CHAPTER VIII

Regulation Of Nitric Oxide Production By Leishmania lipophosphoglycan (LPG) In Murine Macrophages

1.0 Introduction

Leishmania are digenetic parasites that alternate between the sandfly vector as a free-living flagellate form, the promastigote, and the macrophage phagolysosome as obligate intracellular amastigotes (Alexander and Russell, 1992). To resist destruction and to modulate host defense functions, *Leishmania* relies on various virulence determinants, including a family of specialized virulence glycoconjugates that have in common the presence of repeating Gal(β1,4)Man(α1)-PO4 units (Descoteaux and Turco, 1999; Ilg et al., 1999). Lipophosphoglycan (LPG), which consists of a glycophosphatidylinositol-anchored polymer of the repeating units, represents the most abundant promastigote surface molecule and forms a dense glycocalyx around the parasite (Turco and Descoteaux, 1992). This multifunctional virulence factor participates in a variety of processes during the establishment of infection within mammalian hosts: resistance to complement-mediated lysis, inhibition of phagosomal maturation, resistance to oxygen radicals, impairment of macrophages signal transduction pathways, and modulation of cytokine and nitric oxide (NO) production (Chan et al., 1989; Descoteaux et al., 1991; Desjardins and Descoteaux, 1997; McNeely and Turco, 1990; Piedrafita et al., 1999; Proudfoot et al., 1996; Puentes et al., 1988).

Macrophages express inducible nitric oxide synthase (iNOS) following activation by a variety of immunological stimuli such as interferon γ (IFN-γ), tumor necrosis factor α (TNF-α), and bacterial lipopolysaccharide (LPS) (Hibbs et al., 1988; Nathan and Xie, 1994). Inducible nitric oxide synthase (iNOS) catalyses the synthesis of high concentrations of NO from L-arginine and molecular oxygen (Moncada and Higgs, 1993), and NO is involved in the killing of a range of microorganisms (Liew and Cox, 1991).

Nitric oxide (NO) has emerged as an important cytotoxic and cytostatic effector for a number of pathogens, including viruses, bacteria, fungi, and parasites. Nitric oxide is generated by constitutively expressed neuronal and endothelial NO-synthases (NOS-I and NOS-III, respectively). Under pathological conditions (e.g., inflammation and infection), high levels of NO are synthesized following transcriptional induction of the inducible type of NO-synthase (NOS-II) (Moncada, Higgs, and Furchgott, 1997). NO is considered a double-edged weapon (Colasanti and Suzuki, 2000). In fact, constitutive-derived low levels of NO are involved in several physiological processes, including nervous transmission and vasodilation. On the other hand, inducible-derived high levels of NO can exert beneficial effects on the host by acting as an antiviral, antibacterial, antifungal, -parasitic, and tumoricidal agent (De Groote and Fang, 1995; Clark and Rockett, 1996; Wink et al., 1998; Akaike and Maeda, 2000; Brunet, 2001) or be detrimental to the host by acting as a cytotoxic effector (Brunet, 2001; MacMicking, Xie and Nathan, 1997). Both endogenous and exogenous NO inhibits the development of parasites, including intracellular (*Trypanosoma*, *Leishmania*, *Plasmodium*, *Toxoplasma*) and extracellular (*Entamoeba*) protozoa, as well as the helminth *Schistosoma*. However, unregulated production of NO during parasite infection promotes inflammation and induces cell and tissue dysfunction (Clark and Rockett, 1996; Brunet, 2001).

NO produced by human macrophages and released by NO-donors has been demonstrated to be involved in *Leishmania* killing (Romao et al., 1999; Salvati et al., 2001). Interestingly, the therapeutic treatment of cutaneous leishmaniasis with NO-releasing drugs has been recently approached (Zeina, Banfield and al-Assad, 1997; Lopez-Jaramillo et al., 1998; Davidson et al., 2000). However, in order to guarantee invasion, control, and persistence in host organism, *Leishmania* adopts a survival strategy including

the suppression of NOS-II, the induction of NOS-II suppressors (e.g., transforming growth factor-β, interleukin-4 and -10), and the entry into NOS-II negative target cells, such as Langerhan cells (Bogdan et al., 1996).

