CHAPTER IV

Role Of Protein Kinase C In Leishmania donovani Lipophosphoglycan Mediated Signal Transduction In Macrophage

1.0 Introduction

Leishmania donovani is an obligate intracellular parasite that survives inside the macrophages of the vertebrate host, resulting in visceral leishmaniasis in humans. The parasite exists in two forms, a motile extracellular promastigote form within the gut of the sandfly vector and a nonmotile amastigote form within the macrophages of the mammalian host. In order to survive within the host, the parasite has to subvert the immune response of the host. This is brought about by the modulation of the host transductional mechanism in the parasite's favour (Descoteaux and Turco, 1993). This process begins as soon as the parasite binds to the macrophage receptors. The two basic mechanisms of signal transduction are the protein kinase C (PKC) and the protein kinase A (PKA) pathways modulated by phospholipase C and adenylate cyclase, respectively (Alberts et al., 1994). Most intracellular parasites exert their effect on host cells via the PKC pathway of signal transduction (Segwin, Keller and Chadee, 1995; Vieira, de Carvalko and de Sonza, 1994; Ward et al., 1994). *Leishmania* is no expection to this rule (Descoteaux et al., 1991). The parasite alters the PKC pathway and has no effect on the PKA pathway of signal transduction as seen by the use of signal transduction inhibitors and activators (Moore, Labrecque and Matlashewski, 1993; Descoteaux, Matlashewski and Turco, 1992; Descoteaux and Matlashewski, 1989). The portion of the parasite implicated in this effect is lipophosphoglycan (LPG) (Descoteaux, Matlashewski and Turco, 1992).

The parasite leads to inhibition of several macrophage functions by the above means like production of IL-1, expression of the c-fos gene in response to lipopolysaccharide (LPS), the expression of the major histocompatibility complex (MHC) in response to interferon-γ (IFN-γ) and the generation of oxygen radical following stimulation with LPS (Turco and Descoteaux, 1992). In addition, interaction or infection of

the macrophage with the parasite leads to a homeostatic imbalance of calcium within the host cell (Olivier, 1996). Since calcium is required during signal transduction events, it follows that the sustained elevation of cytosolic calcium that is observed in *Leishmania* infected macrophages (Olivier, Baimbridge and Reiner, 1992; Eilam, El-On and Spira, 1985), is probably responsible for the above effects of LPG. Furthermore, it has been reported recently that the activity of a serine-threonine phosphatase is increased in cells infected with *Leishmania donovani* possibly leading to dephosphorylation of not only second messengers but also various cellular proteins (Olivier, 1996). Under such conditions, the cell is limited to some kind of physiological paralysis, abolishing its ability to defend itself. Thus it can be seen that the parasite has devised suitable means to defend itself against host microbicidal responses even prior to its uptake by the macrophages. Once the parasite is internalised into the macrophages, other protective mechanisms come into play.

The signal transduction pathway represents an attractive target for the parasite to result in inactivation of macrophage functions. Several early genes, e.g., c-fos, c-jun, and c-myc proto-oncogenes, are involved in the general process of cell activation (Hamilton and Adams, 1987). The c-fos proto-oncogene is among the immediate early genes that are expressed immediately after macrophage activation with LPS (Introna et al., 1986). Ornithine decarboxylase (ODC) is the first and the rate-limiting enzyme in the biosynthesis of polyamines, which is implicated to have a role in a variety of metabolic processes (Pegg and McCann, 1982; Tabor and Tabor, 1984). The accumulation of ODC mRNA in mammalian cells is associated with the stimulation of a variety of physiological responses, indicating that the ODC gene belongs to a group of early genes, e.g., c-fos, c-myc and c-jun proto-oncogenes (Kaczmarek and Kaminska, 1989). Earlier work has shown that bacterial

lipo-polysaccharide (LPS), an activator of macrophage effector functions (Higuchi et al., 1988), induces ODC mRNA accumulation in human and mouse monocytes and macrophages (Messina et al., 1990). Previous reports on the enhanced accumulation of ODC mRNA by IFN-γ in human monocytes, provides further support to the hypothesis that the gene encoding ODC has a role in activation of macrophages (Messina et al., 1990).

