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Modulation of Signal Transduction in *Leishmania donovani*-infected macrophages by a phorbol ester

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Abstract

Visceral Leishmaniasis is the most severe form of leishmaniasis affecting millions of people every year. After attachment to host macrophages parasites send signals to interior of macrophages, whereupon host-defence mechanisms are initiated to combat the infection. How this signalling mechanism can be controlled to our advantage to disable the parasite from infecting the macrophages, is the aim of this study. The role of 12-O-tetradecanoyl-phorbol-13-acetate (TPA), a stimulator of Protein Kinase C (PKC), a key enzyme in signaling pathways involving both phosphatidyl inositides and phosphatidyl choline has been investigated. TPA has the advantage of being an analogue of Diacylglycerol (DAG), the only known physiological stimulator of PKC.

It has been observed that peritoneal macrophages infected with a pathogenic strain of *L. donovani* (AG- 83), can be inhibited upto 75% against attachment if they are exposed to TPA for 60 minutes prior to infection. The synergistic effect of PKC agonists like Calcium ion and Calcium ionophore A23187 with TPA increases % inhibition to attachment of the parasite to almost 90%. But when used alone, the extent of inhibition were 50% and 61% for Calcium ion and the ionophore respectively. Involvement of PKC was shown by use of a PKC inhibitor, Staurosporine. The inhibitor increased the parasite load in macrophages significantly but its effect could be significantly abolished by TPA, thereby strongly indicating involvement of PKC.

Key words : Singnal Transduction, *Leishmania denovani* Protein Kinase, macrophages, TPA.

Leishmania are protozoan parasites belonging to the family of 'Trypanosomatidae' and cause a wide variety of diseases collectively known as 'Leishmaniasis'. It is this resident capacity of the parasite that appears to protect it from many of the body's responses to infection[19] and as a result, there is no satisfactory treatment for any form of

Leishmaniasis. Of the various cells of the human system, macrophages are of particular interest as they play a significant role in host defence. Thus, in Leishmaniasis, which is a macrophage-associated disease, the macrophages play a dual role of serving as host cell for the parasites as well as an immune regulatory effector in infected animals. Hence, macrophages have been used for studying many aspects of host-parasite interaction in vitro[9]. It has been reported that murine peritoneal macrophages maintain an active alternate signaling pathway through hydrolysis of phosphatidylcholine, a major membrane phospholipid. The pathway can be stimulated by external agents, such as TPA, that results in increased generation of the lipid metabolites, DAG (diacylglycerol) and PA (phosphatidic acid) both of which are now recognized as important signaling molecules[1]. Moreover in preliminary experiments it has been observed that this agent is potential in inhibiting the attachment of the parasite to the macrophages after a short exposure. These observations encouraged me the present work as a novel one by understanding the actual underlying mechanism, which would help to identify the possible target(s) of action of TPA and similar agents.

TPA (12-O-tetradecanoyl-phorbol-13-acetate) binds and activates protein kinase C, causing a wide range of effects in cells and tissues[3,14]. PKC is a well characterized example of calcium and phospholipid dependent cellular kinases, which plays a pivotal role in the regulation of a variety of cell functions[20]. Since leishmania promastigotes must attach themselves to the host cells before entry, the plasma membrane of the macrophage which have several receptors on its surface, plays a important role[10].

Reagents : RPMI 1640 with L-glutamine, M199 and FBS were from GIBCO Laboratories, USA, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), PKC agonist Calcium ion and Calcium ionophore A23187, PKC inhibitor Staurosporine were purchased from Sigma Chemical (St.Louis, MO).

Type of study: Experimental type of study.

Source of Balb/c mice: Animals procured from in-house facility.

Sample size: Five.

Ethical permissions: No ethical permission is required.

Isolation of peritoneal macrophages :

Essentially, Balb/c mice were injected with thioglycollate broth four days prior to the actual isolation of macrophages by the method of Russell *et al.*,[25], in order to obtain a sufficient number of activated macrophages. The cells were isolated by peritoneal lavage of the mice with sterile, ice-cold PBS and collected by centrifugation. They were washed once with RPMI 1640 medium containing 5% fetal bovine serum.

Maintenance and preparation of L. donovani promastigotes :

UR-6 strain, a nonpathogenic strain was routinely maintained at 22ÚC in the promastigote form in our laboratory on solid blood agar slants and were subcultured every fourth day. Before use the stationary phase parasites were suspended in the working

medium, *i.e.* RPMI 1640 with 20% FBS and used for in vitro infection of macrophages.