In the present study, *Leishmania* surface molecule, LPG, a predominant surface molecule of promastigotes was chosen, to study the signaling cascade. LPG is involved in macrophage-parasite interaction especially in the attachment and internalisation of the promastigotes as well as in the protection against microbicidal activities displayed by macrophages. LPG is also known to promote as well as inhibit NO synthesis by the murine macrophages (Proudfoot et al., 1996), thereby playing an important role in the host parasite relationship. Binding of *Leishmania* LPG to macrophages may occur through the lectin-like LPS-binding sites belonging to the CD11/CD18 family of surface receptors, CR3, LF1 p150 and p95 (Puentes et al., 1988). The effect of LPG in *Leishmania* is quite analogous to that of LPS, which has also been known to regulate NO synthesis. LPS has been shown to stimulate a variety of signal transduction elements such as Src-related protein tyrosine kinases (Boulet et al., 1992), phospholipases (Ambs et al., 1995; Fouda et al., 1995), protein kinase C, raf (Geppert et al., 1994; Hambleton et al., 1995), mitogen activated protein kinases (Weinstein et al., 1992; Guthridge et al., 1997; Bhat et al., 1998) in a variety of cellular systems. The present work shows the involvement of signal transduction elements in regulating nitric oxide production during *Leishmania* infection, by using specific inhibitors for the relevant kinases pathway.

2.0 Materials and methods

2.1 Chemicals

Powdered media RPMI-1640 and M-199 medium were purchased from Sigma Chemical Co., USA. Other chemicals like bovine serum albumin (BSA), penicillin-G,

HEPES, sodium bicarbonate, streptomycin etc., were also procured from Sigma Chemical Co., USA. Fetal calf serum (FCS) was purchased from Gibco-BRL Ltd., USA. LPS (lipopolysaccharide) from *Escherichia coli* (serotype 0111:B4) was obtained from Sigma Chemical Co., USA. Staurosporine, phorbol myristate acetate (PMA) was from LC Laboratories (Boston, Massachusetts). The PKC inhibitor, 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine hydrochloride (H7), N- (6-aminohexyl)-5-chloro-1naphthalene sulfonamide (W7), a calmodulin protein kinase antagonist was purchased from Calbiochem Corporation (La Jolla). Mitogen activated protein kinase inhibitors PD98059 and SB203580 were procured from Sigma Chemical Co., USA. All other chemicals were of analytical grade.

2.2 Parasite culture

Leishmania strain AG83, an Indian isolate (MHOM/IN/1983/AG83) was maintained in M-199 medium with 10% FCS at 22°C.

2.3 Preparation of LPG

LPG was isolated from *L. donovani* promastigotes at the stationary phase of growth and purified as previously described (Orlandi and Turco, 1987; Russo et al., 1992). LPG was resuspended in complete medium before being used for the requisite assays.

2.4 Macrophage culture

A murine macrophage cell line J774A.1 (American Type Culture Collection, Rockville, Maryland) was used in this study. The macrophages were maintained at 37°C in RPMI-1640 medium with 10% FCS in a CO_2 incubator (5% CO_2). The macrophages were seeded onto microtitre plates (96 well) at a density of 5 x 10^5 cells/well and incubated for 24 h before being used for the requisite assay.

2.5 Stimulation of macrophages

J774A.1, macrophages were pretreated with different modulating agents like PMA (100 nM), staurosporine (2 μM), H7 (50 nM), W7 (100 nM), PD98059 (20 μM) and SB203580 (5 μM) for the indicated periods of time, followed by the treatment with LPG (10 μg/ml) or LPS (1 μg/ml) for 24 h (unless indicated otherwise).

2.6 Nitric oxide assay

J774A.1, macrophage (5 x 10^5 cells/well) were plated onto 96 well tissue culture plates and kept in a CO_2 incubator (5% CO_2) for 12 h at 37°C. Cells were preincubated with specific inhibitors for the indicated period of time at 37°C. Followed by treatment with LPS (1 μg/ml) or LPG (10 μg/ml) for 24 h. The samples were then harvested for nitrite concentration and supernatants were assayed using an automated procedure based on Griess reagent as described by Stuehr and Nathan (1989). In brief, 100 μl of culture supernatant was mixed with 100 μl of Griess reagent (1% sulfanilamide, 0.1% N- [1-naphthyl]-ethylenediamine dihydrochloride in 2.5% H_3PO_4) and incubated at room temperature for 30 min. The absorbance was measured at 540 nm (Green et al., 1982). These results are representative of triplicate samples. Nitrite levels were determined using sodium nitrite ($NaNO_2$) as a standard.

2.7 Statistical analysis

Statistical analysis was performed by standard t-test (Fisher et al., 1957). Student's test was performed on the data and a probability value (P) of less than 0.05 was considered significant.

3.0 Results

3.1 LPG induced nitrite levels in macrophages

We examined the effect of LPG and LPS on nitrite levels in J774A.1 macrophages (Fig. 1A and B). A murine macrophage cell line, J774A.1 (5 x 10^5 cells/well) was plated onto 96 well-tissue culture plates and kept in a CO_2 incubator (5% CO_2) for 12 h at 37°C. Cells were then treated with different concentrations of LPG for 24 h. Nitrite levels present in the supernatants were quantified by using Griess reagent. LPG resulted in a dose-dependent increase in nitrite level in J774A.1 macrophages ($P < 0.01$)(Fig. 1A). LPS was used as a positive control. LPS treated J774A.1, macrophages exhibited a significant dose-dependent increase in nitrite levels over the unstimulated controls (Fig. 1B).

