Modulation of Rat Peripheral Polymorphonuclear Leukocyte Response by Nitric Oxide and Arginine

By P. Seth, R. Kumari, M. Dikshit, and R.C. Srimal

The effect of nitric oxide (NO) on the luminol-dependent chemiluminescence (LCL) response of rat polymorphonuclear leukocytes (PMNLs) was analyzed by using sodium nitroprusside (SNP), a NO donor, and L-arginine (L-arg), a NO precursor. A significant reduction in the LCL intensity was observed in presence of SNP (100 μmol/L) or L-arg (5 or 10 mmol/L) in arachidonic acid (AA) phorbol ester (PMA) and formyl-methionyl-leucyl-phenylalanine stimulated PMNLs. However, opsonized zymosan-induced LCL was not attenuated significantly. Reduction in hydroxyl radical and superoxide generation was also observed in SNP- or L-arg-pretreated cells. D-Arg (10 mmol/L) pretreatment did not inhibit PMNLs' LCL response. Furthermore, methylene blue (5 μmol/L) and L-N^G-mono methyl-L-arginine (100 or 300 μmol/L) significantly attenuated the LCL response, as induced by various agonists. Cyclic GMP did not alter the reactive oxygen species generation from rat PMNLs. In addition, AA-induced release of myeloperoxidase, a marker of azurophilic granules, was found to be enhanced in L-arg- (10 mmol/L) pretreated PMNLs. The results suggest that NO inhibits free radical generation from rat PMNLs.

NITRIC OXIDE (NO) is known to serve a variety of physiologic functions such as mediating endothelial-derived relaxation of vascular smooth muscle,[1,2] inhibiting platelet aggregation and adhesion,[3] acting as a neuronal messenger,[4] and acting as a cytotoxic moiety in macrophage.[5,6] L-arginine (L-arg) is converted into NO and L-citrulline through the enzyme nitric oxide synthase (NOS). This is of two types, one is calcium-dependent and the other is calcium-independent.[3] Polymorphonuclear leukocytes (PMNLs) and macrophages can synthesize NO from L-arg in presence of calcium-independent NOS.

PMNLs and macrophage, when exposed to soluble or particulate stimuli, generate reactive oxygen species.[7] Superoxide radical and NO can react together to generate peroxynitrite, a relatively long-lasting strong oxidant, and hydroxyl radicals.[8,9] In mice and rats, NOS activity in macrophage can be induced by immunologic stimuli and their cytotoxic potential has been found to be proportional to their nitrite-secreting capacity.[10,11] However, human macrophage (which possesses well-developed antitumor and microbicidal capacities) immunomodulator-mediated alteration of NO synthesis is controversial.[12,13] Similarly, the role of NO in PMNLs is also not well defined. Exogenous addition of NO to human neutrophils in vitro has been found to inhibit superoxide radical production.[14] On the other hand, Rubanyi et al[15] have reported that NO inactivates the superoxide radical by scavenging and have suggested that NO released from human PMNLs performs cytoprotective function. The role of NO as a microbicidal in cytoplasts derived from human PMNLs has been demonstrated by Malawista et al.[16] Involvement of NO has also been proposed in PMNLs chemotaxis,[17,18] aggregation,[19] PMNL-mediated tissue damage,[20] and adhesion to endothelium.[21]

We have earlier demonstrated that circulating PMNLs of rat release NO that was potentiated after thrombosis,[22] whereas Wang et al[23] reported that PMNLs do not release NO and, by adding extracellular arginine, there was no synthesis of peroxynitrite radicals. The role of PMNL-derived NO in their functions is thus still not clear.

The present investigation was therefore undertaken to study the interaction of NO/L-arg pathway and the reactive oxygen species in rat peripheral leukocytes that release NO as well as superoxide radicals, to understand more clearly the involvement of NO in thrombosis.

MATERIALS AND METHODS

Chemicals. Arachidonic acid (AA), ferricytochrome C (Cyt C), superoxide dismutase (SOD), L-arg, D-arginine (D-arg), 2-deoxy-D-ribose, dextran-500, N^G-monomethyl-L-arginine (L-NMMA), N-nitro-L-arginine methyl ester (L-NAME), luminol, hemoglobin (Hb), nitroblue tetrazolium (NBT), phenazine methosulfate (PMS), methylene blue (MB), phorbol myristate-13-acetate (PMA), formyl-methionyl-leucyl-phenylalanine (FMLP), 8-bromo cyclic-GMP (cGMP), thiobarbituric acid (TBA), zymosan particles, micrococcus luteus, and O-dianisidine and N-(1-naphthyl)-ethylenediamine dihydrochloride were purchased from Sigma Chemical Co (St Louis, MO). Leukocyte-separating medium (Organon Tenika Corp, Durham, NC) and all other chemicals used in the present study were of analytical grade (SRL, Bombay, India).

Preparation of the reagents. Stock solution of FMLP (100 μmol/L), luminol (100 mmol/L), and PMA (1 mol/L) were made in dimethyl sulphoxide (DMSO) and were stored at −70°C in aliquot.

Opsonized zymosan (OZ) was prepared by treating the washed zymosan particles with fresh autologous serum (rat) for 30 minutes at 37°C, followed by removal of the serum by centrifugation. The particles were resuspended in Hanks' Balanced Salt Solution (HBSS) and a stock solution of 12.5 mg/mL was stored in aliquot at −70°C.