A major cell surface molecule of the *Leishmania* species is the glycoconjugate, lipophosphoglycan (LPG). LPG is involved in macrophage-parasite interaction (Turco and Descoteaux, 1992), especially in the attachment and internalization of the promastigotes (Descoteaux et al., 1991), as well as in the protection against microbicidal activities of macrophages. One of the most important features of LPG may be its ability to inhibit the activation of PKC as shown earlier in *in vitro* systems (McNeely and Turco, 1987). Since protein kinase C (PKC) is the key enzyme of the cell machinery, which conveys signals from the surface of the cell to the nucleus (Nishizuka, 1986), inhibition of PKC is indeed a crucial breach in the defence mechanism of the cell. The present study was undertaken to investigate the role of protein kinase C (PKC) in *L. donovani* lipophosphoglycan (LPG) mediated signal transduction in macrophages using ODC as a marker.

2.0 Materials and methods

2.1 Chemicals

Powdered media RPMI-1640 and M-199 were purchased from Sigma Chemical Co. (St.Louis, Missouri). Fetal calf serum (FCS) was purchased from Gibco-BRL Ltd., USA. Staurosporine, okadaic acid (OKA), dibutryl cyclic adenosine monophosphate (dBcAMP), phorbol myristate acetate (PMA) were from LC Laboratories (Boston, Massacheusets). Lipopolysaccharide from *Escherichia coli* (Serotype 0111:B4) was obtained from Sigma. The PKC inhibitor, 1-(5-isoquinoline sulfonyl)-2-methylpiperazine hydrochloride (H7),

was purchased from Calbiochem Corporation (La Jolla, CA). ^{14}C-ornithine was from New England Nuclear (Boston, Massacheusets). All other chemicals were of analytical grade.

2.2 Preparation of LPG

Purified LPG from stationary phase *L. donovani*, was isolated as previously described (Orlandi and Turco, 1987; Russo et al., 1992).

2.3 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 tissue culture plates (60 mm) at a density of 1×10^6 cells/plate and incubated for 24 h before being used for the requisite assay.

2.4 Stimulation of macrophages

J774A.1 macrophages were incubated for 1 h (unless indicated otherwise) with the indicated concentrations of LPG or LPS and then stimulated with the different modulating agents for the indicated periods of time. The concentrations used were: 1 mM dBcAMP, 0.2 μM okadaic acid, 2 μM staurosporine, 100 ng /ml PMA and 50 nM H7.

2.5 Ornithine decarboxylase assay

The treated macrophages were scraped off in PBS (pH - 7.4), centrifuged at 5,000 rpm for 15 min at 4°C and resuspended in harvest buffer (50 mM Tris, 10 μM EDTA, and 2.5 mM DTT) (pH-7.5). The cells were homogenized in an all glass homogenizer and centrifuged at 5,000 rpm for 10 min at 4°C to pellet the lysed cells. The supernatant was used for assaying ODC activity (Seely et al., 1982). The activity was expressed in nm/hr/mg protein.

2.6 Protein estimation

Protein was estimated measured in the cell supernatant using Lowry's procedure (Lowry et al., 1951).

2.7 Statistical analysis

All experiments were done in replicate sets with duplicate samples in each set. The ODC activity was expressed as mean ± SD and the data were analysed by the Student's t-test. A value of $P \leq 0.05$ was regarded as statistically significant.

3.0 RESULTS

The effect of LPG on ODC activity in J774A.1 macrophage cell line is shown in Fig. 1. LPG resulted in a dose-dependent increase in the ODC activity in J774A.1 macrophages ($P < 0.001$) at 1 h of treatment. As a positive control, macrophages were treated with different concentrations of LPS. LPS, like LPG, also resulted in a dose dependent increase in the ODC activity of J774A.1 macrophages ($P < 0.001$) (Fig. 2).