The kinetics of induction of nitrite levels was determined by incubating the J774A.1 cells with LPG (10 μg/ml) for different time intervals. After a further 24 h incubation, culture supernatants were collected and nitrite levels were measured. Increase in nitrite levels was observed as early as 30 min and reached a peak at 180 min ($P < 0.01$) (Fig. 2A). A similar time dependent increase in induction of nitrite levels was observed with LPS (1 μg/ml) (Fig. 2B).

3.2 Protein kinase C (PKC) mediated response of LPG induced nitrite levels in macrophages

Protein kinase C stimulation has been closely linked with macrophage activation. To determine whether nitrite levels induced by LPG (10 μg/ml) are through a protein kinase signal transduction event, specific inhibitors of protein kinase were used. Macrophages were pretreated with staurosporine (2 μM), a broad-spectrum inhibitor of protein kinase. Macrophages treated with staurosporine (2 μM) for 15 min did not result in inhibition of nitrite levels over untreated control ($P < 0.01$). Pre-treatment of macrophages

with staurosporine (2 μM) for 15 min followed by treatment with LPS (1 μg/ml) or LPG (10 μg/ml) for 24 h showed inhibition of nitrite level in both LPG and LPS-treated groups ($P < 0.01$) (Fig. 3).

Effect of protein kinase C inhibitor, phorbol myristate acetate (PMA) on LPG induced nitrite levels was monitored. Macrophages were pretreated with PMA (100 nM), a specific inhibitor of PKC (Rodriguez-Pena and Rozengurt, 1984; Young et al., 1987) for 4 h, followed by treatment with LPG (10 μg/ml) for 24 h. A significant inhibition of nitrite level over LPG-treated group ($P < 0.01$) was observed (Fig. 4). A similar observation was made when macrophages were pretreated with PMA (100 nM) for 4 h and then treated with LPS (1 μg/ml) for 24 h (Fig. 4). These results show the role of PKC in LPG-induced nitrite levels in macrophages.

We also examined the effect of both H7, a non-specific general kinase inhibitor that inhibits both PKC and PKA (Hidaka et al., 1991; Kawamoto and Hidaka, 1984; Hannun, Mrril and Bell, 1991) and W7, a specific inhibitor of calmodulin protein kinase (CaM-PK) (Hidaka et al., 1981). We pretreated J774A.1 macrophages either with H7 (50 nM) or W7 (100 nM) (Fig. 5 and 6) for 5 min and then stimulated with the LPS (1 μg/ml) or LPG (10 μg/ml) for 24 h. Significant inhibition of nitrite levels was observed with both H7 and W7. The concentrations of inhibitors used in these studies were non-toxic to the parasite growth.

3.3 Extracellular signal regulated kinase (ERK1/2) and p38 mitogen activated protein kinase mediate LPG induced nitrite levels in J774A.1, macrophages

To assess the role of ERK1/2 and p38 MAPK in the regulation of LPG induced nitrate levels, we used PD98059 (20 μM) and SB203580 (5 μM), selective inhibitor of the ERK1/2 kinase (Dudley et al., 1995) and p38 MAPK (Cuenda et al., 1995) respectively.

We preincubated, J774A.1 for 1 h in the absence or the presence of either PD98059 (20 μM) or SB203580 (5 μM) followed by treatment with LPS (1 μg/ml) or LPG (10 μg/ml) for 24 h. As shown in Fig. 7, both PD98059 (20 μM) and SB203580 (5 μM) resulted in inhibition of nitrite levels over LPG- or LPS-treated groups ($P < 0.01$).

4.0 Discussion

Protozoan organisms of the genus *Leishmania* are obligate intracellular parasites of macrophages that are responsible for severe morbidity and mortality in infected people in many parts of the world. A principal function of macrophage is to destroy intracellular pathogens. Hence, the manner in which *Leishmania* and other intracellular pathogens are able to survive and replicate within this ostensibly hostile intracellular milieu is an important question in cell biology and immunology. *Leishmania* and other intracellular pathogens have evolved mechanisms to modulate host-signaling pathways in order to facilitate invasion and survival. Diverse lines of evidence indicate that *Leishmania* interferes with signal transduction in macrophages (Reiner, 1994). It has been reported earlier that *Leishmania donovani* infection of human monocytes selectively attenuates the gamma interferon (IFN-γ)-activated Jak-Stat1 signal pathway by inhibiting tyrosine phosphorylation of Jak1, Jak2, and Stat1 (Nandan and Reiner, 1995). Furthermore, it has also been found earlier that infection of macrophages with *L. donovani* significantly reduces protein phosphorylation in response to phorbol-12-myristate-13-acetate (PMA), which correlates with diminished protein kinase C (PKC) activity (Olivier, Brownsey and Reiner, 1992) and c-fos gene expression induced by PKC is impaired whereas protein kinase A (PKA)-mediated c-fos gene expression is unaffected (Moore, Labrecque and Matlashewski, 1993).