Radiolabeling of Macrophages :

The macrophages were labeled either with [^{3}H]- Palmitic acid or Myristic acid for 1 hour to label the fatty acyl chain of phosphatidylcholine or [Methyl ^{3}H]-choline for 24 hours in order to label the choline part of the molecule at 37°C. After removing the unincorporated counts by washing with the medium, the cells were resuspended in fresh medium for subsequent treatment with agonists/inhibitors or infection by parasites.

Infection of macrophages with the parasite:

Macrophages were allowed to interact with the parasite at 37°C in a definite ratio for different periods of time. The macrophage: parasite ratio and the time of contact were depend upon the extent of infection wanted. Non-ingested parasites were removed by three washes in RPMI 1640 complete medium.

Treatment of macrophages with different agents:

For activation of protein kinase C of the macrophages, labeled cells were incubated with TPA (100ng/ml) for 60 mins at 37°C, prior to infection. Suitable controls, *i.e.* incubations with and without TPA and parasite treatments were included in the experiments.

Extraction of total lipids from macrophages:

Total lipids from macrophages after incubation were extracted by a modification of the Bligh and Dyer procedure. In brief, the macrophage suspension were first extracted with 3.75ml of 2:1 methanol : chloroform(v/v) for 1.5hrs, followed by extraction with 4.75ml of 2:1:0.8 methanol: chloroform : water (v/v) for 1hr.

Characterization of phospholipid metabolites by thin layer chromatography:

In routine, radiolabeled phosphatidic acid (PA) were resolved in solvent system containing chloroform/pyridine/70% formic acid (50:30:7, v/v) while radiolabeled DAG was identified by thin layer chromatography (TLC) in hexane/diethylether/acetic acid (60:40:1, v/v).

Effect of calcium ion upon parasite attachment to macrophages :

It is evident from Table 1 after TPA treatment 79% increased inhibition of parasite attachment compared with macrophage treated with parasite only. Macrophages treated with TPA, Ca^{++} and parasite showed 1.68% increased inhibition of attachment in comparison with macrophages treated with Ca^{++} and parasite only. Synergistic effect of Ca^{++} and EGTA decreased 6.4 × fold decreased inhibition compared with Ca^{++}, EGTA and TPA. Calcium plays crucial roles in different signaling pathways in both animal and plant cells. To evaluate the role of calcium ion on parasite attachment to macrophages 1mM calcium ion and EGTA was treated for 15 minutes. Parasites treated with 1mM calcium ion alone for 15 minutes 50% inhibition of attachment achieved. Synergistic effect of calcium ion and EGTA exhibits only

Table-1 *L. donovani* attachment profile to Macrophages: Effect of Ca^{++} ion

System	% attachment	% inhibition
MΦ+ P	39.5±2.8	---
MΦ+ TPA + P	8.4±0.9*	79±5.2
MΦ + Ca^{++} + P	19.7±1.8	50±3.7*
MΦ + Ca^{++} + TPA + P	6.4±0.8*	84.1±5.6
MΦ + Ca^{++} + EGTA + TPA + P	9.2±0.91*	77.0±4.8
MΦ + Ca^{++} + EGTA + P	34.2±2.5	11.8±1.1*

*The values are expressed as mean ± SD of five independent experiments, P< 0.001

11.8% inhibition as EGTA chelates the function of calcium ion. TPA treatment at 200ng/ml for 60 mins. Parasite infection at MΦ : Parasite ratio for 60 minutes at 37°C, Coverslips stained with Giemsa and at least 10 fields scored/ coverslip, (Table-1).

Effect of calcium ionophore A23187 upon parasite attachment to macrophages :

The calcium ionophore is used in laboratories to increase intracellular Ca^{++} levels in intact cells. To evaluate the role of calcium ionophore on parasite attachment to macrophages 2μM calcium ionophore was treated for 5 minutes, 61.4% inhibition of attachment achieved. Ionophore and TPA treatment showed 80% where ionophore and Ca^{++} showed 82% inhibition respectively. Synergistic effect of calcium ionophore, calcium ion and TPA exhibits upto 89.8% inhibition (Table-2).

