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Effects of prostaglandin E_2, cholera toxin and 8-bromo-cyclic AMP on lipopolysaccharide-induced gene expression of cytokines in human macrophages

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SUMMARY

Prostaglandin E_2 (PGE_2) appears to regulate macrophage cytokine production through the stimulatory GTP-binding protein (Gs protein)-mediated cyclic AMP (cAMP)-dependent transmembrane signal transduction pathway. In this study, we used PGE_2, cholera toxin (CT; a direct $G\alpha s$ protein stimulator) and 8-bromo-cAMP (a membrane permeable cAMP analogue) to stimulate this pathway, and investigated their influence on cytokine gene expression in lipopolysaccharide (LPS)-activated human macrophages. The mRNA expression for interleukin-1α (IL-1α), IL-1β, tumour necrosis factor-α (TNF-α), IL-6 and IL-8 were determined employing reverse transcription polymerase chain reaction (RT-PCR) using specific primers. We demonstrated that PGE_2, CT and 8-bromo-cAMP inhibited the LPS-induced gene activation of TNF-α and IL-1α, and had no effect on the gene activation of IL-1β and IL-8. Further, our data indicate that PGE_2 suppressed the gene activation of IL-6 following LPS stimulation, but neither CT nor 8-bromo-cAMP had an effect. These data suggest that PGE_2 alters LPS-stimulated gene activation of only some of the early macrophage cytokines, and does so either by a Gs transmembrane cAMP-dependent or an independent system.

INTRODUCTION

The mononuclear phagocyte system plays a central role in the regulation of the immune response in both acute and chronic inflammation by releasing proinflammatory mediators including the cytokines. The responsiveness of macrophages to external stimuli generally involve a series of intracellular biochemical changes that alter the cell function. Such transmembrane signalling pathways frequently include the generation of intermediate intracellular signals collectively termed as second messengers. Cyclic AMP (cAMP) is perhaps the best known and understood of the second messengers[1] and generally considered to play a suppressive role in immune regulation and mononuclear phagocyte function.[2] Prostaglandin E_2 (PGE_2) is a potent intracellular cAMP elevator and have been clearly shown to inhibit many *in vitro* and *in vivo* immune responses.[3–6] Although the mechanisms through which the prostaglandins regulate the immune system are not well elucidated, a growing body of evidence suggests that the regulatory effects of PGE_2 on the immune system are accomplished through the activation of the cAMP-dependent intracellular signal transduction pathway of immunocytes.[7,8] This signal pathway appears to be at least one of the major important intracellular regulatory systems of immune cells. Gram-negative bacterial endotoxin (lipopolysaccharide; LPS) is a potent cytokine initiator in severe Gram-negative infection and has been widely used as a macrophage stimulator to investigate the mechanisms underlying cytokine production *in vitro*. In the present study, we investigated the mechanisms underlying cytokine production *in vitro*. We investigated the influence of PGE_2 and the cAMP-dependent transmembrane signal pathway linked to the PGE_2 receptor on the regulation of LPS-induced cytokine gene expression.

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MATERIALS AND METHODS

Reagents

The RPMI-1640 medium and Hanks' buffered salt solution (HBSS) were obtained from Whittaker Bioproducts (Walkersville MD). Human AB serum, PGE_2 and cholera toxin (CT) were purchased from Sigma Chemical Co. (St Louis, MO), fetal bovine serum was from Hyclone (Logan, UT) and LPS (from *Escherichia coli* 055:B5) was from Difco Laboratories (Detroit, MI). Ficoll–Paque was obtained from Pharmacia LKB (Uppsala, Sweden), deoxynucleoside triphosphate (dNTP) and *Taq* DNA polymerase were purchased from Promega (Madison, WI), Moloney murine leukemia virus reverse transcriptase was obtained from Bethesda Research Laboratories (Bethesda, MD). Oligo$(dT)_{12-18}$ was from

Boehringer Mannheim (Indianapolis, IN) and the specific primers for human β-actin, interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α), IL-6 and IL-8 were from Clontech (Palo Alto, CA) and that of IL-1α was from Perkin Elmer-Cetus (Norwalk, CT).