Earlier reports show that in addition to parasite products and virulence factors that facilitate survival and entry of metacyclic promastigotes into the host cell, sandfly saliva suppresses macrophage leishmanicidal activity by inhibiting nitric oxide (NO) production (Hall and Titus, 1995) and accelerates lesion development (Hall and Titus, 1995). This activity has been attributed to the sandfly salivary peptide maxadilan, a selective agonist of the pituitary adenylate-cyclase-activating polypeptide type 1 receptor, which inhibits tumour necrosis factor-α (TNF-α) production by lipopolysaccharide (LPS)-stimulated macrophages (Bozza et al., 1998) and diminishes their ability to produce NO and kill *Leishmania in vitro* (David et al., 1997). Consequently, administration of maxadilan together with *L. major* promastigotes significantly exacerbated disease in resistant mice, which is associated with diminished NO production in draining lymph nodes (David et al., 1997).

It has been reported earlier that in epidermal Langerhan cells, *L. major* parasites survive due to the lack of cytokine-inducible nitric oxide synthase (iNOS, NOS-2) (Blank et al., 1996). Thus these Langerhan cells form a safe habitat for parasite and transporting the parasites from the infected skin to the draining lymph node (Blank et al., 1996). In contrast, cytokine-activated macrophages express iNOS and synthesize high levels of NO from L-arginine. Proudfoot et al. (1995) demonstrated that infection of a macrophage cell line with *L. major* promastigotes prior to stimulation with IFN-γ and lipopolysaccharide partially inhibited the release of NO. This effect was mimicked by addition of glycoinositolphospholipids from *L. major*, which is abundantly expressed on the surface of both the promastigote and amastigote parasite form. Another parasite molecule, which downregulate iNOS activity in infected macrophages, is the LPG-associated kinetoplastid membrane protein-11. At amino acid position 45, this protein contains N^G-monomethyl-L-

arginine, a structural analogue of L-arginine and well-known inhibitor of iNOS (Jardim et al., 1995). Persistence of the parasites in macrophages, dentritic cells was paralleled by a sustained, lifelong expression of iNOS mRNA and protein, which was dependent on $CD4^+$ and not on $CD8^+$ T cells. Furthermore, 30-40% of the parasites detected co-localized with iNOS-positive cells (macrophages and dendritic cells), whereas 60-70% were found in areas negative for iNOS.

Transcription factors, NF-κB and AP-1, play a major role in mediating IL-1-induced cellular responses. While activation of NF-κB seems to be an essential step for iNOS induction in most cell types (Eberhardt et al., 1998; Xie et al., 1994), the role of AP-1 and other transcription factors is less clear. Furthermore, the mechanisms that link IL-1-binding to its cognate receptor to the activation of transcription factors, and subsequent iNOS gene expressions, are still incompletely understood, in all cell types. Several distinct protein kinase system, including protein kinase C (Diaz-Guerra et al., 1996; Paul et al., 1997), cAMP-dependent protein kinase (Oddis et al., 1996), protein tyrosine kinases (PTK) (Corbett et al., 1994; Cruz et al., 1999; Hirasawa et al., 1999; Kleinert et al., 1998; LaPointe and Sitkins, 1996; Lee et al., 1997), and members of the mitogen-activated protein kinase (MAPK) family (Cruz et al., 1999; Badger et al., 1998; Chan et al., 1999; Chen and Wang, 1999; DaSilva et al., 1997; LaPointe and Isenovi, 1999)-which includes the extracellular signal-regulated kinases 1 and 2 ($p42/44^{MAPK}$), the c-Jun NH_2-terminal kinase (JNK), and the $p38^{MAPK}$ -have been reported to participate in the expression of iNOS, in response to a variety of stimuli, including IL-1. Nevertheless, a marked difference is evident considering the protein kinase families that are required for iNOS expression in different cell types and in response to distinct stimuli, suggesting that the iNOS promoter is differentially regulated depending on the cell type and the stimulus considered. On the

other hand, there is some evidence, suggesting that MAPK cascades, namely the JNK and other pathways, may be involved in the activation of NF-κB and subsequent iNOS expression (Chen and Wang, 1999; Janssen-Heininger, Macara, and Mossman, 1999; Lee et al., 1997). Recent reports also suggest that PTK may be required for iNOS induction mediated by NF-κB activation (Cruz et al., 1999; Lee et al., 1997).

