3′, R-5′TCTCTAAGGAAGTGATGGTTGAC3′), inducible NOS (F-5′ GTTCTCAAGGCACAGGTCTC3′, R-5′GCAGGTCACTTATGT CACTTATC3′) endothelial NOS (F-5′ATTATATCCTACACAAGACTCCAG3′, R-5′ TCTTCAAGTTGCCCATGTTAC3′) [28] and β-actin (F-5′AACT GGAACGGTGAAGGTG3′, R-5′CTGTGTGGACTTGGGAGAGG3′) [29], which amplified 165 bp, 210 bp, 160 bp and 208 bp products respectively. Real-time RT-PCR was performed with a Maxima SYBR Green RT-PCR Kit (Fermentas) on Roche light cycler. Reaction volume was 25 μl volumes, which included 12.5 μl of 2× Maxima SYBR Green RT-PCR Master Mix, 1 μl cDNA template, and 0.2 μmol/l primers designed to amplify a part of each gene. Three-step PCR protocol applied for nNOS reactions consisted of 35 cycles of 95 °C for 15 s, 57 °C for 30 s, and 72 °C for 30 s, for iNOS of 35 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s, and for eNOS of 35 cycles of 95 °C for 15 s, 56 °C for 30 s, and 72 °C for 30 s. After PCR, a melting curve analysis consisting of 1 cycle: 95 °C for 0 s, 70 °C for 10 s, 95 °C for 0 s, cooling 1 cycle: 40 °C for 3 min was performed to demonstrate the specificity of the PCR product as a single peak. The amplification reactions for β-actin were 30 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. The differences in the threshold cycles for β-actin and NOS isoforms were used to calculate the expression levels of NOS mRNA.

2.5. NOS immunoprecipitation and Western blotting

PBMCs, monocytes and lymphocytes were suspended in ice-cold radio IP assay (RIPA) buffer [PBS containing 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 μg/ml aprotinin, 100 μg/ml PMSF, 20 μg/ml pepstatin, 5 mM di-isopropyl fluorophosphates (DFP)] were lysed with 1% Triton X 100 and 0.1% SDS at 4 °C, and were exposed to rabbit IgG (1 μg for nNOS and eNOS) and mouse IgG (1 μg for iNOS) for 1 h at 4 °C and then 'precleared' using protein-G agarose beads. Following centrifugation, the supernatant was collected and incubated with 1 μg of nNOS, iNOS or eNOS antibodies [23]. Western blotting (WB) was performed with primary (at 1:500 in 1.5% skimmed milk TBST) and HRP-conjugated anti-rabbit IgG (for nNOS) or anti-mouse (for iNOS and eNOS) secondary antibodies (at 1:20,000 in 1.5% skimmed milk TBST) followed by chemiluminescence detection (Millipore, USA). β-actin was used to normalize the NOS expression, which was assessed in the protein sample at 1:25 dilution. It was done to avoid the dispersion of the band which otherwise would hinder in densitometry analysis. NOS immunoprecipitation was performed in the undiluted sample.

2.6. NO generation by flow cytometry

NO production in human PBMCs, monocytes and lymphocytes was assessed in the presence or absence of NOS cofactors (BH_4, 10 μM, NADPH 1 mM, FAD 5 μM, FMN 25 μM, L-Arg 0.15 μM, ascorbate 1 mM) and NOS inhibitor (L-NAME 10 μM), using DAF-2DA (10 μM), a NO probe by flow cytometry [30]. DAF, yields a green fluorescent triazole (DAF-2T) following binding to NO, offering the advantage of specificity

and sensitivity in NO detection. 10,000 cells from each sample was acquired using a flow cytometer (FACS Calibur, Becton Dickinson, USA) and analyzed by Cell Quest program. Cells without DAF-2DA treatment were used as negative control, and NO generation potential was assessed by comparing the cells in the presence and absence of L-NAME.

2.7. NOS catalytic activity in PBMCs

PBMCs suspended in incubation buffer (Hepes 25 mM; NaCl 140 mM; KCl 5.4 mM; $MgCl_2$ 1 mM; pH 7.4) were sonicated and incubated with tetrahydrobiopterin (BH_4) (100 μM), NADPH (1 mM), FAD (5 μM), FMN (25 μM), and calmodulin (10 μg/ml) in the presence of $CaCl_2$ (2 mM) or EGTA (5 mM). Reaction was initiated by adding L-[^{3}H] arginine, continued for 30 min at 37 °C and was stopped by adding ice-cold stop buffer (NaCl, 118 mM; KCl, 4.7 mM; KH_2PO_4, 1.18 mM; $NaHCO_3$, 1 mM; EDTA, 4 mM; N^{ω}-Nitro-L-arginine methyl ester (L-NAME), 5 mM; pH 5.5). The reaction mixture was passed through Dowex 50WX8-400 columns. Radioactivity in the eluent was measured using β-scintillation counter (LKB Wallace 1409, Liquid Scintillation counter, USA) as described elsewhere [23]. Calcium independent activity was evaluated in the presence of EGTA (5 mM). Calcium dependent NOS catalysis was evaluated in the presence of $CaCl_2$ (2 mM). NOS catalysis has been reported as pmol of L-[^{3}H] citrulline formed/30 min/10^7 cells.