The time-dependent effect of LPG and LPS on ODC activity is shown in Figures 3 and 4, respectively. Maximum stimulation of ODC activity with LPG (10 μg/ml) was observed at 3 h after treatment of macrophages. The ODC activity came down at later time points (data not shown) (Fig. 3). LPS (100 ng/ml) also caused maximum stimulation at 3 h with a similar decline in ODC at later time intervals (Fig. 4).

To determine whether the pattern of ODC activation in response to LPG occurred through a protein kinase signal transduction event, protein kinase was inhibited prior to LPG treatment. First, J774A.1 macrophages were pretreated with staurosporine, a broad-spectrum inhibitor of protein kinase. Macrophages treated with staurosporine (2 μM) for 15 min showed an inhibition of ODC activity over untreated macrophages ($P < 0.001$). Pretreatment of macrophages with staurosporine (2 μM) for 15 min followed by treatment

with LPG (10 μg/ml) or LPS (100 ng/ml) for 1 h resulted in inhibition of ODC activity over LPG or LPS treated groups ($P < 0.001$) (Fig. 5).

An attempt was made to see if the inhibitory effect of staurosporine could be overcome by okadaic acid (OKA), a specific inhibitor of protein serine-threonine phosphatase 1 and 2A that has little effect on protein tyrosine phosphatases (Reiner, 1987; Reiner et al., 1987). It was observed that when macrophages were treated with 0.2 μM OKA for 15 min, a significant inhibition of ODC activity over macrophage alone values was observed ($P < 0.001$). Next, macrophages were pretreated for 15 min with 2 μM staurosporine and 0.2 μM OKA followed by treatment with LPG (10 μg/ml) or LPS (100 μg/ml) for 1 h. In this case, the inhibition of ODC activity caused by staurosporine was not abrogated by OKA in LPG treated groups (Fig. 6). However, it was observed that OKA could partially abrogate the inhibition of ODC caused by staurosporine in LPS-treated group (Fig. 6).

Long-term treatment of macrophages with 100 ng/ml phorbol myristate acetate (PMA), a specific inhibitor of PKC (Rodriguez et al., 1984; Young et al., 1987) is known to exhaust the supply of PKC in macrophages (Murray, 1982). When macrophages were pretreated with PMA for 18 h and subsequently treated with LPG (10 μg/ml) for 1 h, a significant inhibition of ODC activity over LPG-treated groups ($P < 0.001$) was observed. This hinted at the role of PKC in LPG induced activation of ODC within macrophages (Fig. 7). A similar observation was made when macrophages were pretreated with PMA (100 ng/ml) for 18 h and then treated with LPS (100 ng/ml) for 1 hr (Fig. 7).

To further indicate that the LPG-induced activation of ODC in macrophages occurred through a PKC signal transduction event, J774A.1 macrophages were pretreated

with the PKC inhibitor, H7 (50 nM) (Kambe et al., 2000) for 5 min and then stimulated with LPG (10 μg/ml) or LPS (100 ng/ml). In H7-pretreated cells, ODC activity was inhibited at 1 h by 20% and 84% in response to both LPG and LPS, respectively (Fig. 8).

To determine if LPG was capable of impairing the protein kinase A (PKA) associated signal transduction pathway, macrophages were pre-incubated for 1 h with or without LPG, followed by subsequent treatment with 1 mM dBcAMP for 30 min (Fig. 9). As shown in Fig. 9, macrophages incubated with LPG had no significant difference in ODC activity when compared to macrophages pre-incubated with LPG and subsequently treated with dBcAMP.