Effect of PKC regulation upon parasite

Table- 2. *L. donovani* attachment profile to Macrophages: Effect of Ca^{++} ionophore A23187

System	% attachment	% inhibition
MΦ + P	48.5± 3.26	—
MΦ + TPA + P	10.6±0.93*	78.1±4.89
MΦ + I + P	18.7±1.74	61.4±3.67*
MΦ + I + TPA + P	10.1±0.92*	80.0±5.02
MΦ + I + Ca^{++} + P	8.5±0.86	82.4±5.26*
MΦ + I + Ca^{++} + TPA + P	5.0±0.61*	89.8±5.84

*As compared with the respective control P < 0.001, n= 5.

attachment in presence or absence of A23187 & TPA :

TPA treatment with staurosporine showed 78.5% inhibition as compared with staurosporine only. Staurosporine, (PKC inhibitor) treatment for 15 minutes at 500nM followed by Ionophore and TPA treatments shows 88% inhibition of attachment of parasites to macrophages shows approximately 2 × fold increased inhibition as compared with staurosporine and ionophore only clearly indicates the PKC regulation of attachment (Table-3). At least 10 fields were scored per coverslip. Infection carried out at MΦ : Parasite ratio of 1:10 for 60 minutes at 37°C.

Effect of PKC regulation upon parasite attachment in presence and absence of Ca^{++} & TPA:

Table-3.Effect of PKC regulation on the attachment of *L. donovani* to Macrophages in presence or absence of A23187 & TPA

System	% attachment	% inhibition	No. of att. P per 100 MΦ
MΦ + P	45.9±3.42	---	153±8.0
MΦ + TPA + P	12.1±1.22*	73.6±4.36	100±6.0
MΦ + S + P	52.7±3.86	---	205±9.0
MΦ + S + TPA + P	11.3±0.98*	78.5±4.88	105±6.0*
MΦ + S + I + P	27.8±1.89	47.2±3.25*	127±7.0
MΦ + S + I + TPA + P	6.3±0.76*	88.0±5.78	73±5.0*

*As compared with respective control $P < 0.001$, $n = 5$

Data in Table-4 shows 71% inhibition of attachment after macrophages treated with TPA and parasite compared to macrophages treated with parasite only. Addition of TPA with macrophage treated with parasite and staurosporine showed 78% inhibition of attachment compared with macrophages treated with parasite and staurosporine only. Ca^{++} augmented inhibition of attachment 67.5% but after EGTA treatment 6.75 × fold decrease of inhibition observed. as EGTA chelates Ca^{++}. Synergistic effect of Ca^{++} and TPA shows 1.2 × fold increased inhibition of attachment as compared with macrophages treated with Ca^{++}, parasite and staurosporine. Staurosporine treatment same as in table-3 (Table-4).

Modulation of PC- Hydrolytic pathway by TPA and Staurosporine during macrophage-Parasite interaction :

PKC agonist TPA had significant stimulatory effect on phosphatidylcholine (PC) breakdown that was sensitive to staurosporine, as evidenced by increased formation of lipid metabolites DAG & PA. TPA used at 200ng/ml for 60 minutes and staurosporine at 500nM for 15 minutes at

Table-4. Effect of PKC regulation on *L. donovani* attachment to Macrophages in presence and absence of Ca^{++} & TPA

System	% attachment	% inhibition
MΦ + P	30.4±2.14	---
MΦ + TPA + P	8.7±0.92*	71.4±4.2
MΦ + S + P	46.7±3.16	---
MΦ + S + TPA + P	10.2±0.88*	78.2±4.84
MΦ + S + Ca^{++} + P	15.2±1.3*	67.5±3.86*
MΦ + S + Ca^{++} + EGTA + P	41.9±3.06	10.0±0.85*
MΦ + S + Ca^{++} + TPA + P	8.2±0.86*	82.4±5.08

*As compared with respective control $P < 0.001$, n = 5.

37°C. Data in Table-6 indicates that normal macrophages possess an endogenous DAG and PA counts that could be detected even in absence of TPA. DAG counts stimulated 66% and 116% following treatment of macrophages with parasite and TPA respectively where PA counts stimulated 32% and 57% respectively. Macrophage treated with staurosporine exhibits no stimulation in DAG and PA counts with respect to macrophage control. Macrophage treated with TPA and parasite exhibits more than 4 × fold stimulation in DAG counts but no significant changes observed in PA counts. Macrophages treated with staurosporine, TPA and parasite showed 153% stimulation in DAG counts compared with macrophage treated with staurosporine and parasite only, but 6.5 × fold stimulation in PA counts. $P < 0.02$ indicating macrophage treated with staurosporine compared with macrophage control only (Table-5).