Isolation and culture of human monocyte-derived macrophages

Venous blood was collected from healthy adult male volunteers and peripheral blood mononuclear cells (PBMC) were isolated after Ficoll–Paque gradient centrifugation. Cells were plated in 6-well polystyrene plates (at 10×10^6 cells/well in 5 ml of RPMI-1640 supplemented with 10% human AB serum, 100 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine and 25 mM HEPES buffer) and after 24 hr of incubation the non-adherent cells were removed by changing the medium. The adherent cells were then cultured for 10 days to allow differentiation[9] under the same conditions by changing the medium every 3 days. At the end of the differential culture, almost all the cells that remained adhering on the plastic surface of the culture dishes had morphology changes, i.e. the cells spread and had a fibroblast-like appearance which is characteristic of monocyte-derived macrophages.[9] In randomly selected cultures more than 80% of the cells were non-specific esterase positive by an α-naphthyl acetate esterase determination.[10]

Modulation of LPS-induced macrophages activation by PGE_2, CT and cAMP analogue

After 10 days in culture, the medium was removed and the cells were washed twice with HBSS. Five millilitres of RPMI-1640-supplemented media with 2% fetal bovine serum was added to each well and incubated for 4 hr. The cells were preincubated in the presence or absence of PGE_2 (10^{-5} M), CT (10 μg/ml), or 8-bromo-cAMP (10^{-4} M) for 10 min and then stimulated without (control) and with LPS (1 ng/ml) for 4 hr. The optimal physiological and pharmacological concentrations of LPS and other modulators were obtained from the results of our previous studies and studies conducted by other investigators using similar experimental conditions to get an optimal biological response without or with minimal cytotoxicological effect on different immunocytes.[11–13] At the end of these treatments the cells were washed twice with HBSS and RNA was extracted immediately.

RNA isolation

The total RNA was isolated from macrophages by the method of acid guanidium–thiocyanate, phenol/chloroform extraction.[14] The isolated RNA pellet was washed with 75% ethanol, dried in a vacuum oven and dissolved in deionized distilled water.

Reverse transcription (RT)

The RNA was reverse transcribed to the first chain of complementary DNA (cDNA) [in 50 mM Tris–HCl, pH 8·3, 75 mM KCl, 10 mM dithiothreitol (DTT), 3 mM $MgCl_2$, 1 mM each of dGTP, dATP, dCTP and dTTP (dNTP), 2·5 mM random hexamers or oligo$(dT)_{12-18}$ and 1 U/μl of placental ribonuclease inhibitor] using Moloney murine leukemia virus reverse transcriptase (2·5 U/μl). This incubation step was carried out, first, at 25° for 10 min and then at 42° for 60 min. The reaction was stopped by heating to 99° for 5 min and then quick chilled on an ice bath.

Amplification of specific cDNA by polymerase chain reaction (PCR)

Aliquots of first-chain cDNA were amplified specifically by PCR at a final concentration of 50 mM KCl, 10 mM Tris–HCl, pH 9·0, 1·5 mM $MgCl_2$, 0·01% gelatin (w/v), 0·1% Triton X-100, 0·2 mM of each of the dNTP, 0·15 μM each of 5′ and 3′ specific primers and 25 U/ml *Thermus aquaticus* DNA polymerase (*Taq* polymerase) in a total volume of 50 μl. The mixture was overlaid with mineral oil and then amplified in a Perkin-Elmer/Cetus Thermal Cycler (model 480). The amplification profile involved denaturation at 95° for 30 seconds, primer annealing at 60–65° for 1 min (according to the primer sequence) and extension at 74° for 1 min, for a total of 20–25 cycles. Following this, an extended incubation at 74° for 7 min was carried out. From the results of our previous study, at this experimental condition, the amplified DNA signals of each cytokine gene expression are optimal in terms of comparison to each other.[11] All amplifications were repeated at least twice and on two separate reverse transcription products. Negative controls included amplification of reverse transcription mixtures incubated without reverse transcriptase to assess DNA contamination in the RNA samples, and amplification of the PCR mixture without cDNA to assess contamination at later stages. A 5–10-μl aliquot of PCR product was electrophoresed in 2% agarose gel stained with 0·5 μg/ml ethidium bromide, visualized under an ultraviolet light and photographed with Polaroid Type 55 positive/negative film. Specific amplification was determined by the size of the product on the gel relative to known markers from *Phi*X174 phage DNA cut with restriction endonuclease *Hae*III. The amount of PCR product was semiquantitatively determined by measuring the density of the specific bands on the negative film with a densitometer. β-Actin was expressed at a constant level in most cells and served as a control for both the cell viability and multiple steps in sample preparation. The density of a specific cytokine PCR band was compared with that of β-actin in the same sample and expressed as a ratio to normalize the data.