4.0 Discussion

Leishmania donovani is an obligate intracellular parasite that resides within mammalian macrophages. The molecular basis of this host-parasite interaction is not understood. Macrophages require activation for killing any intracellular pathogen. Several early genes, like c-fos, c-jun, and c-myc, are known to express during the development and activation in mature macrophages (Hamilton and Adams, 1987). Earlier reports have also indicated the role of the gene encoding ornithine decarboxylase (ODC) in activation of mononuclear phagocyte function (Kaczmarek and Kaminska; 1989, Butler et al., 1991). Bacterial lipopolysaccharide is known to induce a marked and rapid increase in the accumulation of the ODC gene transcript (Messina et al., 1990). It has also been reported that recombinant human interferon- γ (IFN-γ) induced ODC mRNA accumulation in human monocytes (Messina et al., 1990). These observations indicate that accumulation of ODC mRNA is a marker for macrophage activation. The present study was designed to investigate the effects of a major surface molecule of *L. donovani*, lipophosphoglycan, on ornithine decarboxylase activity in J774A.1 macrophages. Involvement of protein kinase C

(PKC) in the regulation of ornithine decarboxylase has been reported earlier (Mustelin et al., 1987; Butler et al., 1991). However, other factors in addition to PKC may be required for ODC induction (Butler et al., 1991). The present study is focused on the ability of protozoan lipophosphoglycan (LPG) to affect macrophage signal transduction.

Lipophosphoglycan was found to stimulate ODC in J774A.1 murine macrophages in a dose-and time-dependent manner, confirming the possible role of ODC as an early marker of macrophage activation. LPG stimulated ODC activity within 60 min after exposure, suggesting that the interaction of LPG with its receptor stimulated a specific signal transduction pathway within the macrophages. The ODC gene, like the c-fos gene, is among the early genes that are expressed immediately after macrophage activation with LPS and thus represents a useful marker to examine signal transduction in macrophages (Hamilton and Adams, 1987).

LPG was found to stimulate ODC through protein kinase signal transduction as judged by the use of a broad-spectrum inhibitor of protein kinase, staurosporine. The use of OKA along with staurosporine showed that a protein serine-threonine kinase might not be the relevant target for staurosporine within macrophages. Another possibility could be that 15 min pretreatment with OKA may not have been sufficient to cause changes in the degree of protein phosphorylation. However, OKA under similar conditions partially abrogated the inhibition of ODC caused by staurosporine in LPS-treated macrophages. Specific inhibitors of protein kinase C were used to determine if PKC present in the macrophage is the relevant target for staurosporine. Previous studies have shown that PKC signal transduction pathway is a possible intracellular target for LPG (Moore et al., 1993; Descoteaux et al., 1992; Descoteaux and Turco, 1993). Depletion of PKC by PMA or H7 treatment greatly

reduced ODC activity in response to LPG. This demonstrated the involvement of PKC-dependent stimulus response in macrophages exposed to LPG.

Lipophosphoglycan (LPG) did not inhibit induction of ODC activity by dibutyryl cyclic AMP (dBcAMP). This result ruled out the role of PKA in LPG stimulation of ODC activity. This is consistent with the previous finding that LPG has no *in vitro* effect on PKA activity (McNeely and Turco, 1987). Since the ODC activity is stimulated immediately after macrophage activation with LPG, it represents a useful marker to examine signal transduction in macrophages.

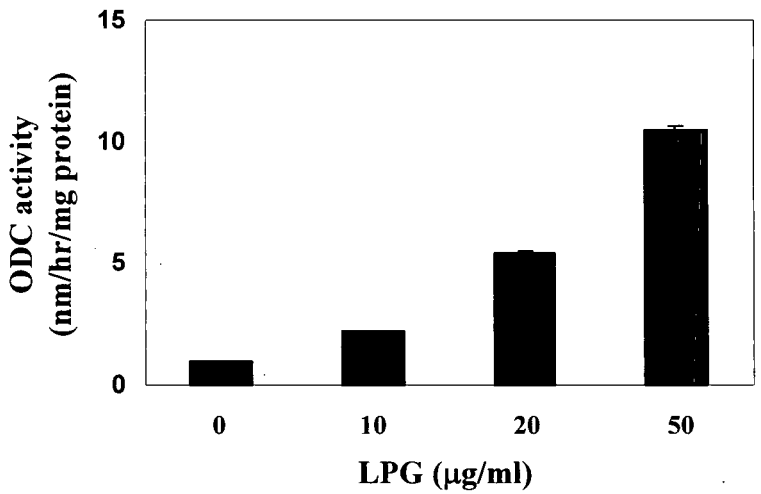

Fig. 1: Dose-dependent effect of lipophosphoglycan (LPG) on ornithine decarboxylase (ODC) activity in J774A.1 macrophages. Macrophages were treated with different concentrations of LPG for 1 h at 37°C and the ODC activity was measured. Values are representatives of duplicate samples in replicate sets.