PMNLs isolation. Male Sprague-Dawley albino rats (130 to 150 g) were obtained from CDRI (Lucknow, India) animal house colony. Rat polymorphonuclear leukocytes were prepared by the method of Boyum.[24] Blood was collected in sodium citrate (0.129 mol/L, pH 6.5, 9:1) by cardiac puncture under ether anaesthesia. Platelet-rich plasma was removed by centrifugation at 150*g* for 20 minutes, and the buffy coat was subjected to dextran sedimentation as described in detail previously.[22] In some experiments on luminol chemiluminescence response, PMNLs further purified by leukocyte-separating media were also used. Cells obtained were suspended in HBSS and were counted. The viability of the cells was tested by the trypan blue exclusion test in some experiments and was never less than 95%. PMNLs were stored at 4°C until the time of experimentation, which never exceeded 2 to 3 hours.

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Measurement of luminol-dependent chemiluminescence (LCL). Free radical generation from PMNLs, stimulated with various agonists such as AA (5×10^{-5} mol/L), FMLP (1 μmol/L), OZ (1.25 μg), and PMA (3×10^{-8} mol/L), was measured, at 37°C with constant stirring at 900 rpm, using a dual-channel Lumiaggregometer (Model 560; Chronolog Corp, Havertown, PA). Free radical generation has been reported as LCL units, which have been defined as maximum output (obtained from stimulated PMNLs) divided by the "gain setting" of the instrument at the time of response. Assay mixture (1000 μL) contained 1 to 5×10^6 PMNLs, 10 μmol/L luminol, the test substance, and the agonist.

Superoxide anion generation. O_2^- generation was monitored by the SOD-inhibitable reduction of cytochrome C.[25] PMNLs were incubated in the presence or absence of L-arg along with 60 μmol/L of Cyt C at 37°C for 5 minutes. Control was run in presence of SOD (30 U/mL). The production of superoxide radicals was initiated by AA (2.5×10^{-4} mol/L) and followed for 20 to 30 minutes at 550 nm at 37°C.

Nonenzymatic generation of superoxide radical. The test substances were preincubated with 10 μmol/L phenazine methosulphate (PMS) and 25 μmol/L nitroblue tetrazolium (NBT) in phosphate buffer (0.1 mol/L, pH 7.4) for 5 minutes at room temperature. The reaction was started by adding 78 μmol/L NADH and the absorbance of the reaction mixture was read at 560 nm against the blank without PMS for 2 minutes at room temperature.[26]

Quantitation of malonaldehyde (MDA). Levels of MDA were measured in the PMNLs (1×10^7 cells) with or without L-arg (10 mmol/L), after 1 hour of incubation at 37°C with the inducers used for LCL response, as TBA-reactive material.[27]

Estimation of hydroxyl radical. The estimation was performed by the method of Greenwald et al.[28] Reaction mixture contained 10 mmol/L deoxyribose, 0.1 mmol/L Fe(III)-EDTA, 5×10^6 cells, and PMNL's agonists (equivalent concentrations used for chemiluminescence). Blanks without the agonists were also run. Degradation of the deoxyribose was measured at 532 nm after development of color with TBA and trichloroacetic acid (TCA).

Nitrite measurement. Nitrite content of the suspending medium of the PMNLs was measured by diazo formation according to Bennett et al.[29] PMNLs (1×10^7 cells) were suspended in HBSS and incubated in a shaking water bath at 37°C for 60 minutes with or without L-arg, PMA, AA, FMLP, or OZ (in the equivalent concentrations used for obtaining the chemiluminescence response). Supernatant was collected after centrifugation and was kept on ice. Supernatant (500 μL) was mixed with 500 μL water and 500 μL 1% (wt/vol) sulfanilic acid in 2 N HCl. After 5 minutes, 500 μL of 1% (wt/vol) aqueous N-(1-naphthyl)-ethylenediamine dihydrochloride was added and the absorbance was measured at 548 nm. A sodium nitrite standard curve was prepared with each experiment.

Determination of myeloperoxidase and lysozyme activities. Release of the granular enzymes was measured in the PMNLs suspending medium with and without L-arg. Myeloperoxidase[30] and lysozyme[31] were measured according to standard procedures in the supernatant after PMNLs were stimulated with various secretogogues and incubated for 15 minutes at 37°C. Total enzyme activity was measured after PMNLs were lysed by sonication (Ultrasonics, Farmingdale, NY). Release of the cytoplasmic enzyme lactate dehydrogenase[32] was used as a marker of cell viability and was not more than 6% in any experiment.

Statistical analysis. Values reported in the results are mean ± SE. Comparisons of the differences between the groups were performed by the Mann-Whitney-U-test. $P < .05$ was considered significant. The number of experiments performed in each set was never less than three.

RESULTS

FMLP, AA, PMA, and OZ induced luminol-dependent chemiluminescence in rat neutrophils. Free radical generation, as measured by luminol-dependent chemiluminescence, in rat PMNLs (1 to 5×10^6 cells/mL) has been summarized in Table 1. The profile of FMLP- (1×10^{-6} mol/L) and AA- (5×10^{-5} mol/L) induced chemiluminescence response was similar. An initial peak of light generation was observed within 23.85 ± 0.9 seconds and 41.87 ± 2.3 seconds, after the addition of FMLP and AA, respectively (Fig 1). However, on addition of PMA (3×10^{-8} mol/L) and OZ (1.25 μg) to the PMNLs, luminol chemiluminescence started after a lag phase of 180.0 ± 21.6 seconds and 29.27 ± 1.3 seconds, and reached its maximum in 16.0 ± 1.6 minutes and 2.94 ± 0.1 minutes, respectively (Fig 1). PMA in the concentration used was found to be the most potent inducer of free radical generation (Table 1). Generation of free radicals by AA, OZ, and FMLP was 53% ($P < .05$), 38% ($P < .01$), and 25% ($P < .01$), respectively, in comparison to PMA (Table 1).