Mitogen-activated protein (MAP) kinases are downstream targets of PKC (Seger et al., 1992), suggesting the possibility of impaired signaling through these enzymes in *Leishmania*-infected cells. Two closely related MAP kinases, extracellular signal regulated-protein kinases 1 and 2, function as essential relays in many signal transduction processes. Also, survival of *Leishmania* parasite in macrophages has also been shown to be associated with the ability of the LPG to regulate production of nitric oxide (NO) (Proudfoot et al., 1996) and IL-12 (Piedrafita et al., 1999). NO, which mediates many of the inflammatory responses of macrophages following infection by pathogens is generated following the up-regulation of expression of inducible NO synthase (iNOS) (Liew et al., 1991). These immunomodulatory proteins have been shown to play a crucial role in the development of immunity to intracellular pathogens such as *Leishmania*. Expression of these immunomodulatory proteins appears to be regulated primarily at the level of transcription (Martin, Nathan and Xie, 1994; Xie et al., 1994; Kamijo et al., 1994; Ma et al., 1996). The kinases are involved upstream of the macrophage effector molecules. Thus cytokines, nitric oxide production might all be controlled by the direct action of the parasite through the induction of the endogenous signaling pathway leading to the activation/inactivation of transcription factors. The molecular mechanism underlying the induction/inactivation of these effector molecules is not fully understood. In the present study, we examined

regulation in induction /inactivation of nitric oxide activation via PKC, and MAP kinase signaling pathways using specific inhibitors.

It was observed from the present study that LPG (10 μg/ml) resulted in a significant dose-dependent and time-dependent increase in nitrite levels. Studies using selective inhibitors of ERK (PD98059) and p38 (SB203580) showed that both ERK and p38 MAP kinases play an essential role in the induction of nitric oxide. In order to determine the involvement of protein kinase signal transduction pathway in nitric oxide induction, broad-spectrum inhibitor of protein kinase, staurosporine was used. Furthermore, specific inhibitors of protein kinase C namely H7 and PMA were also used to study effect on LPG induced nitrite levels. The involvement of PKC in LPG stimulated nitrate levels in macrophages was demonstrated.

Thus, differential sensitivity of the macrophage effector responses to pharmacological modulators would suggest that such reagents could be used as potential immunomodulators to generate the required immunity to combat a particular pathogen or alternatively reduce inflammation/pathology associated with certain disease status.

A

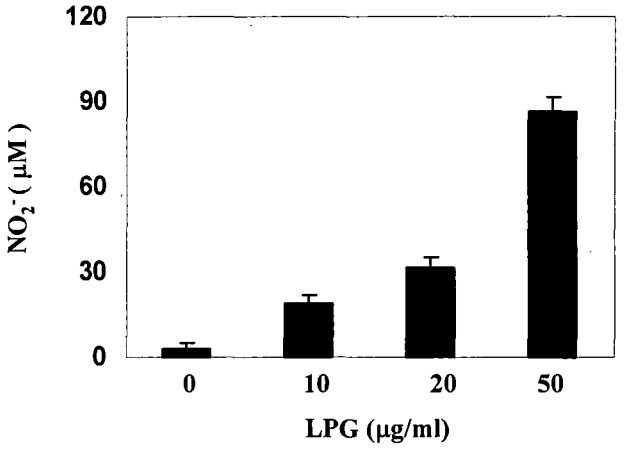

Fig. 1A: Effect of LPG on nitrite levels in J774A.1 macrophages. J774A.1 macrophages ($5x10^5$) cells/well were seeded onto 96 well tissue culture plates and kept in a CO_2 incubator at 37°C. Cells were then treated with different concentrations of LPG and again incubated at 37°C for 24 h. Nitrite levels present in the supernatants were quantified by Griess reagent. Data are mean ± standard deviation (S.D.) of triplicate values.