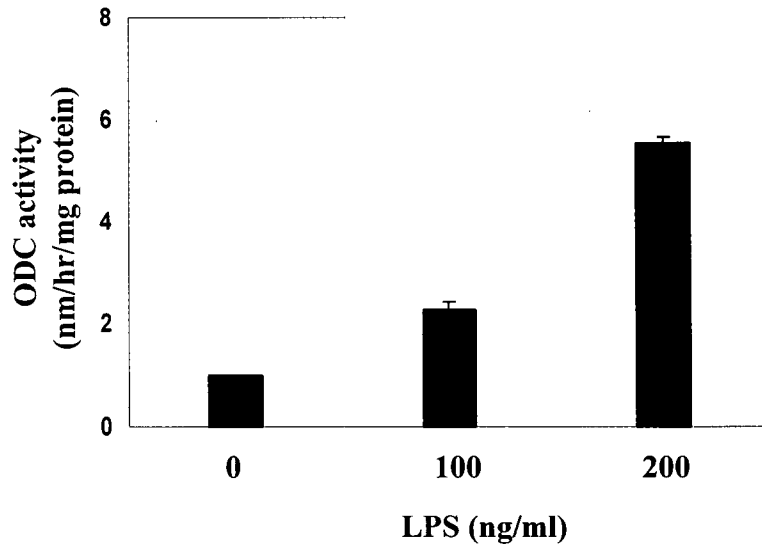

Fig. 2: Dose-dependent effect of lipopolysacchride (LPS) on ornithine decarboxylase (ODC) activity in J774A.1 macrophages. Macrophages were treated with different concentrations of LPS for 1 h at 37°C and the ODC activity was measured. Values are representatives of duplicate samples in replicate sets.

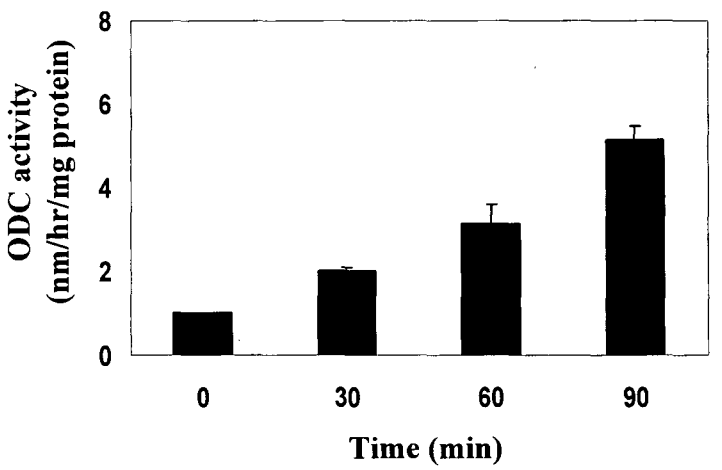

Fig. 3: Time-dependent effect of 10 μg/ml lipophosphoglycan (LPG) on ornithine decarboxylase (ODC) activity in J774A.1 macrophages. LPG was added onto macrophages, followed by incubation for different time periods, after which the ODC activity was measured. The results are mean ± SD of 4 samples.