Alteration of luminol-dependent chemiluminescence by SNP and arginine analogues. SNP, a nitric oxide donor, added to the PMNLs in 10 to 100 μmol/L concentration, 5 minutes before AA (5×10^{-5} mol/L), reduced the free radical generation in a concentration-dependent manner (Fig 2). Maximum inhibition (73%) was observed at the 100 μmol/L concentration (Table 1). LCL response induced by FMLP and PMA in presence of SNP (100 μmol/L) was reduced by 59.5% ($P < .05$) and 59.6% ($P < .05$), respectively (Table 1). However, response of OZ was not reduced (Table 1).

To investigate the effect of endogenous NO on the LCL response, the effect of NO precursor, L-arg was evaluated on AA-induced luminol chemiluminescence. As shown in Fig 2A, prior addition of L-arg (1 to 10 mmol/L) reduced the AA-induced LCL intensity in a concentration-dependent manner. Statistically significant inhibition of AA-induced respiratory burst was observed in presence of 5 and 10 mmol/L L-arg (Fig 2A). At 0.5 mmol/L, L-arg exhibited only 8% reduction (data not shown). In addition, the effect of L-arg was also studied in experiments in which it was added after AA (Fig 3). In these experiments also, an inhibitory effect on the LCL could still be observed. To study the effect of L-arg on other inducers, it was used in a concentration of 10 mmol/L. Although a significant inhibition of PMA- and FMLP-induced LCL was obtained, response to OZ was not significantly altered (Table 1). D-Arg (10 mmol/L) was ineffective in modulating the LCL induced by FMLP, OZ, PMA, and AA (Table 1).

MB (5 μmol/L), a guanylate cyclase inhibitor, was preincubated with the cells for 30 minutes before the addition of LCL inducers. It attenuated the free radical generation from rat PMNLs (Fig 4). 8-Bromo cGMP (10 μmol/L), a cell permeable analogue of cGMP, had no effect on the AA-induced chemiluminescence. Surprisingly, NOS inhibitors, L-NAME (50 μmol/L; data not shown) and L-NMMA (100 μmol/L), both decreased the LCL intensity (Fig 5). A higher concentration of L-NMMA (300 μmol/L) completely abolished the LCL response induced by various inducers (Fig 5). Furthermore, oxy-hemoglobin (1 μmol/L), which makes NO unavailable by binding to it, was also found to reduce the LCL response induced by AA, FMLP, and OZ; however, PMA response was not significantly inhibited (Fig 6). Addi-

Table 1. Effect of Different Inducers on Luminol-Dependent Chemiluminescence in the Rat PMNLs and Their Modulation by L-Arg and SNP

	Chemiluminescence Units			
Inducers	Control	SNP (100 μmol/L)	L-Arg (10 mmol/L)	D-Arg (10 mmol/L)
AA (5×10^{-5} mol/L)	560.2 ± 28.3 (n = 84)	149.9 ± 20.9 (n = 11)*	130.8 ± 17.9 (n = 12)*	656.4 ± 125.8 (n = 12)
PMA (3×10^{-8} mol/L)	1,059.1 ± 208.2 (n = 20)	427.8 ± 111.3 (n = 4)†	475.0 ± 103.9 (n = 4)†	1,061.1 ± 226.3 (n = 4)
OZ (1.25 μg)	400.7 ± 31.0 (n = 40)	537.5 ± 68.2 (n = 6)	337.4 ± 76.3 (n = 9)	399.9 ± 65.2 (n = 6)
FMLP (1×10^{-6} mol/L)	260.1 ± 21.7 (n = 55)	105.5 ± 37.9 (n = 7)†	110.2 ± 40.6 (n = 11)†	364.9 ± 122.3 (n = 15)

Values are mean ± SE (n = number of experiments).

* $P < .01$; † $P < .05$ in comparison to their respective controls.

tion of L-Arg with these agents further attenuated the LCL intensity (data not shown).

Effect of NO-modulating agents on nonenzymatic generation of superoxide radical. Effect of Hb (1 μmol/L), L-NAME (100 μmol/L), L-NMMA (300 μmol/L), L- and D-arg (10 mmol/L), MB (5 μmol/L), and SNP (10 and 100 μmol/L) was studied on the nonenzymatic generation of free radicals. Hb (1 μmol/L) reduced the free radical generation by 35.8% ± 5.5% ($P < .01$) and SNP (10 μmol/L) inhibited it by 50.9% ± 10.5% ($P < .02$). Other agents had no significant effect.

Effect of L-arg on superoxide anion generation. L-arg (1, 5, and 10 mmol/L) inhibited the free radical generation from PMNLs in a concentration-dependent manner when added before AA (Fig 7). It also inhibited the superoxide radical production from the cells by 13% when added after AA at a 10 mmol/L concentration.