Data analysis

All data are expressed as an average of three to four experiments ± SEM. Relative mRNA levels (density ratio to actin) of each experimental group were compared to that of the control group alone (cells treated with LPS only) and the statistical significance in the differences was determined by Student's *t*-test.

RESULTS

Effect of LPS on gene expression of cytokines in human macrophages

Ten days after the initiation of the culture, the monocytes differentiated to macrophage-like cells and adhered to the plastic. Trypan blue exclusion test revealed that more than 80% of the cells were viable before the initiation of the experiment. After stimulation with different agents used in the experiment, the gene expression of β-actin was determined for both the cell viability evaluation and control of amount of RNA and experimental procedures. The result of RT-PCR shown in Fig. 1 indicated that for the control and for each of the different experimental conditions the cells expressed relatively

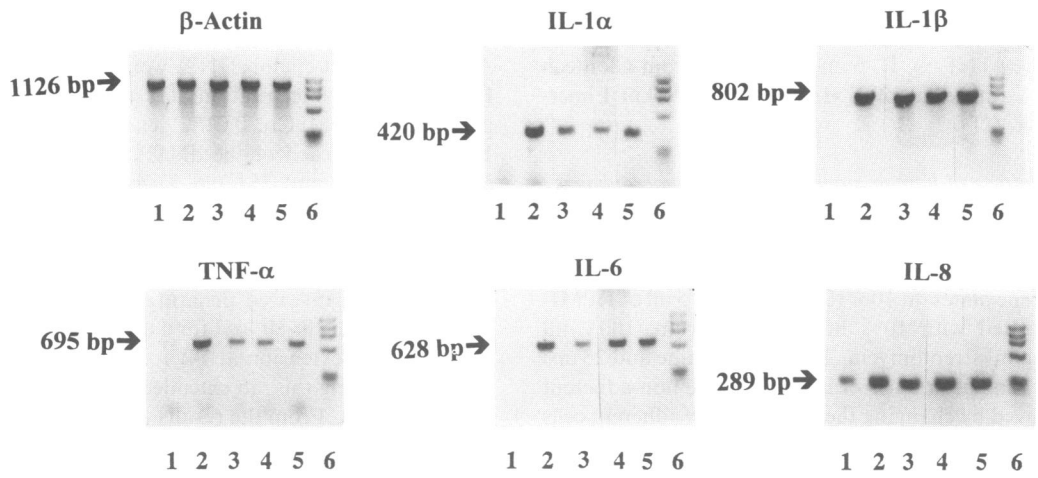

Figure 1. Photographs of representative RNA-PCR products electrophoresed on 2% agarose gel. Human monocyte-derived macrophages were cultured with PGE_2, CT and 8-br-cAMP, and then stimulated with bacterial LPS. Gene expression of different cytokines was determined by RNA-PCR as described in the Materials and Methods. Lane 1, control; lane 2, cells treated with LPS; lane 3, cells treated with PGE_2 and LPS; lane 4, cells treated with CT and LPS; lane 5, cells treated with 8-br-cAMP and LPS; lane 6, DNA marker. bp, base pairs.

the same amount of mRNA for β-actin, which excludes the possibility that the toxic effects of these agents may influence the results of the experiment.

