L-No-Nitroarginine Inhibits the Induction of Nitric Oxide Synthase (iNOS) and Cyclooxygenase-2 (COX-2) by Inhibiting NF-κB and AP-1 Activation in RAW 264.7 Cells

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Abstract

Increased production of nitric oxide (NO) by iNOS (inducible nitric oxide synthase) and PGE2 (prostaglandin E2) by COX-2 (Cyclooxygenase -2) are the key molecular events that occur during the inflammatory process. Previous studies have shown that NO influence the expression of COX-2 in a different manner in different cells. We studied the mechanism by which NO regulates the expression of COX-2 in LPS stimulated murine macrophage cell line RAW264.7. Treatment of the cells with NOS inhibitor L-Nω-Nitroarginine (L-NNA) in the presence of LPS (1µg/ml) reduced the production of NO in cultured macrophages. Moreover, significant inhibition of COX-2 was observed when the cells were treated with L-NNA (8.1µM) for 18 h in the presence of LPS. Reporter gene analysis and mRNA analysis by RT-PCR showed that NO also regulates COX-2 expression at transcription level. Additional experiments suggested that L-NNA mediated inhibition of COX-2 is due to blocking of NF-κB, ERK, C-Jun, AKT as well as pro-inflammatory cytokines like TNF-α and IL-1β.

Keywords: Inflammation; NO; iNOS; COX-2; L-NNA; Raw 264.7 cells

Introduction

Many biological, chemical and physical agents can lead to chronic inflammation with increased risk of human cancers [1]. Inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) are the specific enzymes that play a key role in an inflammatory process. These enzymes produce NO and prostaglandins respectively [2]. Constitutive NOS (cNOS) produces a small amount of NO that is essential for maintaining cellular function [3]. Since inducible nitric oxide synthase is produced in a higher amount, iNOS induction is one of the most important inflammatory signals in activated macrophages. Overproduction of NO has detrimental roles including tissue injury, septic shock and apoptosis [4,5]. Cyclooxygenases convert arachidonic acid into endoperoxides (PGG2 and PGH2) which further get converted into prostaglandins and thromboxanes by their specific synthases [6,7]. COX-1 is present in normal human tissue and maintains the lining of a stomach, kidney and functioning of platelets while COX-2 is mainly responsible for inflammation [8]. COX-2 is also induced in response to lipopolysaccharide (LPS) and several cytokines [9]. Thus, COX-2 is one of the major target for the prevention and treatment of inflammatory disease and specific inhibitors such as Celecoxib are an important advancement in the drug development [10].

Several studies suggest an important link between iNOS and COX pathways. NO is the key molecule that is responsible for their correlation [11]. There are many conflicting reports on the regulation of COX-2 by NO. For example, NO up-regulates COX-2 activity in LPS activated RAW 264.7 cells and fibroblast [12] but inhibits its production in rat kuppfer cells [13] and cultured bovine endothelial cells [14]. L-arginine analogs such as NG-monomethyl-L-Arginine (L-NMMA) block the formation of NO. L-NMMA inhibits the production of NO and PGE2 in LPS treated macrophages [12]. NOS inhibitors also decrease the formation of NO and PGE2 in an in vivo model of acute inflammation [15]. These earlier findings suggest that NO activates the COX enzymes responsible for PG production, but the mechanism is still unclear. To explore the mechanism, we studied the effect of L-NNA in LPS stimulated RAW264.7 cells hypothesizing that the compound could effectively block the synthesis of both NO and COX-2 following distinct signaling pathways.

L-No-Nitroarginine (L-NNA) is one of the nitric oxide synthase (NOS) inhibitors recognized in the early nineties which show minor selectivity to neuronal NOS and endothelial NOS compared to iNOS [16,17]. Selectivity is based on the difference in the binding site of the L-NNA between inducible and constitutive enzymes which can be exploited to develop potential iNOS inhibitor [18].

In this study we investigated (a) the effect of L-NNA on the expression of COX-2 in LPS stimulated RAW264.7 cells and (b) the mechanism by which the NO regulates the expression of COX-2. We found that L-NNA inhibited the activation of NF-κB, C-jun, ERK and AKT in LPS stimulated macrophage, and this was required for its blocking effect on COX-2.