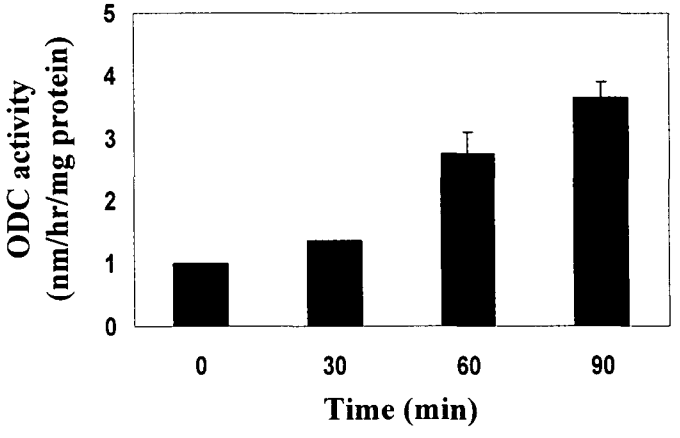

Fig. 4: Time-dependent effect of 100 ng/ml lipopolysaccharide (LPS) on ornithine decarboxylase (ODC) activity in J774A.1 macrophages. LPS was added onto macrophages, followed by incubation for different time periods, after which the ODC activity was measured. The results are mean ± SD of 4 samples.

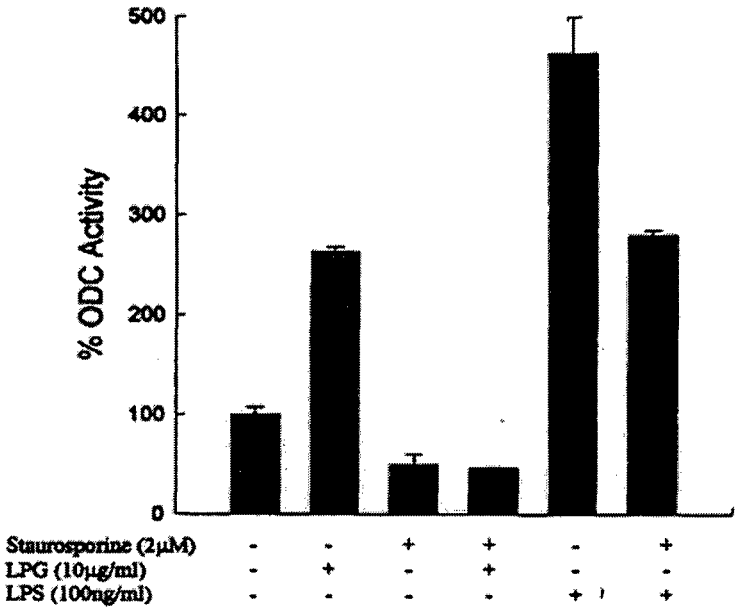

Fig. 5: Effect of the protein kinase inhibitor staurosporine on ornithine decarboxylase (ODC) activity in J774A.1 macrophages. Macrophages were treated with either 2 μM staurosporine for 15 min, or 10 μg/ml lipophosphoglycan (LPG) or 100 ng/ml lipopolysaccharide (LPS) for 1 h. Alternatively, they were pretreated with 2 μM staurosporine for 15 min followed by LPG (10 μg/ml) or LPS (100 ng/ml) treatment for 1 h and assayed for ODC activity. Values are representative of duplicate samples in replicate sets. + represents presence of and - represents absence of LPG, LPS or staurosporine.