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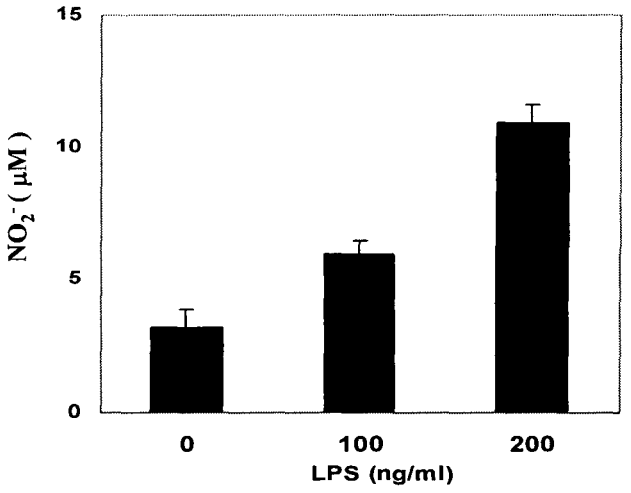

Fig. 1B: Effect of LPS on nitrite levels in J774A.1 macrophages. J774A.1 macrophages ($5x10^5$) cells/well were seeded onto 96 well tissue culture plates and kept in a CO_2 incubator at 37°C. Cells were then treated with different concentration of LPS and again incubated at 37°C for 24 h. Nitrite levels present in the supernatants were quantified by Griess reagent. Data are mean ± standard deviation (S.D.) of triplicate values.

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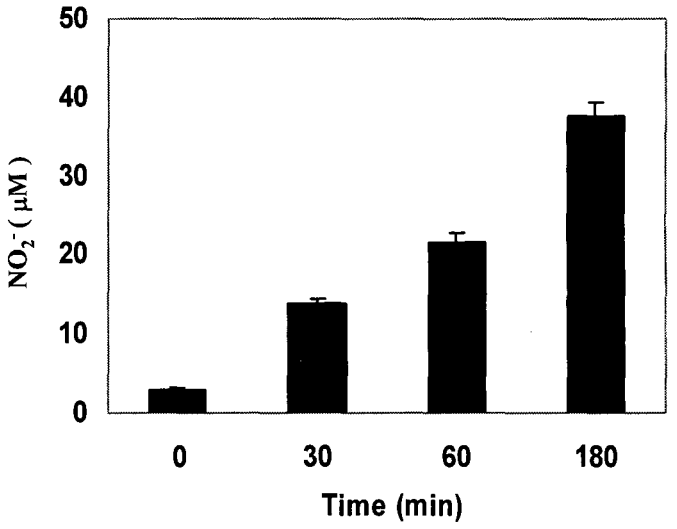

Fig. 2A: Kinetics of LPG induced nitrite levels in J774A.1 macrophages. J774A.1 cells were treated with LPG (10 μg/ml) for indicated times. Nitrite levels present in the supernatants were quantified after 24 h by Griess reagent. Data are mean ± standard deviation (S.D.) of triplicate values.

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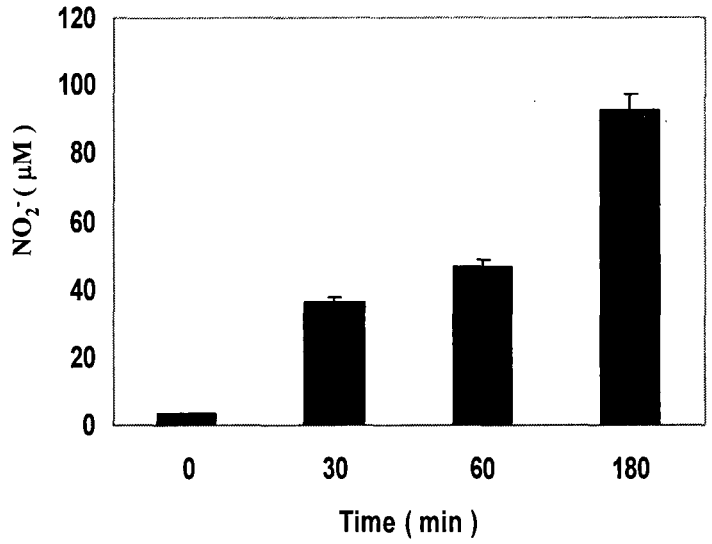

Fig. 2B: Kinetics of LPS induced nitrite levels in J774A.1 macrophages. J774A.1 cells were treated with LPS (1 μg/ml) for indicated times. Nitrite levels present in the supernatants were quantified by Griess reagent. Data are mean ± standard deviation (S.D.) of triplicate values.