Materials and Methods

Materials

Rabbit polyclonal IgG NOS-2/ COX-2, ERK/P-ERK, AKT/P-AKT, Mouse monoclonal IgG1 β actin, goat anti-rabbit IgG- HRP were purchased from Santa Cruz Biotechnology Inc (Dallas, Texas, USA). Goat anti- mouse IgG was obtained from Sigma Life Science, USA. Anti-NF-κB (P65-Rel A) was obtained from Rockland Immunochemicals Inc (Pottstown, USA). C-jun and P-C jun
antibiotics were purchased from Abcam (Cambridge, UK). Antibiotics solution Penicillin-streptomycin 100X liquid, protease inhibitor were purchased from Himedia Laboratories Pvt. Ltd (Vadhanis Ind. East Mumbai). Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine serum (FBS) were purchased from Invitrogen, (California, USA) and Bovine Serum Albumin (BSA), Griess reagent were obtained from a supplier Cayman Chemicals (Ann Arbor, MI, USA), Dimethyl sulfoxide (DMSO), 40% Polyacrylamide/Bisacrylamide TM , Temed (ultra-pure graded), Tween-20 were obtained from AMRESCO (Cochran Rd, Solon, Ohio, US), BioRad Clarity® Western ECL substrate was purchased from BioRad Laboratories USA Stripping Buffer obtained from Thermo Scientific (Meridian Rd, Rockford, USA).Cell Titre Glow® (CTG) reagent was purchased from Promega (Madison, WI, USA), Trypsin (0.25%) and Dulbecco's Phosphate Buffer Solution (DPBS) were from Himedia Laboratories Pvt. Ltd (Vadhanis Ind. East Mumbai). Other chemicals were purchased from Sigma and locals companies (India). Glasswares were obtained from Genetix Biotech Asia Pvt. Ltd, India. Compound Nω-Nitroarginine was purchased from Sigma-Aldrich, USA.

Cell culture

Raw264.7 cells were obtained from National Centre for Cell Science (NCCS) Pune India. The cells were cultured at 37°C in humidified air with 5% CO₂ in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), 100 U/ ml penicillin and 100 µg/ml streptomycin. For an experiment, cells were grown to 80-90% with no cell passage.

Measurement of cell viability

Raw 264.7 cells were cultured in 96 well plate and on the next day the compound L-NNA was added in different concentration (3 µM, 10 µM, 30 µM and 100 µM) and incubated for 24 h. L-NNA was solubilized in 0.1 N HCL. For control, the cells were treated with 0.1N HCL for all the experiments. Cell 'Titer-Glo' reagent was added equal to the volume of cells containing the media (i.e., 100 µl in each well). The plate was kept on the shaker to induce cell lysis. The plate was allowed to incubate at room temperature to stabilize the luminescence signal. The luminescence was recorded in the microplate reader (Biotek, Winooski, USA).

Measurement of NO

Raw 264.7 cells (5 ×10⁵) were cultured at 37°C for 12 h in serum-free media. On the next day the cells were stimulated with LPS (1 µg/ml) along with L-NNA (3-100 µM) and NO production was estimated by measuring nitrite level in culture media at 6, 12, 24 and 48 h after LPS treatment using Griess reagent (1% salicylamine, 0.1% N-1-naphthylenediamine dihydrochloride and 2.5% phosphoric acid). The culture plate was incubated for 10 min and absorbance was measured at 540 nm.

Preparation of nuclear extract

Cells were pre-incubated for 10 min in culture medium in the presence and absence of L-NNA and then stimulated with LPS (1 µg/ml). Nuclear fraction was obtained as described previously [19].

Western blot analysis

Cell lysates were prepared from control and treated cells using lysis buffer and immunoblotted on PVDF membrane as described previously [19].

Reporter gene assay

Cells were plated at a density of 3 × 10⁵ cells/well in a 12-well plate and transfected on the following day. A dual-luciferase reporter assay system (Promega, Madison, WI, U.S.A.) was used to determine promoter activity. Briefly, cells were transiently transfected with 1 µg of pGL-COX-2, iNOS, NF-κB, AP-1 Luciferase plasmid and 20 ng of the pRL-SV plasmid (Promega, Madison, WI, U.S.A.) using the Lipofectamine LTX™ Reagent (Invitrogen) and then exposed to LPS for 18 h. Firefly and Renilla luciferase activities in cell lysates were measured using a luminometer. Relative luciferase activities were calculated by normalizing firefly luciferase activities versus that of Renilla luciferase [19].