Modulation of the hydroxyl radical generation by L-arg. PMNLs were preincubated with L-arg (10 mmol/L) for 5 minutes to determine its effect on OH^- production. In presence of L-arg, the hydroxyl radical generation by AA, PMA, FMLP, and OZ, was significantly decreased (Fig 8).

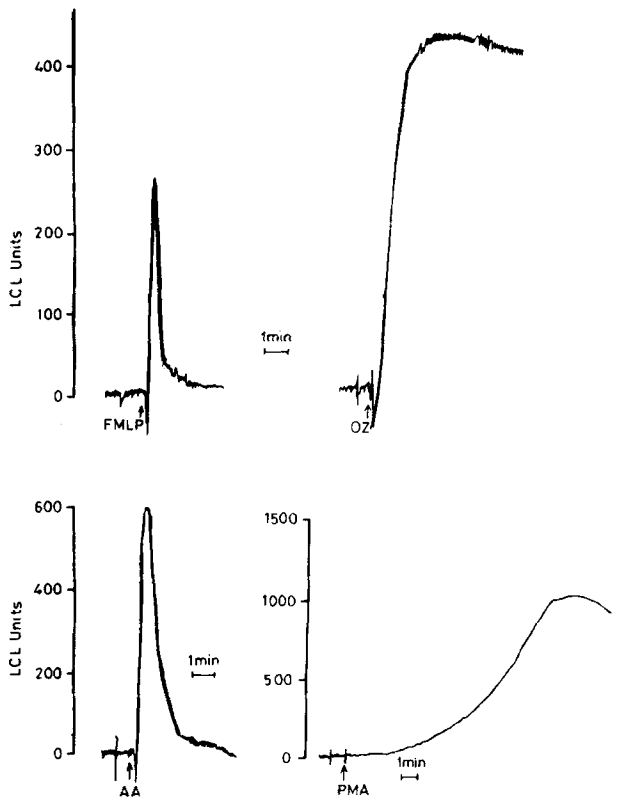

Fig 1. Stimulation-dependent chemiluminescence of activated neutrophils. PMNLs (1 to 5 × 10^6 cells) were kept at 37°C in HBSS (pH 7.4) containing 10 mmol/L glucose, 100 μmol/L luminol, 1 mmol/L $CaCl_2$ and $MgCl_2$. Concentrations of the stimulants used were FMLP (1 μmol/L), AA (5×10^{-5} mol/L), OZ (1.25 μg), and PMA (3×10^{-8} mol/L). Scale of LCL units in the upper panel is same for both FMLP and OZ. However, as indicated in the lower panel, scales for AA- and PMA-induced LCL responses are different.

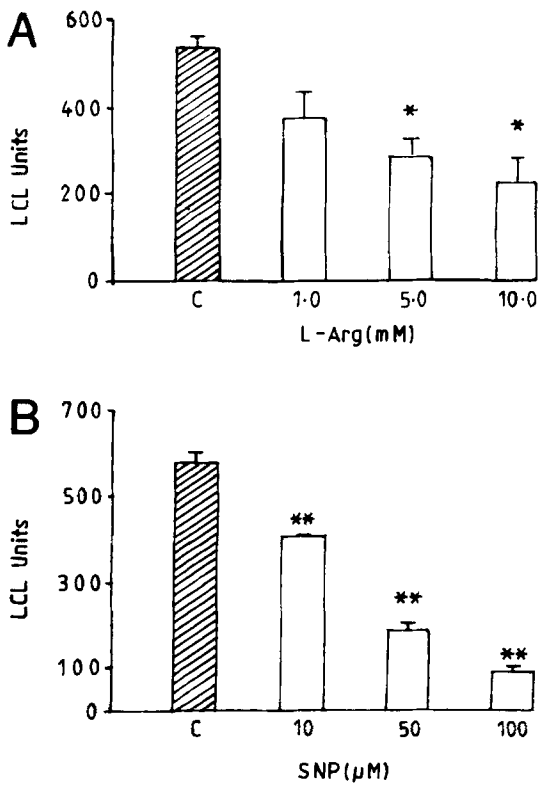

Fig 2. Inhibition of AA-induced LCL in rat PMNLs by L-arg (A) and SNP (B). Experimental conditions were same as described legends of Fig 1 except for the preincubation of cells for 5 minutes with arg or SNP at different concentration. *$P < .05$; **$P < .01$ in comparison to their respective controls (■).

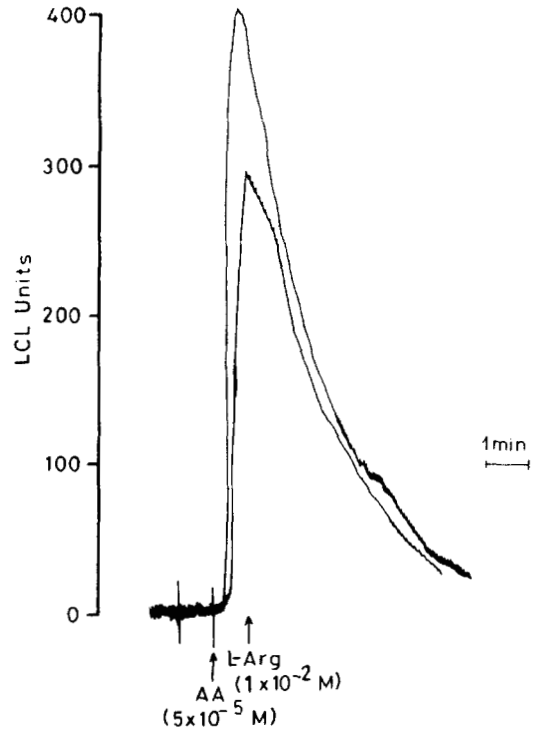

Fig 3. Effect of L-arg (10 mmol/L) on LCL response added to the cells after AA addition. L-arg reduced the LCL intensity.