2.8. Immunogold electron microscopy

Buffy coat obtained after centrifugation of human blood at 800 *g* for 20 min at 25 °C (3 K30 Sigma Centrifuge, Germany), was fixed overnight at 25 °C with 2% (w/v) paraformaldehyde and 0.5% (v/v) glutaraldehyde in PBS (pH 7.4) at 25 °C. Small pieces of buffy coat washed with PBS containing 0.5% (w/v) glycine to quench excess fixative were dehydrated in an ascending series of ethanol (30% for 30 min, 50% for 30 min, 70% for 30 min, 90% for 1 h, absolute alcohol for 1 h), impregnated in London–White resin, and polymerized at 60 °C for 48 h. Ultrathin sections (80–100 nm) collected on nickel grids were blocked in PBS containing 0.1% BSA (w/v) and 1% (v/v) Teleost fish gelatin for 30 min at room temperature and incubated overnight with or without (for negative control) nNOS, iNOS or eNOS antibodies at 1:200 dilution in blocking buffer at 4 °C, washed 10 times with blocking buffer and subsequently, sections were incubated with 10 or 15 nm gold-coupled goat anti-mouse or goat anti rabbit IgG (1:20) for 2 h at 37 °C. Grids were further washed with blocking buffer and then 10 times with water. The grids were stained with 1% uranyl acetate. After washing 10 times with water grids were examined under TEM at 80 kV [23].

2.9. Morphometric analysis

The density of the gold particles in the PBMCs (both lymphocyte and monocyte) was quantitatively evaluated after immunogold labeling with nNOS, iNOS or eNOS antibodies and subsequently with a secondary antibody coupled to 10 or 15 nm gold particles. The total 6 grids from three independent experiments for NOS labeling were used for this analysis. Electron micrographs were captured at 11,000× magnification for the morphometric analysis as has been previously described [23,25]. Morphometric analysis with lymphocytes and monocytes was performed only on those cells exhibiting nucleus in the plane of the section and the relative distribution was ascertained by counting the total number of gold particles in the nucleus, cytoplasm and plasma membrane for both cNOS (nNOS and eNOS) and iNOS in the lymphocytes and monocytes. The labeling density of nNOS, iNOS and eNOS (number of gold particles/μm^2) was calculated using the Scion Image processing software 4.0.3.2 and expressed for different subcellular compartments in monocytes and lymphocytes.

2.10. Statistical analysis

Results have been expressed as mean ± SEM. Statistical analysis between two groups was performed using unpaired 't' test and among multiple groups by one-way ANOVA followed by Newman–Keul's post analysis test. Results were considered significant at $p<0.05$.

3. Results

3.1. NOS expression, and NO generation in human PBMCs, monocytes and lymphocytes

Transcripts for NOS isoforms in the PBMCs, monocytes and lymphocytes were identified by real time PCR which demonstrated constitutive expression of all the three NOS isoforms (nNOS, iNOS and eNOS) in these cells isolated from healthy individuals. Quantitative analysis showed significantly higher expression of iNOS transcript as compared to nNOS ($p<0.001$) and eNOS ($p<0.01$) in PBMCs (Fig. 1A). Relative copy number of eNOS was significantly more as compared to nNOS ($p<0.01$) and iNOS ($p<0.001$) in the resting monocytes (Fig. 1D). However, relative copy number of iNOS was significantly higher than nNOS ($p<0.001$) in resting lymphocytes (Fig. 1G). These results clearly indicate constitutive expression of cNOS (nNOS and eNOS) and iNOS in human PBMCs, monocytes and lymphocytes. Level of NOS was found to be 10^6 folds lesser than β-actin as measured by real time PCR. Hence, the normalized values were presented in multiples of 10^6.

Expression of NOS isoforms at protein level was detected by immunoprecipitation followed by Western blotting. The presence of nNOS (160 kDa), iNOS (130 kDa) and eNOS (130 kDa) has been shown in PBMCs (Fig. 1B), monocytes (Fig. 1E) and lymphocytes (Fig. 1H). Densitometric analysis showed that iNOS expression was more than nNOS ($p<0.05$) and eNOS ($p<0.05$) (Fig. 1B), which correlated with the relative abundance of iNOS transcript over that of nNOS and eNOS as was observed with real time PCR in PBMCs. In monocytes eNOS expression was more than that of nNOS ($p<0.05$) (Fig. 1E). However iNOS expression was higher than nNOS ($p<0.01$) in lymphocytes (Fig. 1F). Band intensity of NOS and β-actin were compared by using 25 fold diluted protein for β-actin than NOS. Hence, the normalized values were presented as multiple 1/25.

