ROLE OF PROTEIN KINASES IN THE INDUCTION OF INDUCIBLE ISOFORM OF CYCLOOXYGENASE-2 (COX-2) BY ENDOTOXIN-ACTIVATED ENDOTHELIAL CELLS

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ABSTRACT

Cyclooxygenase (COX) exists in at least two isoforms. COX-1 is present constitutively under physiological condition. COX-2 is induced in various cell types by mitogens and cytokines including endotoxin (lipopolysaccharides, LPS). Recently, we have shown that COX-2 can be induced by endotoxin in endothelial cells. The signal transduction mechanism of COX-2 induction is still unclear. Some cell membrane receptors have an intracellular protein kinase domain, activation of which results in the phosphorylation of proteins following ligand binding. In the present report, protein kinase inhibitors (erbstatin and genistein for tyrosine kinase inhibitors, staurosporine and calphostin C for protein kinase C inhibitors) were used as pharmacological tools to investigate the potential role of protein kinase in COX-2 induction in bovine aortic endothelial cells (BAEC) activated with endotoxin. The predominant COX metabolite, 6-oxo-prostaglandin (PG) $F_{1\alpha}$ was measured by radioimmunoassay under the following experimental conditions: (i) accumulation of COX metabolites of endogenous arachidonic acid was measured at 24 h after addition of LPS (1 μg/ml); (ii) determination of "COX activity" by measuring COX metabolites generated by LPS-activated BAEC after incubation with exogenous arachidonic acid (30 μM) for 15 min. Erbstatin (0.25 to 25 μM) or genistein (0.15 to 150 μM) caused a dose-dependent inhibition of the accumulation of COX metabolites in the supernatant of LPS-activated BAEC. Erbstatin or genistein also caused a dose-dependent inhibition of "COX activity" in BAEC. Western blot analysis with a specific antibody to COX-2 which determined the expression of COX-2 protein induced by LPS in cell extracts showed that erbstatin (25 μM) or genistein (150 μM) inhibited the expression of COX-2 protein in LPS-activated BAEC. In contrast to tyrosine kinase inhibitors, COX-2 induction in BAEC stimulated with LPS was not inhibited by protein kinase C (PKC) inhibitors, either staurosporine (0.0002-0.2 μM) or calphostin C (0.0015-1.5 μM). These results showed that tyrosine phosphorylation, not protein kinase C, was part of the signal transduction mechanism that mediated the induction of COX-2 elicited by LPS in BAEC.

Key words : lipopolysaccharide, prostaglandins, tyrosine kinase, protein kinase C

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INTRODUCTION

Prostaglandins (PGs) have numerous cardiovascular and inflammatory effects[1]. Cyclooxygenase (COX) is the first enzyme in the pathway in which arachidonic acid is converted to PGs[2-3]. COX exists in at least two isoforms. One is the constitutive enzyme, COX-1, producing regulatory prostanoids under physiological conditions[4], whereas the other, COX-2, is induced by mitogens[5-6], and proinflammatory cytokines[7] during pathological states such as inflammation. Recently, we have shown that COX-2 can be induced by endotoxin (lipopolysaccharides, LPS) in endothelial cells[8]. Little is known about the cellular mechanisms of these events as the signal transduction pathways involved in LPS induced alterations in cell function are not completely understood. Changes in the number of intracellular signal transduction systems have been observed after exposure of cells to LPS. Activation of the G-protein system has been im-plicated in intracellular signal transduction in cells exposed to LPS[9-12]. Also increased phosphorylation of certain proteins occurs after exposure of murine peritoneal macrophages to LPS, raising the possibility that protein kinases may be involved in the signal transduction process[13]. Activation of calcium and phospholipid-dependent protein kinase C has been observed after exposure of cells to either LPS or lipid A[14-15]. More recently, the phosphorylation of tyro-sine residues on cellular proteins leading to liberation of arachidonic acid metabolites has been observed in macrophages[16-17], neutrophils[18-21], smooth mus-cle cells[22], platelets[23], basophilic cells[24-25] and rat Kupffer cells[26] in response to agents such as endotoxin and IL-1. Therefore, the involvement of tyrosine kinase and of protein kinase C in the LPS-induced changes in COX in endo-thelial cells has been evaluated by using specific inhibitors such as erbstatin[27-29] or genistein[30] and calphostin C[31] or stauro-sporine[32-33].

METERIALS AND METHODS

Cell Culture

Bovine aortic endothelial cells (BAEC) were obtained from fresh bovine aortae as previously described[34] and cultured in 96-well plates with Dulbecco's Modified Eagle's Medium (DMEM; 200 µl/well) containing 10% foetal calf serum (Gibco) and 4 mM L-glutamine. The agents, dissolved in distilled water, were sterilized by filtration through a filter (pore size: 0.22 micron) before being added to the cells under sterile conditions. Cells were incubated at 37^0C in a humidified incubator.