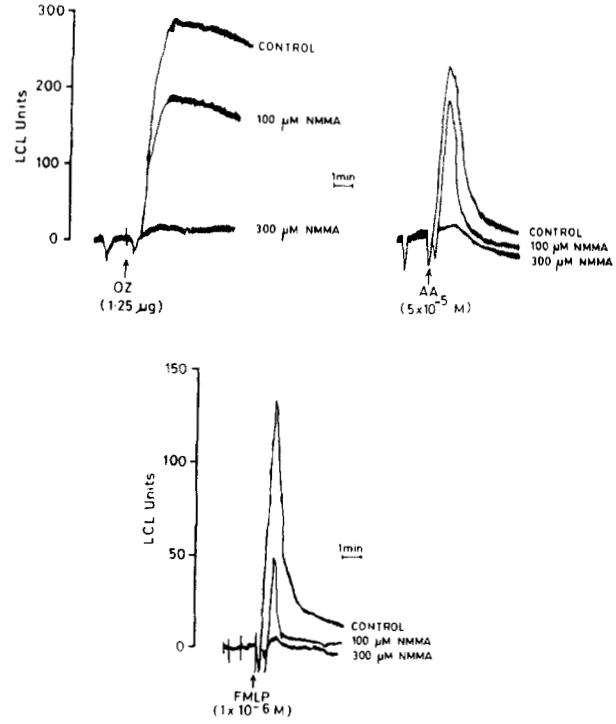

Fig 5. Reduction in the LCL intensity of activated PMNLs pretreated with L-NMMA (100 and 300 μmol/L) for 1 hour at 37°C. LCL units scale is common for OZ and AA.

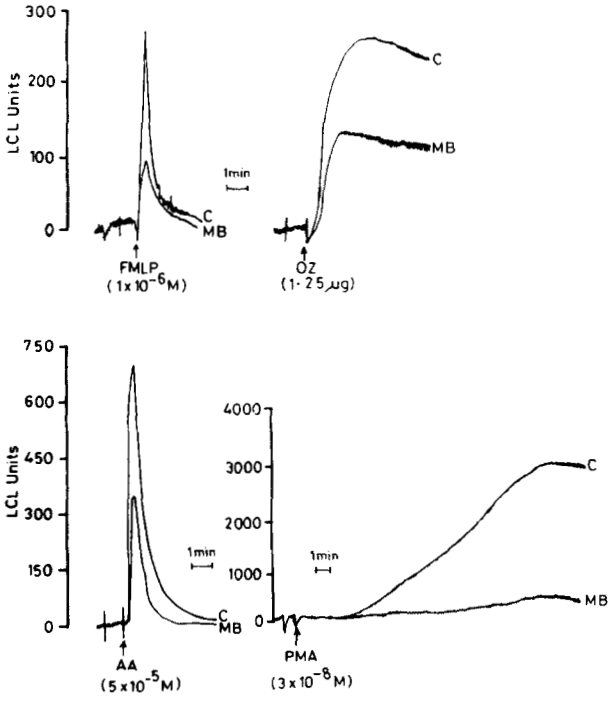

Fig 4. Alterations in the rat PMNL-dependent LCL in MB (5 μmol/L for 30 minutes at 37°C) pretreated PMNLs. Scale representing the LCL units in the upper panel is same for FMLP and OZ, whereas it is different in the lower panel for AA and PMA.

Alteration in the release of MPO and lysozyme in presence of L-arg. L-arg (1 and 10 mmol/L) itself did not affect the enzyme release from rat PMNLs (Fig 9). However, in presence of AA, L-arg–pretreated cells released lysozyme, present in the specific as well as azurophilic granules and MPO, a marker of azurophilic granules (Fig 9). No change in LDH activity in the supernatant was observed after AA and L-arg treatment (data not shown).

Effect of different inducers on nitrite release from PMNLs. The level of nitrite in the supernatant released from rat PMNLs after AA, PMA, FMLP, and OZ treatment is shown in Fig 10. Surprisingly, in SOD-treated control PMNLs, the level of nitrite was less than in the untreated cells (1,032 ± 105 nmol/L/10^7 cells), suggesting interaction of O_2^- and NO. However, in presence of free radical inducers, it was not different than their respective controls. Nitrite content in AA-treated cells was more in comparison to control. Further, after FMLP, PMA, and OZ addition, nitrite contents in the supernatant were not significantly altered (Fig 10). Nitrite contents in L-arg–pretreated cells after the addition of the inducers was found to be further enhanced (Fig 10).