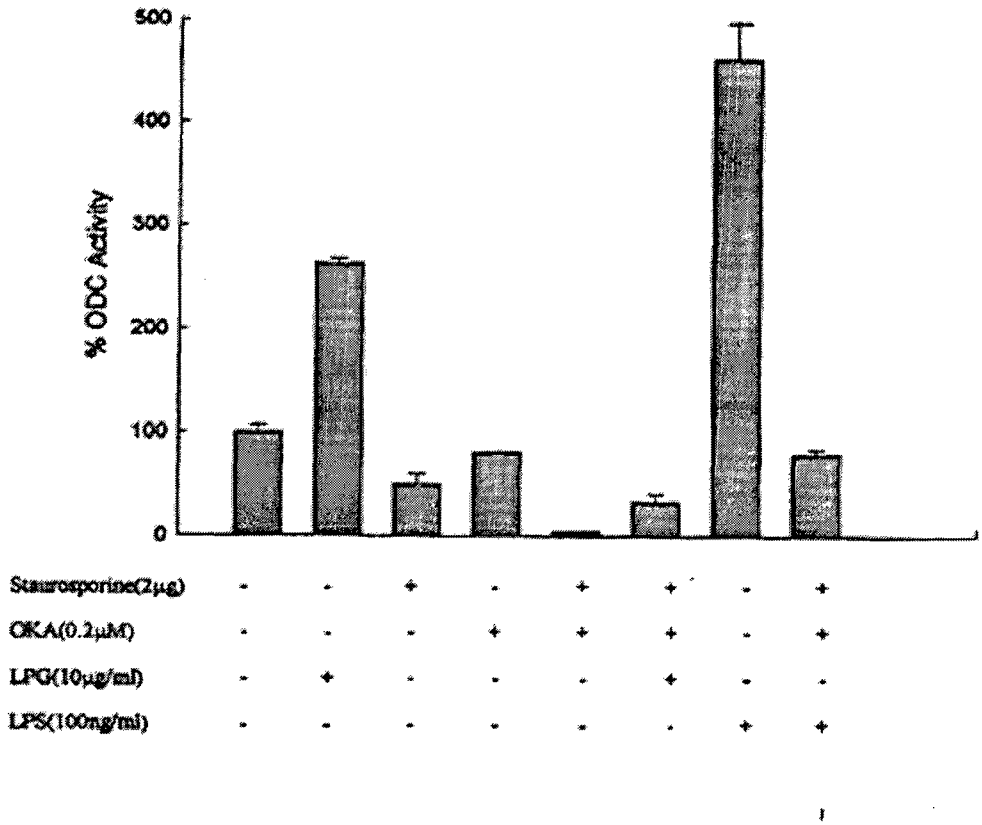

Fig. 6: Effect of okadaic acid (OKA) on ornithine decarboxylase (ODC) activity in J774A.1 macrophages. Macrophages were treated with 2 μM staurosporine for 15 min, 0.2 μM OKA for 15 min, or 10 μg/ml lipophosphoglycan (LPG) or 100 ng/ml lipopolysacchride (LPS) for 1 h. In other sets, macrophages were pretreated with 2 μM staurosporine for 15 min followed by 10 μg/ml LPG or 100 ng/ml LPS treatment for 1 h, or pretreated with 2 μM staurosporine and 0. 2 μM OKA for 15 min followed by LPG or LPS treatment for 1 h. They were then assayed for ODC activity. Values are representative of duplicate samples in replicate sets. + represents presence of and - represents absence of LPG, LPS, staurosporine or OKA.

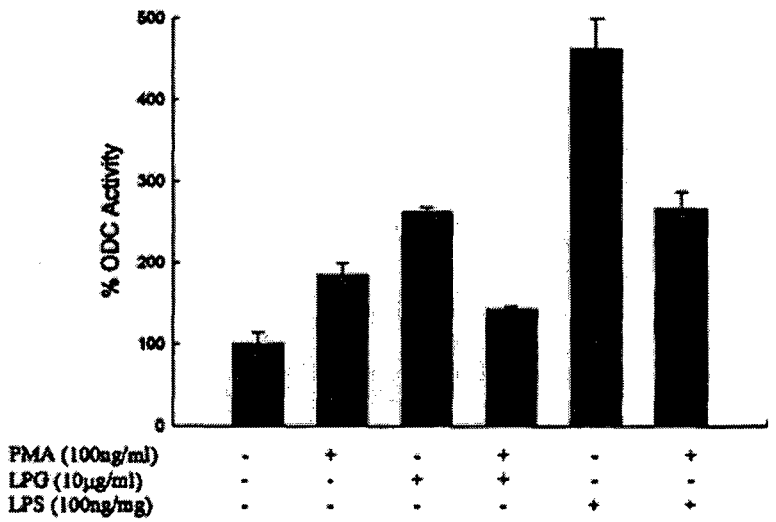

Fig. 7: Effect of protein kinase C (PKC) inhibitor phorbol myristate acetate (PMA) on ornithine decarbboxylase (ODC) activity in J774A.1 macrophages. Macrophages were pretreated with PMA (100 ng/ml) for 18 h and then stimulated with 10 μg/ml lipophosphoglycan (LPG) or 100 ng/ml lipopolysacchride (LPS) for 1 h. The cells were then harvested to measure ODC activity. Values are representative of duplicate samples in replicate sets. + represents presence of and – represents absence of LPG, LPS or PMA.