Measurement of the release of COX metabolites

The main COX metabolites released after activation of cells with LPS (6-oxo-$PGF_{1\alpha}$for BAEC[8]) were measured by radioimmunoassay[35]. In experiments to measure the effects of tyrosine kinase inhibitors on the release of COX metabolites from endogenous arachidonic acid, cells were treated with LPS (1 µg/ml) together with the tyrosine kinase inhibitors erbstatin (0.25, 2.5 and 25 µM) or genistein (0.15, 1.5, 15 and 150 µM) or the protein kinase C inhibitors staurosporine (0.0002, 0.002, 0.02 and 0.2 µM) or calphostin C (0.0015, 0.015, 0.15 and 1.5 µM) for 24 h, and the medium was subsequently removed for radioimmuno-assay. In separate experiments designed to measure the effect of protein kinase inhibitors on "COX activity", cells were treated with LPS

(1 μg/ml) together with the respective protein kinase inhibitors (as above) for 12 h, after which time the cells were washed and fresh medium containing AA (30 μM) was added for 15 min at 37^0C. The formation of COX metabolites was then assessed by radioimmunoassay of the cell culture supernatant.

Immunoblot (Western blot) Analysis

BAEC which were untreated (control), treated with LPS alone (1 μg/ml), treated with LPS (1 μg/ml) plus erbstatin (25 μM) or with LPS plus genistein (150 μM) were cultured in 6-well culture plates (37^0C; for 24 h). After incubation, cells were extracted and analysed by immuno-blotting for COX-2 protein as previously described[8].

Measurement of cell viability

Cell respiration, an indicator of cell viability, was assessed by the mitochondrial dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan[36]. At the end of each experiment, cells in 96-well plates were incubated (37^0C; 1 h) with MTT (0.2 mg/ml) dissolved in culture medium, after which time, the medium was removed by aspiration and cells were solubilized in DMSO (200 μl). The extent of reduction of MTT to formazan within cells was quantitated by the measurement of optical density at 650 nm (OD_{650}) using a Molecular Devices microplate reader (Richmond, CA, USA).

Statistical analysis

The results are shown as mean ± SEM of triplicate determinations (wells) from 3 separate experimental days (n=9). Student's paired or unpaired *t*-tests, as appropriate, were used for the determination of significance of differences between means and a p -value of less than 0.05 was taken as statistically significant.

RESULTS

Effect of tyrosine kinase inhibitors on the release of COX metabolites from endogenous stores of arachidonic acids by BAEC activated by LPS

LPS enhanced the accumulation of 6-oxo $PGF_{1\alpha}$ in cultures of BAEC[8]. This accumulation was inhibited by erbstatin or genistein in a dose-dependent manner (Figure 1). A significant inhibition was achieved with 2.5 μM for erbstatin and 0.15 μM for genistein.

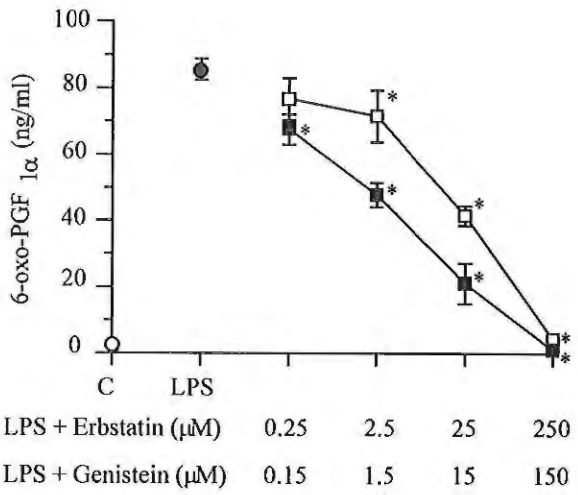

Figure 1 The effects of erbstatin (open squares) and genistein (closed squares) on the stimulation of COX in BAEC activated with LPS (1 μg/ml) measured at 24 h by the accumulation of 6-oxo-$PGF_{1\alpha}$ in culture medium. Data are expressed as mean ± SEM of triplicate determinations (wells) from 3 separate experimental days (n=9). * $p < 0.05$ when compared to LPS-treated cells at 24 h (LPS).

Neither of the tyrosine kinase inhibitors used (up to 250 μM for erbstatin and 150 μM for genistein) had any effect on cell viability, either alone or in combination with LPS (cell viability 84 ± 1 % of control untreated cells).

Effect of tyrosine kinase inhibitors on COX activity

In BAEC, the increase in COX activity caused by LPS, measured in the presence of exogenous arachidonic acid, was almost four-folds that of the control cells at 12 h (Figures 2 and 3). These figures also show the dose-dependent inhibition of COX activity by erbstatin or genistein to values almost as low as those of untreated cells at the highest concentrations of inhibition. The addition of either erbstatin (25 μM) or genistein (150 μM) to cell culture during the 15 min incubation with exogenous arachidonic acids did not affect the formation of 6-oxo $PGF_{1\alpha}$.