Effect of the inducers on MDA levels. MDA was estimated in PMNLs (1×10^7 cells/mL) preincubated with L-arg (10 mmol/L) for 5 minutes before the addition of AA (5×10^{-4} mol/L), PMA (3×10^{-7} mol/L), OZ (12.5 μg), or FMLP (1×10^{-5} mol/L). OZ and FMLP had no effect on the MDA levels. However, AA and PMA significantly increased MDA content of the PMNLs (Fig 11). In the presence of L-arg, PMA-induced elevation in MDA was not

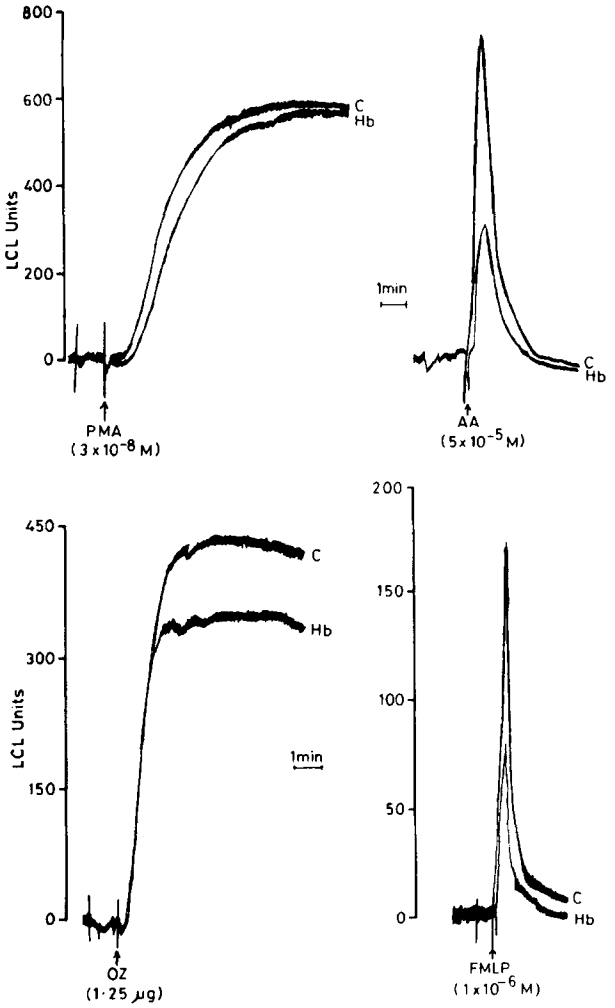

Fig 6. Attenuation in the LCL response of stimulated PMNLs in presence of Hb (1 μmol/L for 30 minutes for 37°C). Inhibition by Hb was more prominent against AA- and FMLP-induced LCL response.

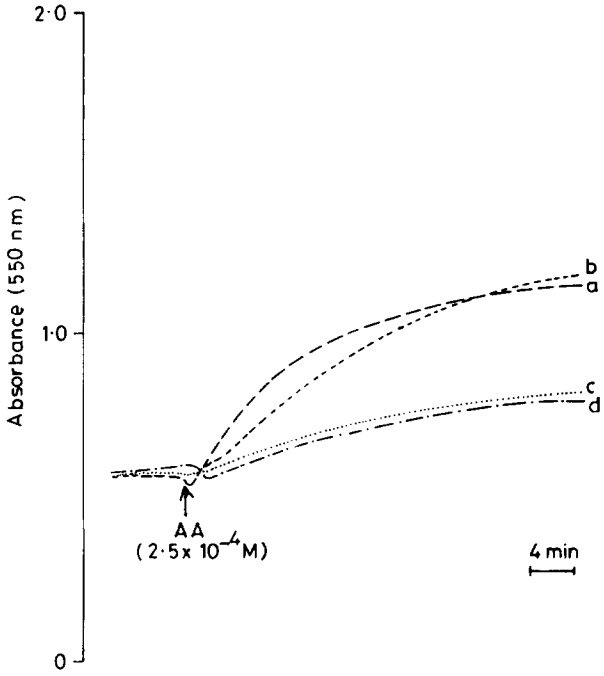

Fig 7. Effect of L-arg on the rate of Cyt C reduction by the rat PMNLs (1 × 10^6 cells). The effect of different concentrations of L-arg on the reduction of Cyt C has been determined at 550 nm in presence of AA (2.5 × 10^{-4} mol/L) activated cells. (a) Control; (b) in presence of 1 mmol/L arg; (c) 5 mmol/L arg; (d) 10 mmol/L arg.

altered, whereas AA-induced increase was further potentiated ($P < .05$, Fig 11). In another series of experiments, PMNLs were pretreated with indomethacin (5 μmol/L for 5 to 10 minutes) before the addition of L-arg. AA was added after 5 minutes and MDA content in these cells was measured and found to be 359 ± 6 nmol/L (n = 3). Interestingly, L-arg–induced potentiation of MDA was prevented in indomethacin-pretreated cells.

DISCUSSION

The results of the present study provide evidence that NO is an important regulator of rat PMNLs functions. L-arg, a precursor of NO, and SNP, a donor of NO, have been found to inhibit the luminol-dependent chemiluminescence induced by AA, FMLP, and PMA. In addition, AA-induced superoxide anion generation was attenuated in the presence of L-arg. Furthermore, a decrease in the hydroxyl radical production induced by AA, PMA, OZ, and FMLP in the presence of arginine was also observed.