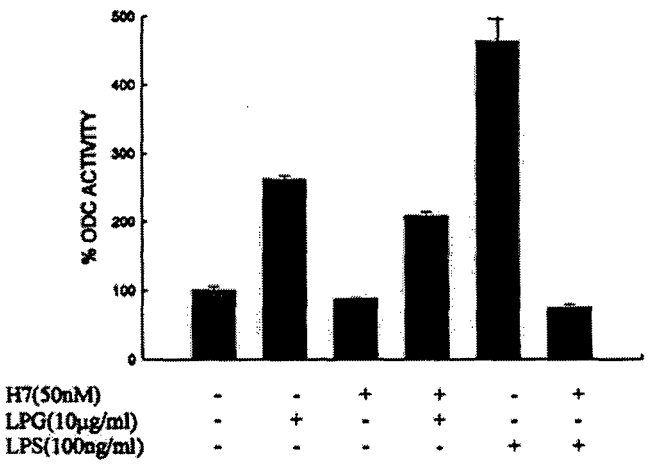

Fig. 8: Effect of protein kinase C inhibitor H7 on ornithine decarboxylase (ODC) activity in J774A.1 macrophages. Macrophages were pretreated with 50 nM H7 for 15 min and then stimulated with 10 μg/ml lipophosphoglycan (LPG) or 100 ng/ml lipopolysacchride (LPS) for 1 h. The cells were then harvested to measure ODC activity. Values are representative of duplicate samples in replicate sets. + represents presence of and – represents absence of LPG, LPS or H7.

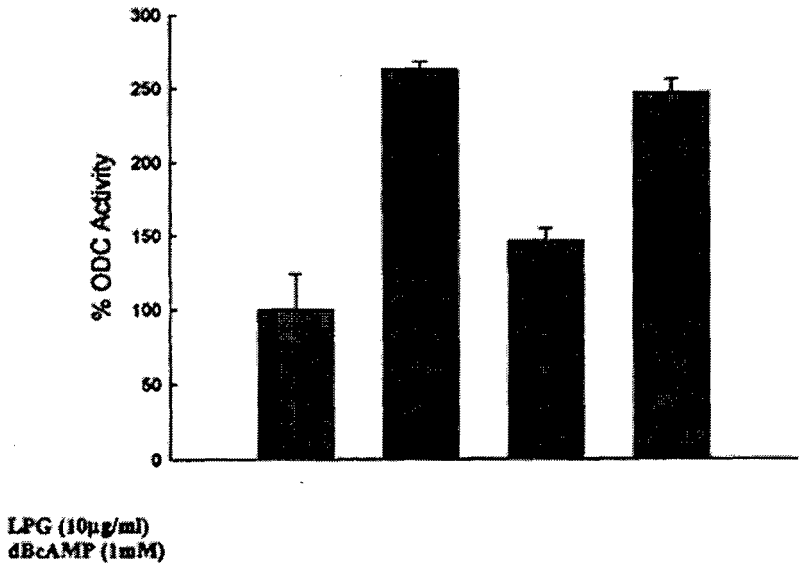

Fig. 9: Effect of dibutyryl cyclic AMP (dBcAMP) on lipophosphoglycan (LPG)-induced ornithine decarboxylase (ODC) activity in J774A.1 macrophages. Macrophages were treated with 1.0 mM dBcAMP for 30 min or with 10 µg/ml LPG for 1 h. Alternatively they were pretreated with LPG (10 µg/ml) for 1 h and then treated with 1 mM dBcAMP for 30 min. They were then assayed for ODC activity. Values are representative of duplicate samples in replicate sets. + represents presence of and – represents absence of LPG or dBcAMP.