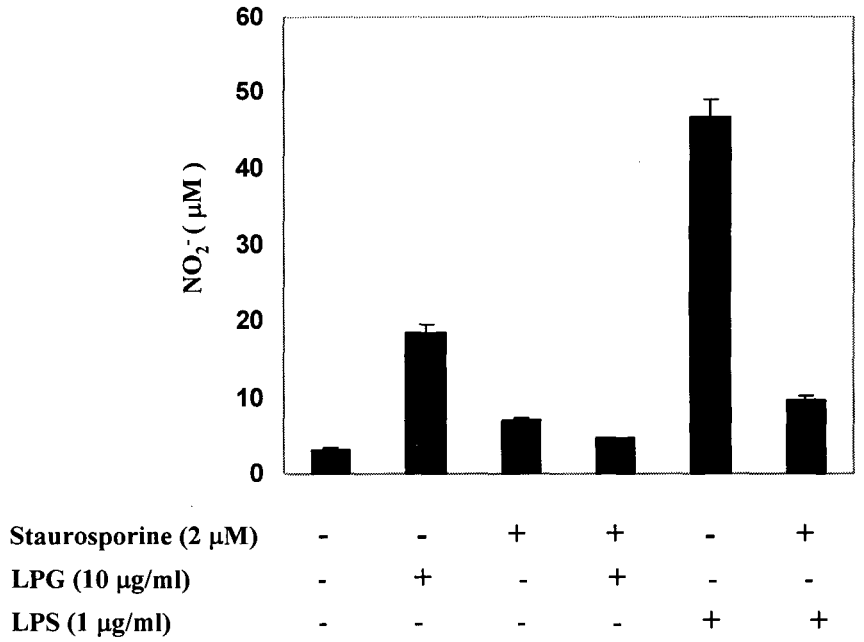

Fig. 3: Effect of the protein kinase inhibitor staurosporine on nitrite levels in J774A.1 macrophages. Macrophages were pretreated with 2 μM staurosporine for 15 min followed by treatment with LPS (1 μg/ml) or LPG (10 μg/ml) for 24 h and assayed for nitrite levels. Values are representatives of triplicate samples. + represents presence of and – absence of LPG or LPS or staurosporine.

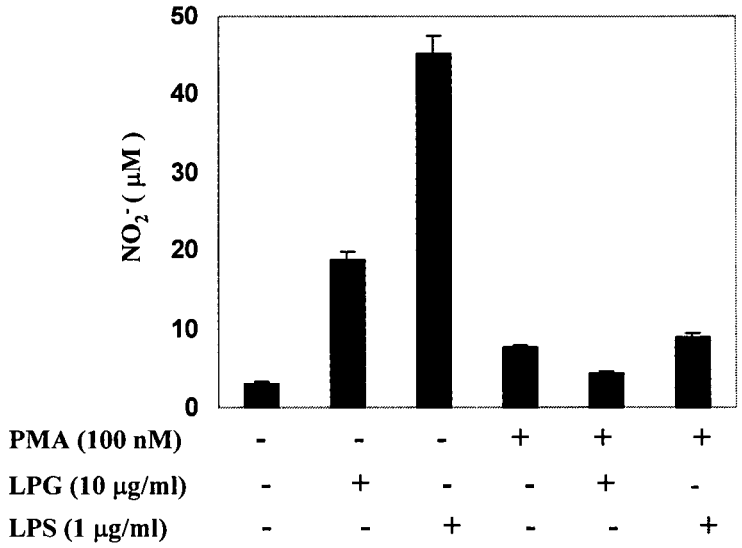

Fig. 4: Effect of PMA on LPG induced nitrite levels in macrophages. Macrophages were pretreated with PMA (100 nM) for 4 h and then stimulated with LPS (1 μg/ml) or LPG (10 μg/ml) for 24 h. Nitrite levels were quantified using Griess reagent. Data are representative of triplicate samples. + represents presence of and – absence of LPG or LPS or PMA.

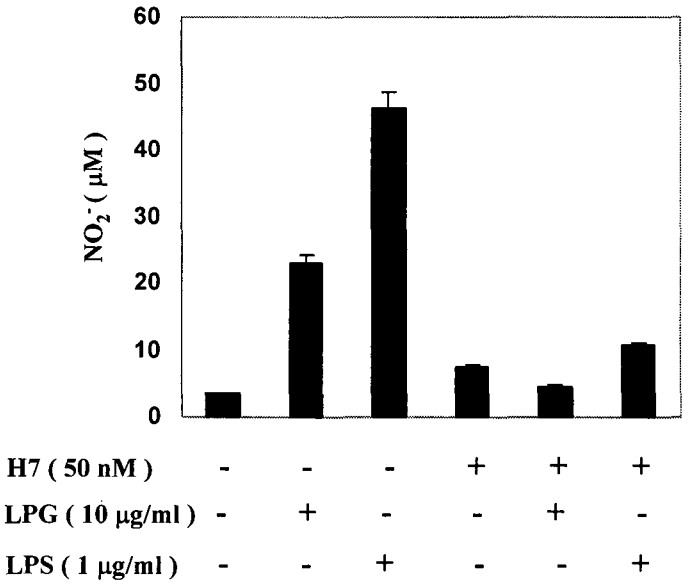

Fig. 5: Effect of H7 on LPG induced nitrite levels in macrophages. Macrophages were pretreated with 50 nM H7 for 15 min and then stimulated with LPG (10 μg/ml) or LPS (1 μg/ml) for 24 h. Nitrite levels were quantified using Griess reagent. Data are representative of triplicate samples. + represents presence of and – absence of LPG or LPS or H7.