A variety of stimuli were used to induce luminol-dependent chemiluminescence. FMLP-mediated response is receptor-mediated, whereas OZ is a phagocytic ligand. AA and PMA cause membrane perturbation via increase in the intracellular Ca^{+2} or protein kinase C activity. LCL responses

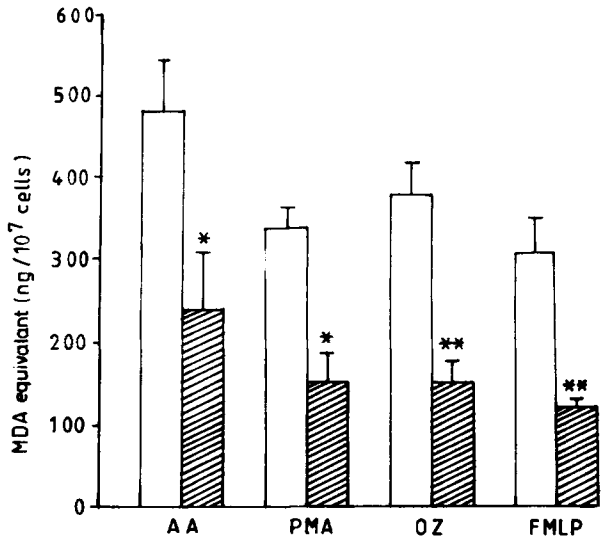

Fig 8. Effect of L-arg pretreatment on the degradation of deoxyribose by PMNLs after stimulation with FMLP, OZ, AA, and PMA. *$P < .05$, **$P < .01$ in comparison to their respective controls.

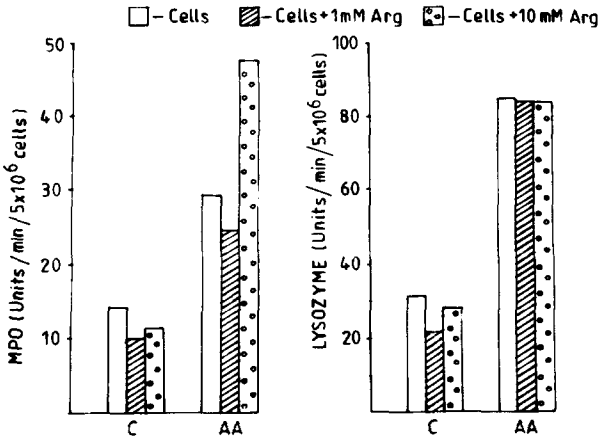

Fig 9. Effect of L-arg (1 and 10 mmol/L) on the granular enzyme release (MPO and lysozyme) from AA-stimulated rat PMNLs (n = 2).

(Fig 1) obtained in this study are in accordance with the reported values.[33]

L-arg and SNP both significantly attenuated the AA-, FMLP-, and PMA-induced LCL intensity. Rubanyi et al[15] have reported scavenging of the superoxide radicals generated by FMLP in presence of NO from human PMNLs. Later, Clancy et al[14] also demonstrated in human PMNLs that NO inhibited NADPH oxidase, the enzyme responsible for superoxide generation, and the inhibition was at the plasma membrane level, which could only be achieved when the cells were incubated with NO, before the addition of FMLP. In the present investigation with rat PMNLs, in the presence of NO, significant inhibition of AA-, FMLP-, and PMA-induced LCL response was observed. We have studied the effect of NO on rat PMNLs by using SNP, which augments the extracellular concentration of NO, and by using L-arg, a precursor of NO, which elevates intracellular concentration of NO. Thus, extracellular as well as intracellular concentrations of NO were raised, to elucidate the physiologic significance of NO effects on PMNLs function. Maximal inhibition of NO was on AA-induced free radical generation, and AA is also known to be released from PMNLs after activation. Therefore, most of the studies were performed with AA as PMNLs' inducer for free radical generation. SNP as well as L-arg were found to inhibit the AA-induced response in a concentration-dependent manner (Fig 2). The possibility of involvement of cGMP in this response was investigated by using MB, a guanylate cyclase inhibitor. Surprisingly, MB at the concentration known to inhibit the guanylate cyclase, exerted significant inhibition on the AA-, PMA-, OZ-, and FMLP-induced luminol-dependent chemiluminescence. Therefore, cGMP permeable analogue was used;[34] however, LCL response remained unaltered. Similarly, NOS inhibitors, L-NMMA and L-NAME, were also found to significantly suppress the luminol-dependent chemiluminescence. The effect of these agents on the nonenzymatic superoxide anion generation demonstrated that only Hb had inhibitory effect, which could be explained on the basis of the thiol groups. It was, therefore, concluded that the decrease in the LCL by L-NMMA, L-NAME, and MB was not due to the scavenging of active oxygen metabolites. It is known that NOS uses only L-arg. The involvement of NO in arginine pathway was confirmed by using D-arg, an enantiomer of L-arg. The inhibitory effect of L-arg on LCL (Table 1 and Fig 2) was found to be enantiospecific (Table 1) and therefore mediated by NO. In addition, elevation in nitrite content in L-arg–pretreated cells also supports the role of NO.

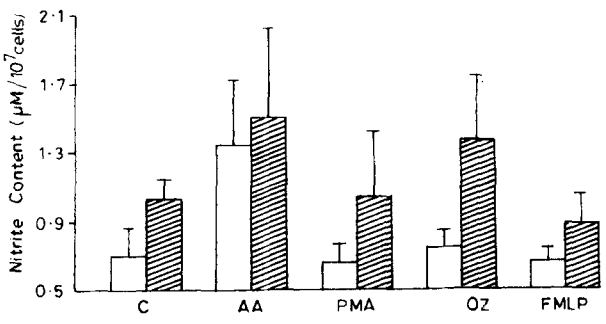

Fig 10. Histogram representing changes in the nitrite content following stimulation. (▩) Nitrite level in arginine-pretreated cells after agonist stimulation. Values are mean ± SE.