NO generation in PBMCs, monocytes and lymphocytes was measured by using DAF-2DA, a NO reactive and sensitive fluorescent probe, exhibiting NO formation in the presence of substrate and cofactors, which was significantly inhibited by L-NAME, the NOS inhibitor (Figs. 1C, F and I). NOS functionality, was also confirmed by using yet another method in total PBMC lysate, in the presence/absence of calcium, NOS substrate L-^{3}H-arginine and cofactors, so as to assess the relative contribution of calcium independent (5 mM EGTA) and calcium dependent NOS (2 mM $CaCl_2$) activity by monitoring the formation of L^{3}H-citrulline. Despite a relative abundance of iNOS at transcript and protein levels, both calcium dependent and independent isoforms were equally active in PBMCs the activity being 0.22 ± 0.08 pmol/30 min/10^7 cells and 0.20 ± 0.003 pmol/30 min/10^7 cells, respectively. These results thus demonstrate that NOS isoform specific transcripts were actively and constitutively translated into NOS proteins and were functional in the resting PBMCs, monocytes and lymphocytes.

3.2. NOS subcellular localization by electron microscopy

Monocytes and lymphocytes were distinguished by their nuclear morphology. Monocytes were identified on the basis of kidney/horse shoe shaped nuclei as described by Sambuy et al. [31] and Sandborn [32]. Lymphocytes were readily identifiable in the electron microscope on the basis of large, round nuclei as reported by Tokuyasu et al. [33]. NOS localization in PBMCs (lymphocytes and monocytes) was