Table-5. Modulation of PC-Hydrolytic pathway by TPA, a PKC agonist and Staurosporine, a PKC antagonist, during Macrophage-Parasite interaction
Counts per minute/1.25×10^6 cells

System	DAG	% Stimulation	PA	% Stimulation
MΦ only	427±18*#	---	13613±56*	---
MΦ+P	709±28*	66±5*	17958±61*	32±2.6*
MΦ+TPA	923±32	116±8.0	21414±75	57.3±4.6
MΦ+S	438±24	---	10675±53	---
MΦ+TPA+P	1672±40	290±15	18684±64	37±3.2
MΦ+S+P	436±21*	---	14491±58*	6.4±0.56*
MΦ+S+TPA+P	1080±38	153±10	19305±68	42±3.8

*As compared with respective control $P < 0.001$, n = 5
#As compared with respective control $P < 0.02$, n = 5

The biochemical mechanisms involved in cell signaling by phosphoinositide breakdown are now well established[1,8,16]. This classical scheme involving phosphoinositide as the sole source of agonist induced DAGs has since been challenged. Other investigations have produced ample evidence in favour of cellular phosphatidyl choline as an alternate and steady source of second messengers such as PA and DAG, for the activation of the signaling pathway in various cells[2,6,9,15,25]. PLD is regulated by ADP-ribosylation factor (ARF), a second small GTP-binding protein called Rho and lastly, protein kinase C [5,26].

In terms of geographical spread and the number of human beings suffering from some form of Leishmaniasis or the other, this disease has been identified by WHO as the second major parasitic disease in the world, next to malaria[30]. There was a severe epidemic in the entire eastern part of the subcontinent during the period 1917- 1937 [26], with an intensified situation in Bihar from 1935-1937 [27]. The other major drawback to the use of antimonials is emergence of resistance to these drugs which according to the Report of Research Training in Tropical diseases[21], is reaching alarming proportions in India. The second-line drugs Amphotericin B [15] and Pentamidines are too toxic to be used as first-line therapy on a large scale. Importance of protein kinase C in host-parasite interplay in Leishmaniasis was reported in 1966 where it was shown that attachment of the promastigote form of *L. donovani* to the macrophage elevated the level of PKC whereas the same enzyme was down regulated once the parasites get internalized within the host[4]. So obviously plays a key regulatory role in the infection process and my aim will be to use agent(s) which will be able to modulate the function of this regulatory enzyme in a manner so as to block the extent of attachment by the parasite on to the host surface. One such appropriate agent is TPA (12-O-tetradecanoyl phorbol - 13-acetate) which is known PKC activator and there are a number of reports that several facets of cell membrane lipid metabolism are modified in response to TPA treatment[7]. The phorbol ester tumor promoter, 12- O- tetra-decanoylphorbol 13- acetate (TPA), stimulates cell proliferation through rapid activation of protein kinase C (PKC), followed by gradual degradation of the kinase. Signal Transduction is a process by which extracellular signals are received and transduced across the cell membrane to the nucleus resulting in elicitation of receptor-mediated characteristic responses. These responses are coupled to various second messengers that are generated from interaction between surface receptors on macrophages and various agonists or extracellular signals[16]. 1,2-diacylglycerol (DAG) is one such messenger molecule, which takes part in many of the macrophage associated responses. DAG can be produced in the host cell by hydrolysis of either phosphatidylinositol (PI) or phosphati-dylcholine (PC). Accordingly, the PI- hydrolysis constitutes the Classical Pathway[2] and PC-hydrolysis, the Alternate Pathway[18] of Signal Transduction. It has been explored in this study the means by which formation of DAG from PC- hydrolysis can be influenced in a way to hamper attachment of parasite itself on to the macrophage surface. The rationale behind choosing the alternate pathway of signal transduction for our study, is that the second messenger DAG unlike the classical pathway, is produced in a pronounced manner over a

longer period of time, as a consequence to a sustained stimulation of the key enzyme Protein Kinase C by external stimuli[13,14].

In conclusion, the present study clearly demonstrates that murine macrophages are capable of hydrolyzing their major phospholipid PC to generate the TPA- induced second messengers PA and DAG. Since macrophages are the principal phagocytic cells and play a crucial part in host defence against various pathogens, it would be interesting to find out how the signals sent by the pathogens influence this alternate pathway of signal transduction in these cells. Incidentally altered signal transduction in the macrophages infected with *L. donovani* has been noticed in relation to a selective impairment of PKC-mediated C- fos gene expression[22]. Unpublished observations from my laboratory also shows that TPA can provide macrophages with a protective role against parasite infection such as, by *L. donovani*. In light of this, the present study is important as it demonstrates the possible mechanism of sustained DAG formation from PC hydrolysis in response to an external stimulus, such as TPA, which may be a prerequisite for conferring a protective function to the macrophages against such infections. Thus, future studies in this direction might provide valuable informations regarding control and regulation of various types of infections.

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