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using QIzOL Lysis reagent (Qiagen, Hilden, Germany). The total RNA (1.0 µg) was isolated and reverse transcribed using an oligo (dT) 18mer as a primer and M-MLV reverse transcriptase (Thermo Scientific) to produce cDNA. PCR was performed using selective primers for mouse iNOS (sense primer 5’ CTCGACGACTTGGATCCAGAACCCTG 3’, antisense 5’GGGAGTAGCCTGTGTGCACCTGGAA 3’), mouse COX-2 (sense primer 5’CCCCCACAGTCAAAGACACT 3’, antisense primer 5’ GAGTCCCCATGTTCCAGGAGGA 3’), mouse TNF-α (sense primer 5’ ATGAGCACAGAAGAGCATGCCG 3’, antisense 5’ TCACAGAGAATTGCCTAAAGTGAT 3’), mouse IL-1β (sense primer 5’ ATGGCAAGCTTCTCTGACATC 3’, antisense 5’ CAGGACAGATGATGTCTTCTTT 3’), microsomal prostaglandin synthase 1 (MPGES-1) (sense primer 5’ ATGACTTCCCCTGCGTGTGATGGAG 3’, antisense primer 5’ ACAGATGGTGCCGCACTTCCAGGA 3’), mouse S16 ribosomal protein (S16r genes sense primer 5’ TCCAAGGGTCCGCTGCAGTC 3’, antisense 5’ CGTTCACCCTTGATGAGGAGC 3’), Cell lysates were incubated for 10 min and absorbance was measured at 540 nm.

Preparation of nuclear extract

Cells were pre-incubated for 10 min in culture medium in the presence and absence of L-NNA and then stimulated with LPS (1 µg/ml). Nuclear fraction was obtained as described previously [19].

Statistical analysis

A paired t-test was used to determine significant intergroup differences. P value < 0.05 and < 0.01 were considered significant.

Results

Inhibition of iNOS induction by L-NNA

Figure 1A shows the chemical structure of L-NNA [20]. First the cytotoxicity of L-NNA to Raw264.7 cells was measured using a cell titer glo assay. The cell viability was not significantly affected by L-NNA up to 100 µM (Figure 1B). To study the promoter activation of iNOS in the presence of L-NNA, reporter gene analysis was carried out using iNOS luciferase plasmid. Raw264.7 cells were transfected with luciferase containing iNOS promoter and stimulated for 18 h
with LPS in the presence and absence of L-NNA. Significant inhibition of luminescence was observed at 8.1µM (Figure 1C).

To access the NO blocking effect of L-NNA, we treated the cells with LPS (1 µg/ml) in the presence or absence of L-NNA in different concentration range (3 µM-100 µM) in different time interval (6 h-48 h). The nitrite level in the culture media was measured by Griess reagent, and it was found that its production was significantly reduced by L-NNA (Figure 1D).

We then investigated whether the decrease in NO production was due to the inhibition of iNOS gene expression. The cells were treated with LPS (1 µg/ml) in the presence and absence of L-NNA (2, 3.5, 5 and 8.1 µM concentration) and Western blot analysis showed that L-NNA potently suppressed the induction of the iNOS protein compared to only LPS treated one (Figure 1E). β actin was used as an internal control. The result suggested that L-NNA effectively blocks the iNOS induction and NO production in macrophages.

The expression of iNOS is mainly regulated at the transcriptional level in response to agents like LPS, cytokines and other compounds [21]. We further studied the expression of iNOS gene using RT-PCR analysis. Raw264.7 cells were incubated with 5 µM and 8.1 µM of L-NNA for 1 h, and inhibition of iNOS gene was observed which was in correlation with Western blot result (Figure 1F).