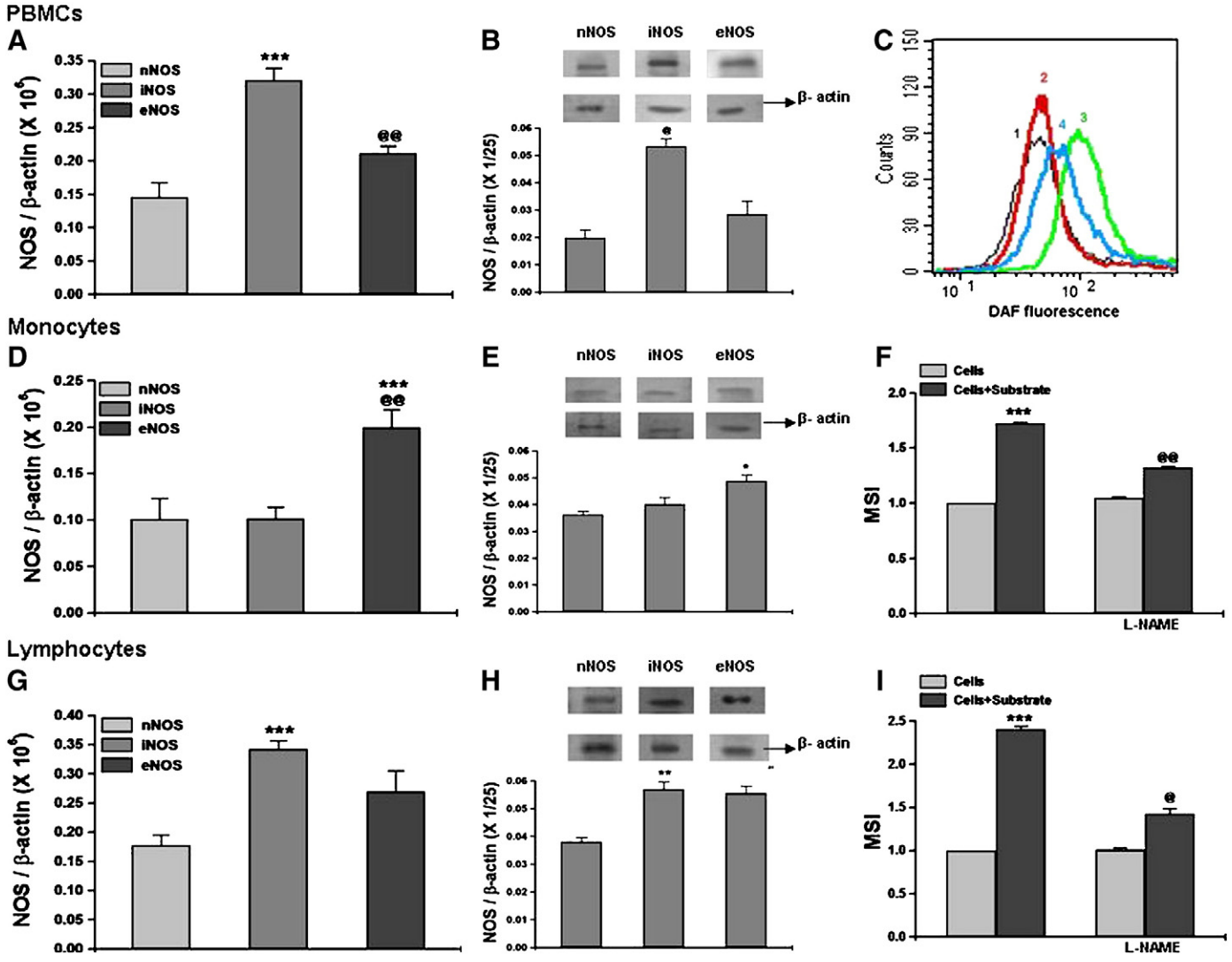

Fig. 1. Expression of NOS isoforms and NO generation in human PBMCs, monocytes and lymphocytes. Bar diagrams in the left panel represent the real time PCR based relative copy number of nNOS, iNOS and eNOS as the ratio of respective NOS isoforms with housekeeping gene β-actin as a multiple of 10^6 in PBMCs (A) monocytes (D) and lymphocytes (G). The data is a representation of 3 independent experiments, and have been expressed as Mean ± SEM (***$p<0.001$ for iNOS Vs nNOS, @@$p<0.01$ for iNOS Vs eNOS in resting PBMCs, @@$p<0.01$ for eNOS Vs nNOS, ***$p<0.001$ for eNOS Vs iNOS in monocytes and ***$p<0.001$ for iNOS Vs nNOS in lymphocytes). Protein content of nNOS, iNOS and eNOS following immunoprecipitation/Western blot in PBMCs (B), monocytes (E) and lymphocytes lysates (H) has been shown in the middle panel. Band 1—nNOS protein at 160 kDa, band 2—iNOS protein at 130 kDa, and band 3—eNOS protein at 130 kDa. Densitometric analysis of nNOS, iNOS and eNOS protein expression in PBMCs, monocytes and lymphocytes as the ratio of respective NOS isoforms with β actin as a multiple of 1/25. The data is a representation of 3 independent experiments, and presented as Mean ± SEM (*$p<0.05$ for iNOS Vs nNOS, @$p<0.05$ for iNOS Vs eNOS in PBMCs, *$p<0.05$ for eNOS Vs nNOS, in resting monocytes and **$p<0.01$ for iNOS Vs nNOS, in resting lymphocytes). Right panel represents (C) histogram overlay representing NO generation (DAF-2DA) in PBMCs (1—cells, 2—L-NAME + cells, 3—cells with cofactors and substrate and 4—L-NAME + cells with cofactors and substrate). Bar diagram representing the mean stimulation index (MSI) of NO generation (DAF-2DA) in monocytes (F) and lymphocytes (I). The data is a representation of 3 independent experiments, and are presented as Mean ± SEM (***$p<0.001$ for cells vs cells with cofactors and substrate, @@$p<0.01$ for cells with cofactors and substrate vs L-NAME + cells with cofactors and substrate in resting monocytes and ***$p<0.001$ for cells vs cells with cofactors and substrate, @$p<0.05$ for cells with cofactors and substrate vs L-NAME + cells with cofactors and substrate in resting lymphocytes).

assessed only in those cells which were distinctly identifiable on the basis of the above mentioned criteria. Experiments were conducted with specific nNOS, iNOS or eNOS antibodies. Moreover under the same condition used, no significant binding was observed with isotype matched control antibodies or secondary antibody alone. The sparse binding with the secondary antibody has even been subtracted, while performing morphometric analysis to assess specific NOS antibody binding.

3.2.1. nNOS, iNOS and eNOS localization in monocytes

Figs. 2A and B demonstrate nNOS immunolabeling in the cytoplasm, nucleus and nuclear membrane as well as at the sites adjacent to the plasma membrane. The presence of nNOS as dimers and monomers were seen in the nucleus which could be suggestive of catalytically active NOS configuration (Fig. 2A). The presence of iNOS and eNOS in the cytoplasm, nucleus and nuclear membrane was also evident (Figs. 2C, D, and E, F). We also analyzed the labeling densities of nNOS, iNOS and eNOS (number of gold particle/μm^2) in the different sub-cellular compartments by counting in 20 representative cells (Table 1). Morphometric analysis revealed that the number of gold particles/μm^2 in a human monocyte representing nNOS, iNOS and eNOS were 2.8 ± 0.2, 3.5 ± 0.4 and 5.4 ± 0.2 respectively. The amount of nNOS labeling in the nucleus (1.8 ± 0.1) was less as compared to the cytoplasm (4.2 ± 0.3, $p<0.001$), indicating that most of nNOS was confined to the cytoplasm, while iNOS and eNOS seems to be equally distributed in the nucleus and cytoplasm.

3.2.2. nNOS, iNOS and eNOS localization in lymphocytes

NOS intracellular distribution studies in the lymphocyte exhibited the presence of both constitutive (nNOS and eNOS) and inducible NOS