Bacterial LPS is a potent stimulator for macrophage activation. In the present study we used LPS at a dose of 1 ng/ml to initiate the cytokine gene expression to investigate further the regulation of this gene expression by activating the cAMP signal transduction pathway. As we expected, in our experimental conditions, LPS stimulation initiated the gene activation of IL-1α, IL-1β, TNF-α, IL-6 and IL-8, as evidenced by an increase in their mRNA levels (lane 2 for each cytokine in Fig. 1).

Effects of PGE_2, CT and 8-bromo-cAMP on cytokine gene expression

We employed PGE_2, which binds to specific receptors and via the Gs-protein and adenylate cyclase, activates the cAMP-dependent pathway, to investigate the effects of this transmembrane pathway on cytokine gene expression. We used CT which directly stimulates the α subunit of Gs protein (Gαs) to activate the cAMP-dependent pathway at the post-receptor level. Cell permeable cAMP analogue 8-bromo-cAMP can act as intracellular cAMP and activate cAMP-dependent protein kinase (protein kinase A, PKA). In this study, we used 8-bromo-cAMP to mimic the effect of cAMP and activate the signal pathway at the intracellular second messenger level. After the selective activation of the cAMP-dependent pathway at different levels by these agents, we investigated their effects on gene expression of some important cytokines produced by macrophages and the data are summarized in Figs 1–6. Following LPS stimulation, the cells pretreated with PGE_2, CT or 8-bromo-cAMP significantly ($P < 0.05$) reduced mRNA expression of IL-1α and TNF-α compared to those stimulated with LPS alone (Figs 1–3). These data indicate that the effect of PGE_2 is likely through a G protein-mediated intracellular cAMP increase. Pretreatment of cells with PGE_2, CT, or 8-bromo-cAMP showed no significant differences in the LPS-induced gene expression for IL-1β and IL-8 compared to the cells stimulated with LPS alone (Figs 1, 4 and 5). These data indicate that stimulation of the receptors of PGE_2 or the Gs protein to increase intracellular cAMP and thus activate PKA are not involved with the gene regulation of these cytokines. For IL-6, pretreatment of the cells with CT or 8-bromo-cAMP showed no significant difference, but preincubation with PGE_2 significantly ($P < 0.05$) decreased LPS-induced IL-6 gene expression compared with those treated with LPS alone (Figs 1 and 6). These data suggest that a mechanism other than direct activation of the cAMP-dependent signal transduction pathway is responsible for the PGE_2 inhibitory effect on IL-6 gene expression.

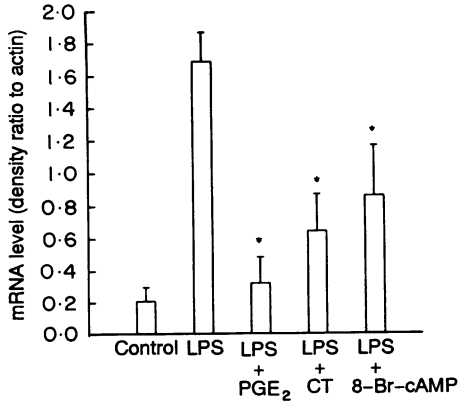

Figure 2. Effects of PGE_2, CT and 8-br-cAMP on bacterial LPS-induced IL-1α gene expression in human monocyte-derived macrophages. The level of gene expression was demonstrated as the ratio of the optical density (OD) of the band of RT-PCR product of IL-1α to that of actin. See the Materials and Methods for further details. *$P < 0.05$ compared to LPS group, Student's *t*-test.