**Figure 1:** Inhibition of iNOS by L-NNA. (A) Structure of L-Nω-Nitroarginine (L-NNA). (B) Effect of L-NNA on the cell viability. Raw264.7 cells were incubated in the presence or absence of 3-100 µM of L-NNA. The cell viability was determined by a CTG assay. The data is reported as a means ± SD two separate experiments. (C) Effect of L-NNA on iNOS gene transactivation. Inhibitory effect of L-NNA on iNOS gene transactivation. Raw264.7 cells were transiently transfected with the pGL-miNOS1588 construct (firefly luciferase), which contained a 1588 bp iNOS promoter sequence and pRL-SV (Renilla luciferase) (in the ratio of 100:1) and stimulated with LPS (1 µg/ml) in the presence or absence of L-NNA (5 and 8.1 µM) for 18 h. The data shown here represent the ratio of firefly luciferase activity to Renilla luciferase activity. The results shown represent the mean ± SD of 4 separate experiment (significant versus LPS treated group **p<0.01, *p<0.05). (D) The effects of L-NNA on LPS-induced NO production. Raw264.7 cells were stimulated with LPS (1 µg/ml) in the presence and absence of L-NNA (3-100 µM). The nitrite produced in the media was measured at 6, 12, 24 and 48 h by Griess reagent. The result shown represents mean ± SD of 4 different samples. (E) Inhibition of LPS inducible iNOS protein expression by L-NNA. Raw264.7 cells were stimulated with LPS (1 µg/ml) in the presence and absence of L-NNA (2, 3.5, 5, 8.1 µM) for 18 h. (F) Effect of L-NNA on iNOS mRNA expression. Inhibitory effect of L-NNA on iNOS mRNA was studied by RT-PCR analysis.16s RNA was used as an internal control.
Inhibition of COX-2 induction by L-NNA

COX enzymes contain an iron heme centre at their active site and are thus the potential target of NO [22,23]. We further studied the effect of L-NNA on the induction of COX-2 protein. The LPS induced COX-2 protein was also significantly reduced in the presence of L-NNA as shown by Western blot analysis (Figure 2A). To further study the effect of L-NNA on the activation of the COX-2 gene, reporter gene analysis was performed using the luciferase reporter plasmid containing the COX-2 promoter in LPS treated macrophages in the presence and absence of L-NNA. The test showed the significant decrease in the activation of the COX-2 promoter in the presence of L-NNA (Figure 2B). This result suggested that the iNOS inhibitor L-NNA may have a direct or indirect effect on the expression of COX-2 in LPS activated macrophages.

We also investigated whether L-NNA has an inhibitory effect on COX-2 at the transcriptional level; we carried RT-PCR analysis as mentioned above. COX-2 mRNA was also significantly reduced in the presence of L-NNA (Figure 2C). This result confirmed that L-NNA not only inhibits iNOS but also inhibits COX-2, which paved the way for finding the mechanism of its inhibition and further study its anti-inflammatory role.

PGE2 is a biologically active molecule responsible for different aspects of inflammation and function of immune cells [24]. Three different forms of PGE synthases viz. cytosolic PGE synthase (cPGES) and membrane bound or microsomal PGE synthase, mPGES-1 and mPGES-2 catalyse the isomerisation of PGH2 to PGE2. mPGES-1 is an inducible enzyme responsible for PGE2 production during inflammation and cancer, and others have housekeeping functions [25,26]. To study the effect of L-NNA on PGE2 production, RT-PCR analysis of mPGES-1 in the presence of L-NNA in LPS stimulated cultured macrophage cells was done. We observed that at 8.1 µM of L-NNA, mPGES-1 was significantly inhibited compared to LPS alone (Figure 2C). This observation led us to the conclusion that L-NNA not only inhibits NO production but also inhibited PGE2 production.

Figure 2: Inhibition of COX-2 by L-NNA. (A). Inhibition of LPS inducible COX-2 protein expression by L-NNA Cell lysate was prepared as mentioned in figure 1 (E) and Western blot was carried out. (B) Inhibitory effect of L-NNA on COX-2 gene transactivation. Raw264.7 cells were transiently transfected with the pCOX-2-Luc plasmid and reporter gene analysis was performed as mentioned in figure 1 (C). The results shown represent the mean ± SD of 4 separate samples (significant versus LPS treated group **p<0.01, *p<0.05). (C) Effect of L-NNA on COX-2 and MPGES-1 mRNA expression. Inhibitory effect of L-NNA on COX-2 and MPGES-1 mRNA was studied by RT-PCR analysis.16s RNA was used as an internal control.
Inhibition of NF-κB activation by L-NNA