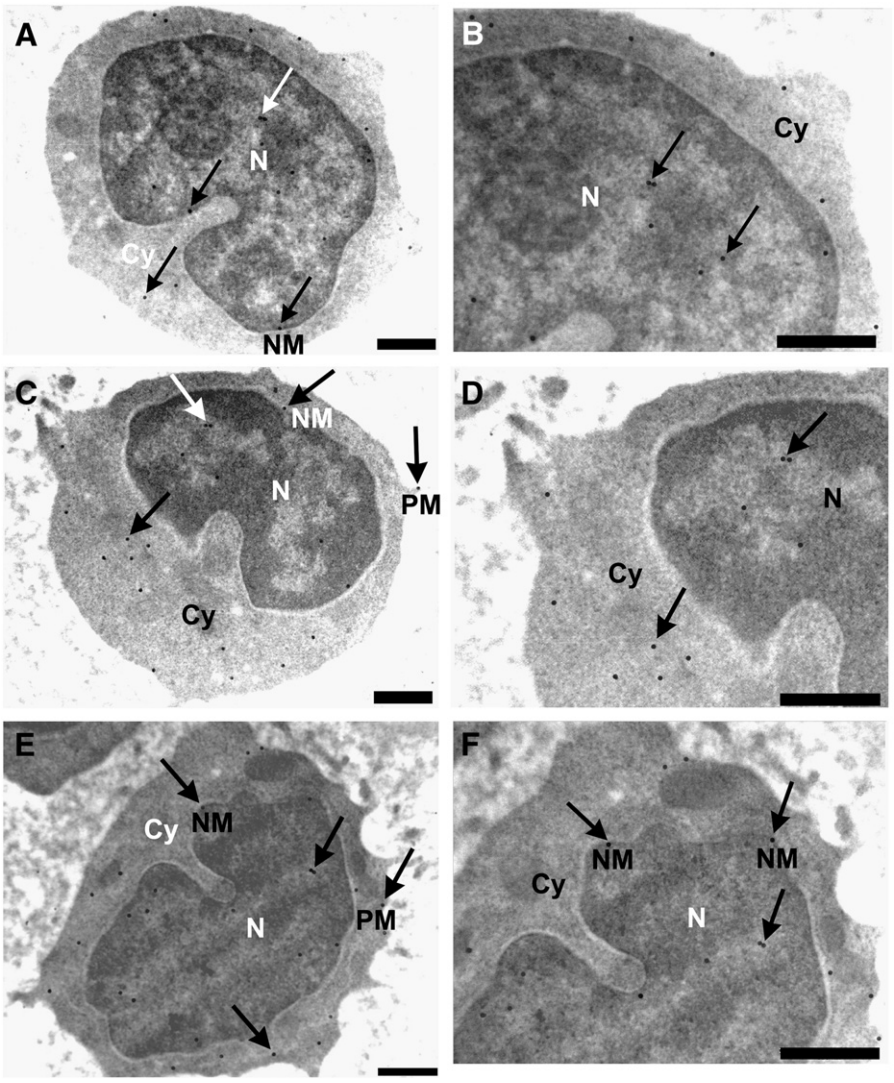

Fig. 2. nNOS, iNOS and eNOS intracellular distribution in human monocytes. Distribution of NOS isoforms in human monocytes. (A) nNOS labeling in the subcellular compartments: (i) nucleus (N), (ii) nuclear membrane (NM) and (iii) cytoplasm (Cy). (B) Magnified view of (A) indicating prominent nNOS labeling seen in the nucleus (N) and cytoplasm (Cy) as shown by arrows. (C) iNOS distribution in nucleus (N), cytoplasm (Cy), nuclear membrane (NM) and plasma membrane (PM). (D) Magnified view of (C) indicating iNOS labeling in nucleus and cytoplasm. (E) eNOS labeling in the nucleus (N), cytoplasm (Cy), nuclear membrane (NM) and plasma membrane (PM) of human monocyte. (F) Magnified view of (E) indicating prominent labeling of eNOS in the nucleus and cytoplasm. Bars represent 500 nm (A–F). The image is a representation of at least 3 independent experiments.

isoforms (iNOS) in the nucleus, nuclear membrane, cytoplasm and plasma membrane. nNOS was mostly localized in the cytoplasm, nucleus and adjacent to plasma membrane (Figs. 3A and B). The presence of iNOS monomers and dimers was seen clearly in the cytoplasm, nucleus, nuclear membrane and along with the plasma membrane (Figs. 3C, D, E and F). Morphometric analysis revealed that the amount of eNOS labeling represented as gold particles/μm^2 was 3.6 ± 0.3 ($n = 20$), which was more than nNOS (2.6 ± 0.3, $p < 0.05$) but was similar to iNOS (3.1 ± 0.3) in lymphocytes. Moreover, eNOS seemed to be equally distributed in nucleus (4.1 ± 0.4) and cytoplasm (4.2 ± 0.3), while nNOS and iNOS were much more in the cytoplasm than the nucleus (Table 2).

Table 1
Labeling densities of NOS isoform (number of gold particles/μm^2) in the subcellular compartments of human monocytes.

Subcellular compartment	Number of gold particles/μm^2in monocytes		
	nNOS	iNOS	eNOS
Nucleus	1.8 ± 0.1	3.0 ± 0.5	5.0 ± 0.4[a,b]
Cytoplasm	4.2 ± 0.3[c]	4.1 ± 0.5	6.2 ± 0.4[d,e]
Total	2.8 ± 0.2[f]	3.5 ± 0.4[g]	5.4 ± 0.2

Values are Mean ± SEM. Data were analyzed by one-way ANOVA followed by Newman–Keul's post analysis test.
[a] $p < 0.001$ for nNOS in nucleus Vs eNOS in monocyte nucleus.
[b] $p < 0.001$ for iNOS in nucleus Vs eNOS in monocyte nucleus.
[c] $p < 0.001$ for nNOS in nucleus Vs nNOS in cytoplasm.
[d] $p < 0.001$ for eNOS in cytoplasm Vs nNOS in cytoplasm.
[e] $p < 0.001$ for eNOS in monocyte cytoplasm Vs iNOS in cytoplasm.
[f] $p < 0.001$ for eNOS Vs nNOS.
[g] $P < 0.01$ for eNOS Vs iNOS.