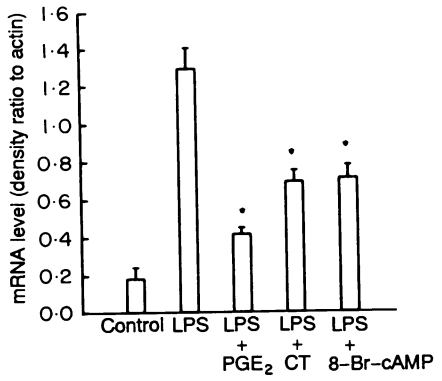

Figure 3. Effects of PGE_2, CT and 8-br-cAMP on bacterial LPS-induced TNF-α gene expression in human monocyte-derived macrophages. The level of gene expression was demonstrated as the ratio of the OD of the band of RT-PCR product of TNF-α to that of actin. See the Materials and Methods for further details. $*P < 0{\cdot}05$ compared to LPS group, Student's *t*-test.

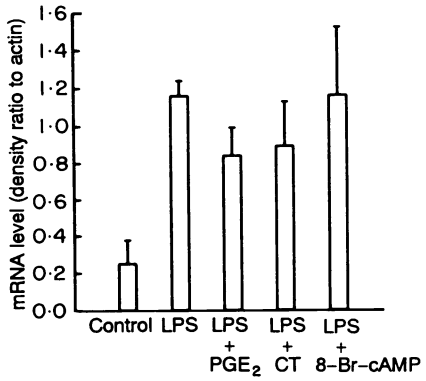

Figure 5. Effects of PGE_2, CT and 8-br-cAMP on bacterial LPS-induced IL-8 gene expression in human monocyte-derived macrophages. The level of gene expression was demonstrated as the ratio of the OD of the band of RT-PCR product of IL-8 to that of actin. See the Materials and Methods for further details.

DISCUSSION

The ability of the E series of prostaglandins to modulate the monocyte/macrophage serves as an important autocrine regulation of this immunocyte's response to an external stimuli. The E series of prostaglandins inhibits a variety of immunocyte responses including mitogen-induced proliferation[15–17] and cytotoxicity[18,19] of different subsets of T lymphocytes, antibody production of B lymphocytes,[20–22] phagocytosis, antigen presentation and secretion of various cytokines of macrophages.[23–25] The production of the E series of the prostaglandins is by the enzyme activity of cyclo-oxygenase on arachidonic acid, a membrane constituent of the eukaryotic cell. Almost any external stimulus will alter the cell membrane usually through a receptor event, but will in doing so also activate the release of the E series of prostaglandins early in the process. Although the mechanism of the inhibitory influence of these eicosanoids on the function of immune cells at the molecular level is not well defined, there is increasing evidence to support the hypothesis that the effects of the E series of prostaglandins are the result of their ability to activate the cAMP-dependent intracellular signal transduction pathway.[26,27] PGE_2 appears to activate the cell through binding to specific receptors on the cell surface. These receptors activate the α subunit of stimulatory GTP-binding protein (Gαs) which is located in the inner leaf of the plasma membrane. This regulatory Gs protein activates the effector enzyme adenylate cyclase which catalyses the reaction that converts ATP to cAMP thus increasing the intracellular cAMP level.[28] Intracellular cAMP activates cAMP-dependent protein kinase (PKA) which in turn phosphorylates various proteins in the cytosol and cell membranes initiating a cascade of events which ultimately regulates the gene expression of the cells.

Macrophages play an important role in the regulation of the immune response. As part of the macrophage response to LPS stimulation cytokines are secreted. This cytokine secretion is associated with cytokine gene activation and an increase in the cytokine mRNA. The cytokine gene activation secondary to LPS is dose and time dependent with early expression of cytokines such as IL-8 and IL-1β, while cytokines such as Il-6

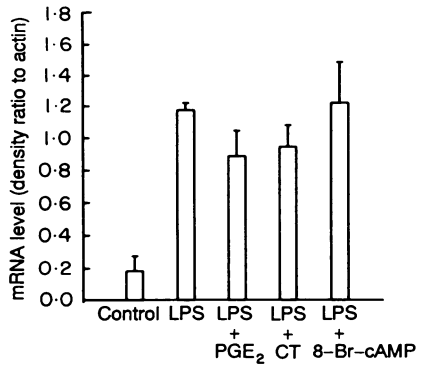

Figure 4. Effects of PGE_2, CT and 8-br-cAMP on bacterial LPS-induced IL-1β gene expression in human monocyte-derived macrophages. The level of gene expression was demonstrated as the ratio of the OD of the band of RT-PCR product oof IL-1β to that of actin. See the Materials and Methods for further details.