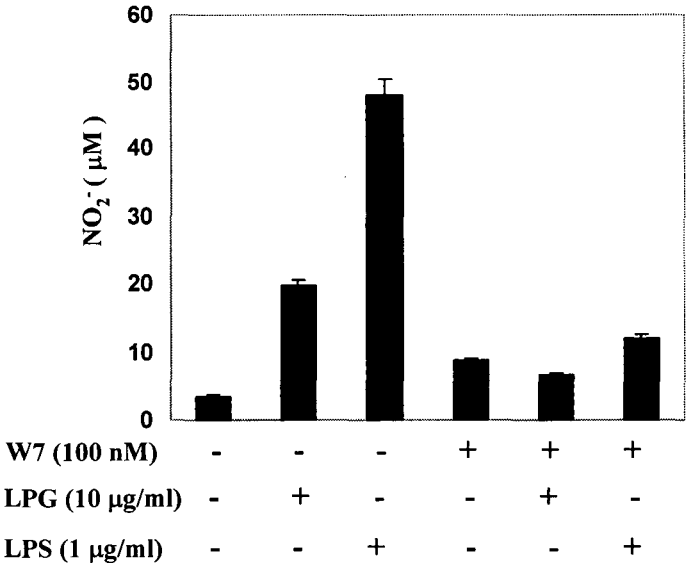

Fig. 6: Effect of CaM-PK inhibitor,W7 on LPG induced nitrite levels in macrophages. Macrophages were pretreated with 100 nM W7 for 15 min and then stimulated with LPS (1 μg/ml) or LPG (10 μg/ml) for 24 h. Nitrite levels were quantified using Griess reagent. Data are representative of triplicate samples. + represents presence of and – absence of LPG or LPS or W7.

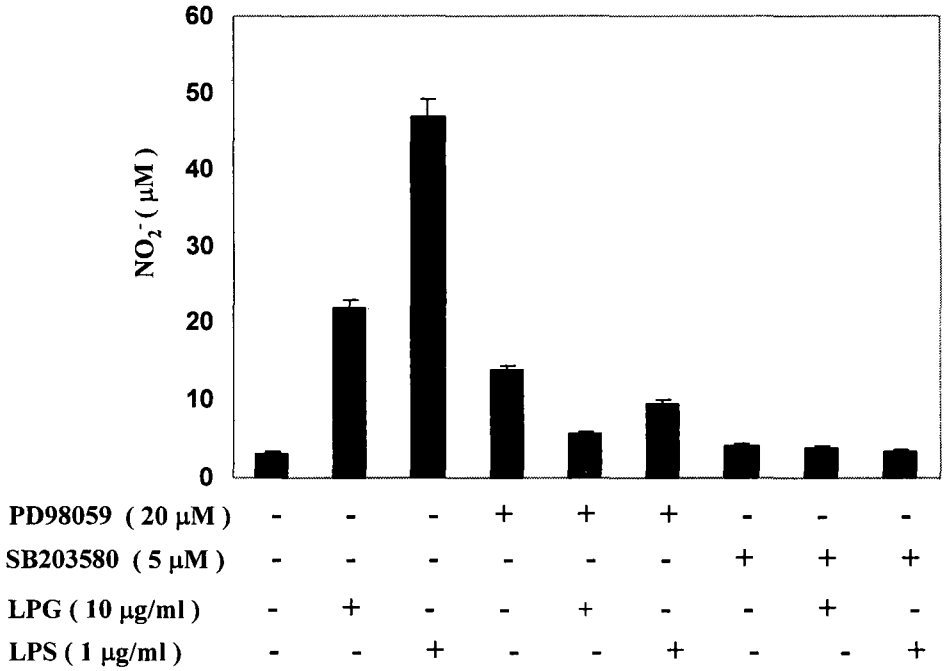

Fig. 7: Effect of ERK1/2 and p38 mitogen-activated protein kinase inhibitor on LPG induced nitrite levels in macrophages. Selective and potent inhibitor of ERK MAP kinase cascade PD98059 and p38 MAP kinase cascade SB203580, were found to significantly inhibit LPG (10 μg/ml) stimulated nitric oxide levels. Macrophages were pretreated with PD98059 (20 μM) and SB203580 (5 μM) 1 h followed by treatment with LPS (1 μg/ml) or LPG (10 μg/ml) for 24 h. Nitrite levels were measured. Values are representatives of triplicate samples. + represents presence of and – absence of LPG or LPS or PD98059 or SB203580.