3.2.3. Distribution profile of NOS isoforms in the monocytes and lymphocytes

Morphometric analysis exhibited the subtle differences in the pattern of NOS distribution in the monocyte and lymphocyte populations. Labeling of nNOS in lymphocytes and monocytes was comparable, however nNOS labeling was significantly more in the cytoplasm as compared to the monocyte and lymphocyte nuclei (Figs. 4A and B). iNOS labeling was also almost similar in monocytes (3.5 ± 0.4) and lymphocytes (3.1 ± 0.3). Distribution of iNOS in the cytoplasm (4.1 ± 0.5) and nucleus (3.0 ± 0.5) was comparable (Fig. 4A) in monocytes, while iNOS labeling in the cytoplasm (4.5 ± 0.4) was

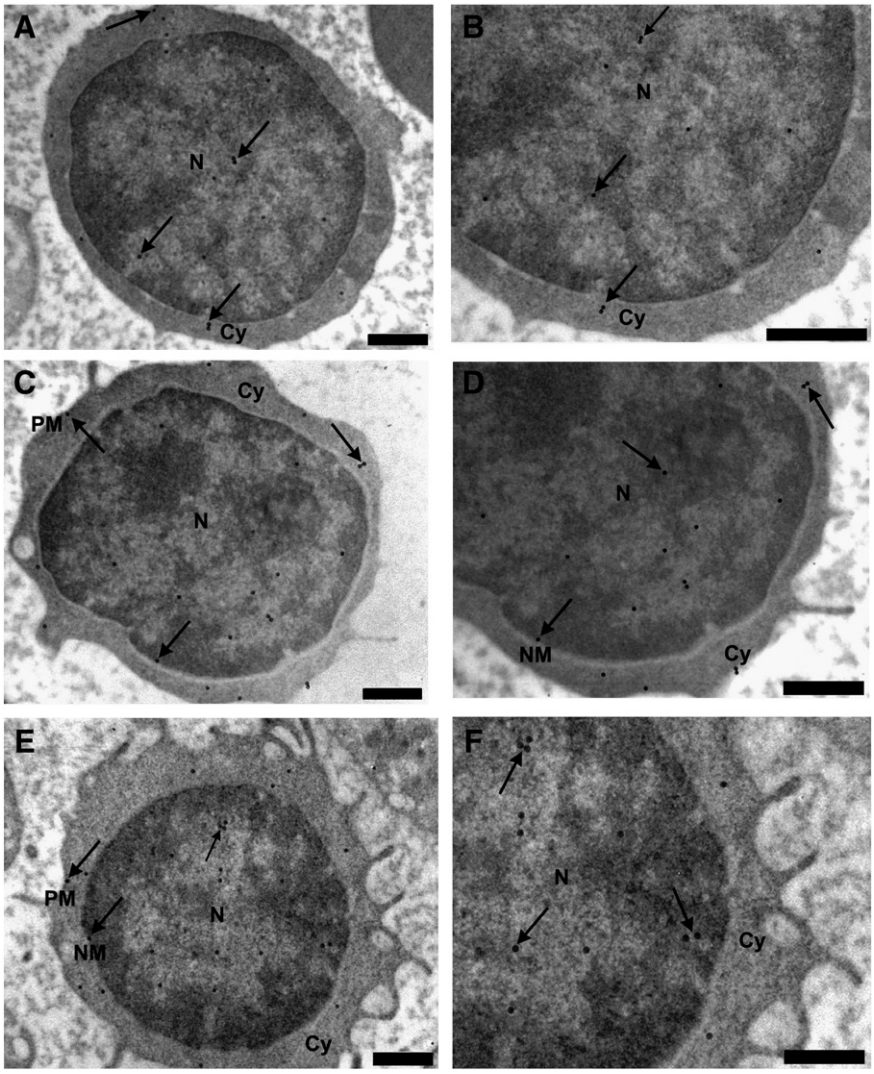

Fig. 3. nNOS, iNOS and eNOS intracellular distribution in human lymphocytes. Distribution of NOS isoforms in human lymphocytes. (A) Presence of nNOS protein at different sub-cellular compartments: (i) nucleus (N) and (ii) cytoplasm (Cy). (B) Magnified view of (A) indicating the presence of nNOS as a dimer in the nucleus and cytoplasm. (C) Sub-cellular distribution of iNOS in a human lymphocyte: (i) the presence of iNOS in nucleus, (ii) cytoplasm and (iii) plasma membrane. (D) Magnified view of (C) indicating the presence of iNOS at the nuclear membrane. (E) Sub-cellular localization of eNOS in (i) the nucleus, (ii) cytoplasm and (iii) plasma membrane. (F) Magnified view of (E) indicating the presence of eNOS at the nucleus and cytoplasm. Bar represent 500 nm (A–F). The image is a representation of at least 3 independent experiments.

more than the nucleus (2.0 ± 0.3, $p<0.001$) among lymphocytes (Fig. 4B). The presence of eNOS was significantly more in monocytes (5.4 ± 0.2) as compared to lymphocytes (3.6 ± 0.3, $p<0.05$).