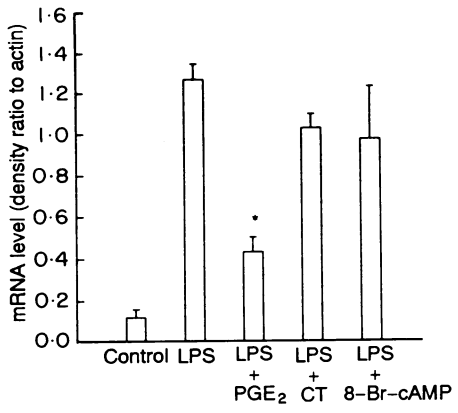

Figure 6. Effects of PGE_2, CT and 8-br-cAMP on bacterial LPS-induced IL-6 gene expression in human monocyte-derived macrophages. The level of gene expression was demonstrated as the ratio of the OD of the band of RT-PCR product of IL-6 to that of actin. See the Materials and Methods for further details. $*P < 0{\cdot}05$ compared to LPS group, Student's *t*-test.

and IL-1α are expressed later.[12] In this study we investigated the role of PGE_2 in regulating cytokine gene activation. Although the mechanisms underlying such activities are multifactorial, it is only by understanding this regulation that there can be attempts to modulate this regulation and consequently the cytokine response. As the macrophage cytokine response is both dose and time dependent one can suggest the hypothesis that different mechanisms regulate the cytokine gene expression, and that they may be very independent of each other.[12]

Our current results support the hypothesis that cytokine gene expressions are regulated by a variety of regulation patterns. It is very likely that the LPS-induced gene expression of IL-1α and TNF-α is suppressed by the PGE_2 transmembrane signal that operates through the Gs protein to increase the intracellular level of cAMP. Although during this study we have not directly demonstrated increased intracellular cAMP levels in monocyte-derived macrophages, the results from the 8-bromo-cAMP study, at least partially, provided evidence to support our hypothesis.

For cytokine TNF-α, increasing intracellular levels of cAMP was shown to have an important inhibitory role in the gene expression and production in cells of the monocyte/macrophage lineage by different investigators[29,30] as well as by this study. However the effect of increased intracellular cAMP on IL-1 (both IL-1α and IL-1β) production is controversial. While there are data to support that an increase in cAMP will potentiate IL-1 gene expression and production,[13,31] other data indicate that cAMP elevation down-regulates or has no effect on IL-1 production.[30,32,33] These variations may be due in part to the different monocyte/macrophage lineage, as well as the conditions of LPS exposure.[34,35] In the present study we have used a lower, more physiological LPS dose in terms of the human macrophage.

In contrast, neither PGE_2, CT nor cAMP analogue had a significant effect on LPS-induced gene expression of IL-1β and IL-8, thus indicating that these cytokine genes are not regulated by PGE_2. Viherluoto *et al.*[36] and Hurme[37] reported that elevation of intracellular cAMP decreased the secretion of IL-1β but both the cell-associated and mRNA of IL-1β remained unchanged. These data are not inconsistent with our findings, in that the mRNA was not altered. Standiford *et al.*[38] demonstrated that PGE_2 can inhibit IL-8 mRNA expression in LPS-stimulated monocytes but not in differentiated alveolar macrophages.[28] This and our current data indicate that, for IL-8 gene expression, either natural or culture-induced cell differentiation changes the responsiveness to the Gs-mediated regulation of PGE_2 in monocyte/macrophage linage.

PGE_2 down-regulated the gene expression of IL-6 in the LPS-stimulated macrophages; however, this does not appear to be because of a Gs–adenylate cyclase cAMP signal. These data are very interesting as they indicate (1) that PGE_2 can operate through a second non-G protein-based transmembrane signal and (2) that IL-6 gene regulation is different from that of other cytokines, particularly TNF-α.