NF-κB is a heterodimeric (p65/p50) protein that remains inactive in the cytoplasm due to its binding with inhibitory protein IκBα. Upon activation by some stimuli, IκBα is phosphorylated and degraded in the cytoplasm, and the released NF-κB translocates into the nucleus [27]. The NF-κB consensus sequence is present in the promoter region of murine iNOS gene [28]. COX-2 gene also contains two putative NF-κB consensus sequences, and binding of NF-κB is also responsible for COX-2 activation [29,30]. To test whether L-NNA inhibits the activation of NF-κB, we measured the nuclear p65 level by cell fractionation followed by western blotting. The level of nuclear p65 increased from 15 min to 30 min after stimulating the cells with LPS (1 µg/ml) and peaked at 30 min after induction with LPS. Pre-treatment of the cells with 8.1 µM of L-NNA 10 min before the LPS exposure significantly inhibited the activation of NF-κB at 60 min (Figure 3A). This result indicated that the blocking effect of L-NNA on iNOS and COX-2 may be mediated by NF-κB pathway.

Reporter gene analysis was also carried out using NF-κB luciferase plasmid as mentioned above. We observed that in the presence of L-NNA (8.1 µM), NF-κB promoter activation was significantly inhibited which suggested that L-NNA blocks iNOS and COX-2 through NF-κB pathway (Figure 3B).

Figure 3: Inhibition of NF-κB by L-NNA. (A) Effects of L-NNA on the p65 nuclear translocation. Raw264.7 cells were treated with 1 µg/ml LPS for 30 min and 60 min with/without 8.1 µM of L-NNA, and the protein levels of nuclear p65 were determined by western blotting. Nuclear lamin A/C was used as an internal control. (B). Raw264.7 cells were transiently transfected with the pNF-κB -Luc plasmid and reporter gene analysis was performed as mentioned in figure 1 (C). The results shown represent the mean ± SD of 4 separate experiments (significant versus LPS treated group **p<0.01, *p<0.05).

Inhibition of C-jun, Erk and Akt activation by L-NNA

AP-1 is one of the transcription factors that is responsible for activation and attraction of immune cell at the site of inflammation caused by different cytokines and chemokines [31]. MAPK signaling cascade has been linked with the biosynthesis of prostaglandins [32,33].

Raw264.7 cells were cultured in 6 well plate and L-NNA (8.1 µM) was treated 10 min before the LPS (1 µg/ml) treatment. The cells were stimulated for 30 and 60 min with LPS. We observed the inhibition of C-Jun activation after 30 min of LPS exposure (Figure 4A). Reporter gene analysis using AP-1 luciferase plasmid also showed that L-NNA exerts its blocking effect on COX-2 through JNK signaling (Figure 4B).
AKT plays a role in NF-κB signaling. It activates nuclear translocation and phosphorylation of p65 at Ser536 [34,35].

Western blotting analysis of nuclear extract showed the significant inhibition of activation of AKT in LPS stimulated macrophages after 60 min (Figure 5A).

Similarly, phosphorylation of ERK was also inhibited at 60 min (Figure 5A). Nuclear lamin was used as an internal control. These results suggest that L-NNA targets MAPK Cascade protein and efficiently reduces the inflammatory reaction in LPS treated RAW264.7 cells.

To further investigate the mechanism of inhibition of inflammation by L-NNA, we studied the level of cytokines like TNF-α and IL-1β produced in the presence of L-NNA by RT-PCR.

The stimulation of macrophage by LPS elicits the production of cytokines such as TNF-α, IL-1β and IL-6, which further augments the original response [36].

RT-PCR analysis showed the L-NNA inhibited LPS induced TNF-α and IL-1β mRNA production in macrophages. S16 mRNA level was used as a loading control (Figure 5B).

The decreased level of TNF-α and IL-1β further supported the action of L-NNA on the signaling pathway induced in response to LPS stimulated macrophages.

**Discussion**

The beneficial, as well as the detrimental role of NO has been well established [3]. NO is an important bioactive molecule that regulates cardiovascular, nervous and immune system [20]. Deregulated production of NO is involved in the pathogenesis of a number of diseases. The development of inhibitors of nitric oxide synthase (NOS), and understanding their mechanism of action needs more attention [37].