Table 2
Labeling densities of nNOS, iNOS and eNOS (number of gold particles/μm^2) in the subcellular compartments of human lymphocytes.

Subcellular compartment	Number of gold particles/μm^2 in lymphocytes		
	nNOS	iNOS	eNOS
Nucleus	1.6 ± 0.1	2.0 ± 0.3	4.1 ± 0.4[a,b]
Cytoplasm	4.6 ± 0.9[c]	4.5 ± 0.4[d]	4.2 ± 0.3
Total	2.6 ± 0.3[e]	3.1 ± 0.3	3.6 ± 0.3

Values are Mean ± SEM. Data were analyzed by one-way ANOVA followed by Newman–Keul's post analysis test.
[a] $p<0.001$ for iNOS in nucleus Vs eNOS in lymphocyte nucleus.
[b] $p<0.001$ for nNOS in nucleus Vs eNOS in lymphocyte nucleus.
[c] $p<0.001$ for nNOS in nucleus Vs nNOS in cytoplasm.
[d] $p<0.001$ for iNOS in nucleus Vs iNOS in cytoplasm.
[e] $p<0.05$ for total eNOS Vs total nNOS in lymphocytes.

4. Discussion

PBMCs constitute an important component of both vascular and immune systems. NO essentially influences both the systems, a detailed insight into the status of NOS might thus be useful to understand diverse pathologies involving PBMCs. NO release from human PBMCs was first demonstrated by Salvemini et al. [10], which was subsequently identified as a mediator of their microbicidal and tumoricidal actions, as well as a regulator (enhancer and suppressor) of T-cell functions [34].

Real time PCR analysis and immunoprecipitation studies indicated constitutive expression of cNOS and iNOS in PBMCs, monocytes and lymphocytes. Higher expression of iNOS was found in PBMCs as compared to eNOS and nNOS (Figs. 1A and B), while monocytes and lymphocytes expressed more amount of eNOS and iNOS respectively (Figs. 1D, E and G, H). Number of lymphocytes in PBMCs is much more than monocytes, we therefore detected higher expression of iNOS in total PBMCs. Existing reports in the literature demonstrate the presence of iNOS and cNOS transcripts in monocytes obtained from severe acute pancreatitis patients complicated with systemic inflammatory response syndrome [36]. Moreover an inverse relationship

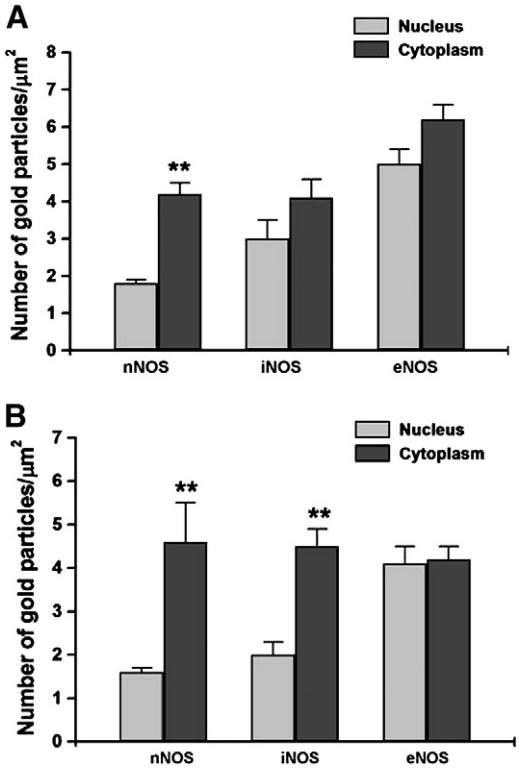

Fig. 4. Morphometric analysis for NOS intracellular distribution, represented as bar diagrams. NOS sub cellular distribution has been shown in the nucleus and cytoplasm of (A) monocytes and (B) lymphocytes. Results have been expressed as Mean ± SEM of the number of gold particles/μm^2 (**$p<0.001$). The data is a representation of at least 3 independent experiments.

was observed between the expression of iNOS and cNOS in monocytes, macrophages and monocytic cell lines upon stimulation with LPS/IFN-γ [37]. Upregulation of iNOS in monocytes from pulmonary tuberculosis patients have also been reported [38]. Germinal center T cells and follicular dendritic cells did not express eNOS mRNA. The presence of eNOS protein was however confirmed in T cells from peripheral blood and in various germinal center cells of frozen tonsillar sections [9]. Thyroid hormones upregulate iNOS gene expression downstream to PKC-zeta in murine tumor T cells [39]. T cell activation induces formation of reactive oxygen intermediates and Ca^{+2}-dependent NO production [35]. Understanding of the differential regulation of NOS expression, catalysis and intracellular localization in PBMCs might be helpful to evolve strategies against graft versus host responses following transplantation, tumor surveillance, inflammation and infectious diseases. The present multiparametric study is the first step in this direction, which demonstrated relative prevalence of NOS isoforms at the transcript and protein levels by real time PCR and immunoprecipitation respectively, NO generation capabilities and NOS sub-cellular distribution in PBMCs, monocytes and lymphocytes. Results obtained by real time PCR and IP/WB indicated the differences in NOS ratio to β-actin (10^6 folds in the case of real time PCR and 25 folds in IP/WB). This could be due to the differences in the sensitivity of the methods used, mRNA stability or protein degradation, which needs to be ascertained in PBMC subsets under physiological and pathological conditions.