Thus at the cellular level, the modification of cytokine production is complicated and has a natural physiological intracellular regulatory system. The cAMP-dependent signal pathway is very unlikely just a general 'turn off switch' for the cells. We speculate that through its interaction with or modulation of other second messenger systems the cAMP-dependent signal transduction pathway regulates the degree of macrophage function at the molecular level. One of the second messenger systems involved in macrophage activation is the phosphatidylinositol–protein kinase C (PKC) system. Stimulation with LPS can initiate hydrolysis of phosphatidylinositol-4,5-biphosphate (PIP_2) through phospholipase C (PLC)[39] and subsequently generate inositol 1,4,5-triphosphate (IP_3) which stimulates the release of calcium from intracellular stores and diacylglycerol (DAG) which activates PKC,[40,41] thus initiating the cascade of intracellular changes leading to cell activation including gene expression of the cytokines. Physiologically, at any given time the cells are exposed to the mediators in the internal environment of the body such as hormones, neurotransmitters and prostaglandins. Some of these mediators can through their specific receptors activate the cAMP second messenger system to activate PKA further. These receptors include β-adrenergic receptors, H-2 histamine receptors and receptors for the E series of prostaglandins. Hence, when macrophages are activated by

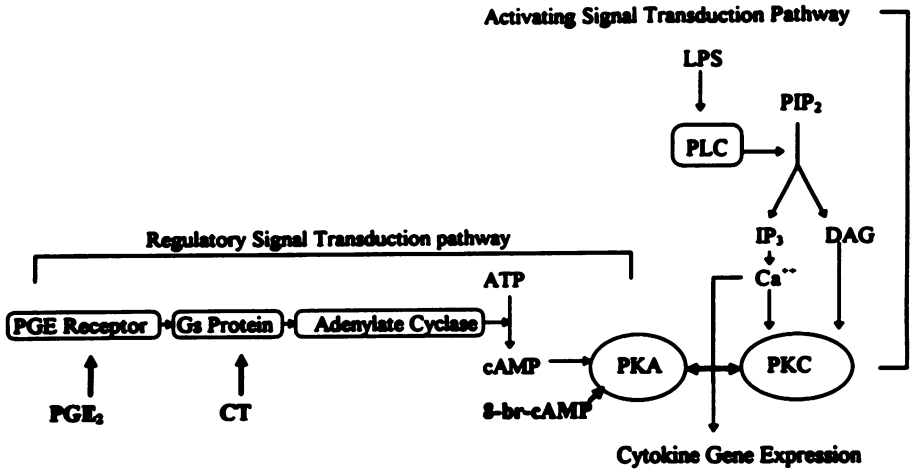

Figure 7. Schematic diagram of proposed mechanism of the interaction between activating signal transduction pathway and regulatory signal transduction pathway for cytokine gene expression during macrophage activation.

external stimuli such as LPS, intracellularly it is very likely that multi-regulatory systems interact with each other and/or co-modulate the cell function. The net outcome of the functional change depends upon the result of the interactions between the second messenger systems of cell activation which are initiated by the external stimuli such as LPS and that of modulation initiated by endogenous modulatory mediators such as PGE_2 (Fig. 7). We speculate that the modulatory systems provide protective mechanisms against over-reaction, which is harmful to the host, rather than shut down the whole immune responses to the infectious agent. For cytokine gene activation, different cytokines have different sensitivity to the two groups of the systems. Some cytokines, such as IL-1β and IL-8, are more sensitive to the second messenger systems for cell activation and relatively insensitive to that for the endogenous modulation compared with other cytokines in macrophages. The discrepancy between individual cytokines in response to different intracellular signals implicate a potential to 'fine tune' modulation of the gene expression of cytokines by manipulation of the signal transduction systems at the molecular level. Thus, further systematical studies of intracellular control of gene activation profiles of different cytokines by selective activation or blockade of different transmembrane signal pathways at different specific levels in different types of immune cells will help us to understand the underlying mechanism of regulation of the immune system at both the cellular and molecular level and will also provide us with a basis from which to develop new immunomodulators for therapeutic applications.

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