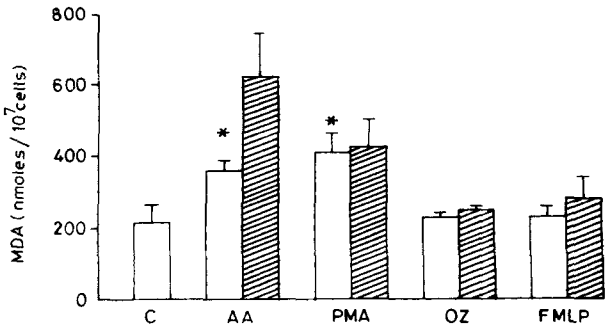

Fig 11. Alterations in the MDA levels (nanomoles/10^7 cells) after activation of PMNLs with various stimuli with (▩) and without (□) L-arg pretreatment. *$P < .05$ and **$P < .02$ in comparison to the control.

It has been reported that NO and superoxide anions can generate peroxynitrite and hydroxyl radicals and possibly mediate the cytotoxic effect of NO[8,9,35] in macrophage. Synthesis of peroxynitrite has been demonstrated by many investigators.[9,23] In an earlier study, we have reported that, after thromboembolism, there is an increase in the release of NO from the neutrophils.[22] The present study is an extension of that study with the objective of evaluating the nature of NO and to explore the possibility of it acting as cytotoxic or cytoprotective moiety. The interaction of hydrogen peroxide and NO to generate LCL has been demonstrated.[36,37] From this report it was expected that PMNLs, which release both NO and H_2O_2, may give LCL and, in this way, LCL could be an important tool to study the modulation of NO in rat PMNLs by its precursor L-arg and SNP, which release NO. However, in the presence of L-arg or SNP there was no increase in the LCL, suggesting that no toxic species, such

as peroxynitrite or hydroxyl radicals, are formed. It appears that NO inhibits NADPH oxidase activity.[14] Therefore, involvement of NO by use of NOS inhibitors, L-NMMA and L-NAME, was explored. However, these inhibitors themselves inhibited the LCL, which indicated the nonspecificity in their action, as reported by other investigators.[38-40] Archer and Hampl[41] have reported even the synthesis of NO from L-NMMA in isolated arterial rings. Our results also suggest the nonspecificity of their action and caution for selecting this intervention to demonstrate the involvement of NO in physiologic responses. It is known that NOS uses only L-arg. The involvement of NO in arginine pathway was confirmed because of the lack of activity by an enantiomer of L-arg. Similarly, MB was also found to reduce the chemiluminescence. MB has been reported to have some nonspecific effects,[42] whereas cGMP has no effect on LCL, suggesting that the effect is not mediated by cGMP. Specificity of these responses was found to be related to NO/L-arg, because D-arg was ineffective and similar response was obtained with SNP. As reported by Wang et al,[23] we have found that 0.5 mmol/L L-arg had no effect on the LCL. This was probably due to low concentration used by them, as has also been seen in our experiment. Higher concentrations of L-arg that were not used earlier, if added, were found to reduce the LCL intensity, hydroxyl radical, and superoxide anion generation.

The results of these experiments suggest that, apart from inhibiting the NADPH oxidase activity, NO also acts as scavenger of active oxygen species. It has also been demonstrated by Clancy et al[14] that preincubation of PMNLs with NO inhibits the superoxide radical generation by preventing the activation of the enzyme. Once the NADPH oxidase is assembled together by the inducers before NO addition, NO had no effect. On the other hand, in our experiments, we could still observe the reduction in LCL when the SNP or L-arg was added after the inducers, suggesting the scavenging activity of NO. Results obtained with AA and L-arg on superoxide radical generation after Cyt C reduction are similar to the reported observations of Rubanyi et al[15] in human PMNLs with FMLP. NO and its toxic mediators can mediate the lipid peroxidation[43]; hence, to further investigate this possibility of any toxic effect of NO on PMNLs, lipid peroxidation product MDA was estimated after AA, PMA, FMLP, and OZ treatment in the cells pretreated with L-arg. Surprisingly, only PMA and AA increased the synthesis of MDA. However, in indomethacin-pretreated cells, the elevation in MDA contents after L-arg treatment was prevented, suggesting the regulation of NOS by cyclooxygenase-derived products. There are some reports in the literature on modulation of NOS activity by AA metabolites[44,45] and this remains to be seen in PMNLs.

LCL response is known to be largely dependent on active oxygen species and MPO. Therefore, we also evaluated the effect of L-arg on the release of MPO, a marker of azurophilic granules. Surprisingly, no inhibitory effect on the release of MPO and lysozyme was obtained. Rather, the release was potentiated from PMNLs preincubated with L-arg. This is in agreement with the report of Wyatt et al.[46]

Our results indicate that inhibition of the release of reactive oxygen intermediates by NO is at enzyme level and it also involves the scavenging of these toxic species. We did not observe synthesis of other toxic species such as peroxynitrite or hydroxyl radicals in L-arg– or SNP-pretreated cells, unlike rat and mouse macrophage, in which NO was reported to be cytotoxic and synthesis of peroxynitrite was demonstrated by using luminol.[8,23]

From the results obtained, it can be concluded that rat PMNLs can be used to predict the effect of NO on human PMNLs, because in both species response of PMNLs to NO is inhibitory to free radicals and, therefore, may play an important role in postthrombotic recovery.

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