NO derived from NOS in human mast cells regulate leukotriene production [14]. Yip et al. [40] have demonstrated the inducible expression of eNOS and iNOS in human mast cells following TNFα, IFNγ and IL1β stimulation. Role of NOS in regulating deformability of human RBC membrane and inhibition of platelet activation has also been proposed [41]. Such studies might be helpful in ascertaining the importance of NO/NOS in PBMCs.

The relative catalytic potential of the calcium dependent and independent NOS in PBMC lysates was almost similar, that was contrary to the relative abundance of iNOS mRNA and protein (Figs. 1A and B). This discrepancy could arise from an inhibitory regulation imposed over the avid production of NO from iNOS despite its constitutive presence. Inhibitory protein–protein interactions with iNOS involving caveolin [23], kalirin, or PIN might exert an inhibitory effect on iNOS activity, which remains to be ascertained in PBMCs. Speculatively iNOS in PBMCs might have non-catalytic roles such as trafficking of cargo proteins to intracellular locales as is demonstrated for nNOS [42] or it might be sequestered in the perinuclear aggresomes [43]. A subset of human iNOS could be sensitive to oscillations in the intracellular calcium reserve thus contributing to both calcium dependent and independent catalysis as has been shown in guinea pigs [44], which warrants further investigations. It has been shown in T lymphocytes that NO synthesis can be triggered by acetylcholine mediated augmentation in $[Ca^{+2}]_i$ dependent NOS activity [45]. It is thus worthwhile to explore the physiological regulators of NOS catalysis governing the extent of NO generation from the calcium dependent and independent isoforms in PBMC subsets.

NOS catalysis could also be influenced by its distinct subcellular localizations, which vary in terms of pH, immediate microenvironment, presence of negative and positive protein regulators, substrate and cofactors availability. Controlled NO generation at the subcellular locales and organelles might be of importance in terms of adequate amount of NO generation at the right place so as to play a pivotal role in modulating organelle specific functions. Biological actions of NO, either beneficial or detrimental are largely concentration dependent and transient due to shorter half life of NO. Characteristically NO liberated from iNOS quickly attains critical concentration and has been implemented in the cytotoxic actions [46]. nNOS and eNOS however generate a low amount of NO that gradually builds up inside the organelles. NOS could also have non-catalytic attributes in subcellular locales which is a vastly unchartered area of research. The present study explored the relative nucleo-cytoplasmic distribution of NOS isoforms in resting human PBMCs by immunoelectron microscopy. The technique used is not considered suitable to assess the quantitative status of protein expression due to exhaustive and time taking steps involved in the sample preparation, which might lead to protein degradation, on the other hand it is also understandable that the distribution of protein might not be similar at all the planes in cell sections. This may partly account for the lack of correlation between relative abundance of iNOS as seen at transcript and protein levels with morphometry data in the lymphocytes. NOS was present in the cytoplasm, nucleus, nuclear membrane and plasma membrane in monocytes and lymphocytes (Figs. 2A–F and Figs. 3A–F). Most of the NOS isoforms were confined to cytoplasm but considerable labeling was also evident in the nucleus of monocytes and lymphocytes. Functional importance of the sub-cellular localization of NOS isoforms however remains to be identified in PBMCs. Morphometric analysis revealed that eNOS was more than nNOS and iNOS in the monocytes. The subtle differences seen in the NOS distribution might be important for the cell/organelle specific functions being regulated by the NO availability and remain to be explored. NOS isoforms interact with a wide array of scaffolding proteins, positive and negative catalytic regulators, signaling intermediates to membrane receptors which might affect both its trafficking and NO generation potential [4]. nNOS having PDZ domain interacts with several other PDZ domain containing proteins. Western blot analysis with antibodies specifically directed against the N-terminal region of nNOS which bears the PDZ domain encoded by exon 2, revealed a full length nNOS protein of 160 kDa in the PBMCs (Fig. 1B). iNOS and eNOS in PBMCs were also full length proteins of 130 kDa. Based on our observations, future endeavors need to be directed to assess the relative expression of NOS isoforms, their subcellular localization, trafficking, nitration and nitrosylation of important proteins in

different pathological conditions where PBMCs play an active role in disease onset and progression.

The present study convincingly demonstrates the presence of functional NOS isoforms, their expression and the differential pattern of subcellular distribution in human PBMCs, monocytes and lymphocytes. The information furnished herein is likely to serve as a template for the future studies directed towards understanding NOS and NO mediated signaling events in various hereditary and acquired immune/inflammatory disorders associated with distinct subpopulations of PBMCs.

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