NO is highly produced by inducible NOS (iNOS) during acute and chronic inflammation [5]. We have studied the effect of L-NNA, one of the NOS inhibitors on the expression of COX-2 and how the level of NO regulates the COX-2 expression in LPS activated RAW264.7 cells. Nω-nitro-L-arginine methyl ester is previously shown to inhibit liver injury induced by interleukin-2 [38]. Studies on murine macrophage cell and cultured rat islet cells showed that NOS inhibitors decrease the biosynthesis of PGE2 [12,39,40]. Oral administration of NOS inhibitors N-iminoethyl-L-lysine or NG-nitro-L-arginine methyl ester decreased both NO2- and PGE2 in the fluid exudates induced by carrageenan in the rat air pouch [15].

Our result also showed that administration of L-NNA to the LPS stimulated macrophages inhibits both the production of NO and COX-2 in macrophage cells. This result suggested that there is a high level of production of NO during inflammation, which directly or indirectly increases the production of prostaglandins and the inhibition of iNOS by L-NNA decreases both NO and COX-2. MPGES-1 level is also decreased in the presence of L-NNA, which further supports that PGE2 level is associated with NO level during inflammation.
To further validate our finding, we transfected luciferase plasmid containing iNOS and COX-2 promoter in Raw264.7 cells and stimulated it with LPS for 18 h in the presence and absence of L-NNA. Reporter gene analysis result also showed that decrease in the iNOS production due to L-NNA caused low luminescence signal indicating a sharp decrease in the COX-2 promoter activation. mRNA analysis by RT-PCR also showed that inhibition of both iNOS and COX-2 mRNA in activated macrophages in the presence of L-NNA. This finding suggested that NO regulates COX-2 at transcription level also.

All the above primary results motivated us to find the signalling pathways and the cross talk between NOS and COX enzymes and the way NO regulates prostaglandin formation in activated macrophages. Previous research also showed that various COX-2 inducing stimuli (e.g. growth factors, cytokines and endotoxin) follows different mechanism to regulate COX-2 expression yet it is not clear what signalling pathways NO follows to influence COX-2 gene expression [41].

Our study clearly shows the different signalling pathways that are triggered in response to LPS and the effect of NOS inhibitor L-NNA on NO production and COX-2 regulation in macrophages.

Since both iNOS and COX-2 contain NF-kB binding site in their promoter region [28,29], we studied the NF-kB activation and its translocation into the nucleus by immunoblotting the nuclear fraction sample prepared as mentioned before. L-NNA inhibited the activation of NF-kB and hence reduced the expression of both iNOS and COX-2. Reporter gene analysis of NF-kB luciferase plasmid in the presence of L-NNA supported our findings. In response to LPS, there is enhanced production of different pro-inflammatory cytokines like TNF-alpha, interleukin-1 (IL-1) and IL-6 as well as IL-10, which has anti-inflammatory properties [42,43].

Early studies on murine macrophages Raw264.7 cell line shows that Raf-1/MEK1-ERK1-Erk2 is involved in TNF-α expression [44,45]. Also, there is a study that shows the involvement of NF-kB mediated TNF-α production causes the release of PGE2 and COX-2 expression in human gingival fibroblast [46]. Our study showed that both TNF-α and COX-2 are inhibited in the presence of L-NNA in activated macrophages. Also, we have shown that there is a decrease in the phosphorylation of ERK as well as C-jun in the presence of L-NNA. AP-1 luciferase signal was also low in the presence of L-NNA. IL1-β is also responsible for induction of COX-2 [47,48]. We showed that decreased production of NO due to L-NNA caused the reduction in the expression of IL-1β and consequently inhibition of COX-2 expression. NF-kB mediated AKT activation has been shown to be responsible for prostaglandin production and COX-2 expression in endometrial cancer cells [49,50]. Also, AKT stimulation by TNF-α is reported in premalignant keratinocytes. We also made it clear that AKT phosphorylation takes place in response to high level of NO and hence may regulate COX-2 via NF-kB pathway.

Conclusion

Dual nature of NO governing both housekeeping functions and pathologies show its importance in animal physiology and research. Till date different iNOS inhibitors viz NG-monomethyl-L-Arginine (LNMMA), L-nitroarginine methyl ester (l-NAME) and their in vitro and in vivo role has been studied but the signalling mechanism remained unclear, and this aspect is well documented in our present study. Our results suggest that L-NNA inhibits NO that further inhibits the expression of COX-2 and prostaglandin formation in macrophages.
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