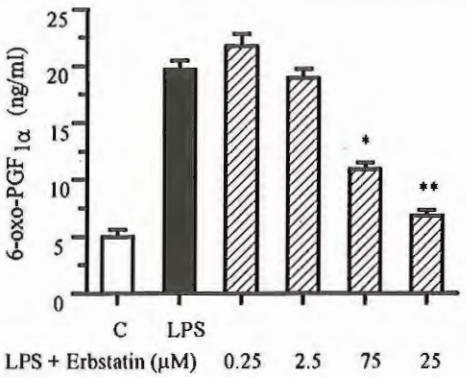

Figure 2 Dose-dependent inhibition of the increase in COX activity by erbstatin in LPS-activated BAEC measured at 12 h by the formation of the 6-oxo-$PGF_{1\alpha}$ in the presence of exogenous arachidonic acid (30 μM; 15 min). Data are expressed as mean ± SEM of 9 determinations from at least 3 separate experimental days. $^{*}p < 0.05$ when compared to LPS-treated cells at 12 h (LPS).

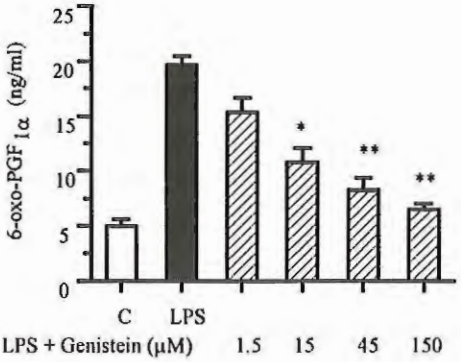

Figure 3 Dose-dependent inhibition of the increase in COX activity by genistein in LPS-activated BAEC measured at 12 h by the formation of 6-oxo-$PGF_{1\alpha}$ in the presence of exogenous arachidonic acid (30 μM; 15 min). Data are expressed as mean ± SEM of 9 determinations from at least 3 separate experimental days. $^{*}p < 0.05$ when compared to LPS-treated cells at 12 h (LPS).

The effect of tyrosine kinase inhibitors on COX-2 protein in LPS-treated BAEC

In these experiments, two control conditions untreated and LPS-treated were repeated to ensure reproducibility. Untreated BAEC contained no COX-2 protein (Figure 4; lane 1). In contrast, BAEC ac-tivated with LPS (1 μg/ml; 24 h) contained a protein of approximately 70 kDa, which was recognised by a specific antibody to COX-2 (Figure 4; lane 2). This induction of COX-2 protein by LPS in BAEC was prevented by erbstatin (25 μM; Figure 4; lane 3) or genistein (150 μM; Figure 4; lane 4).

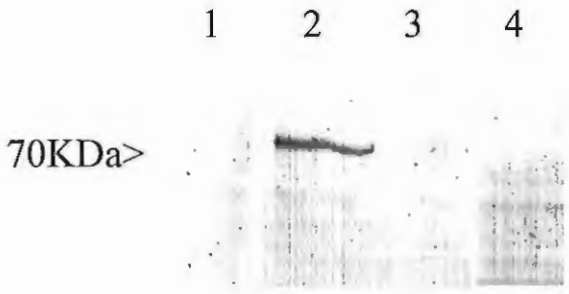

Figure 4 Western blots using polyclonal antibodies to COX-2 of cell extracts from LPS-treated and untreated BAEC. Equal amounts of protein were loaded in all lanes of BAEC (30 μg/lane). Control untreated BAEC (lane 1) contained no COX-2 protein. In contrast, LPS-activated (1 μg/ml for 24 h) BAEC contained COX-2 protein (lane 2). The induction of COX-2 protein by LPS in BAEC was abolished by erbstatin (25 μM; lane 3) and genistein (150 μM; lane 4). Similar results were obtained using cell extracts from 3 separate batches of cells.

Effect of protein kinase C inhibitors on the release of COX metabolites from endogenous stores of arachidonic acid of BAEC activated by LPS

Two protein kinase C inhibitors, calphostin C and stauro-sporine, were used. Either stauro-sporine (0.0002 to 0.2 μM) or calphostin C (0.0015 to 1.5 μM) did not significantly inhibit an increase in the accumulation of 6-oxo $PGF_{1\alpha}$ in BAEC activated with LPS (Figure 5).

Incubation of BAEC with either of the protein kinase C inhibitors (up to 0.2 μM for staurosporine and 1.5 μM for calphostin C) had no effect on cell viability, either alone or in combina-tion with LPS (cell viability 83 ±2 % of control untreated cells).

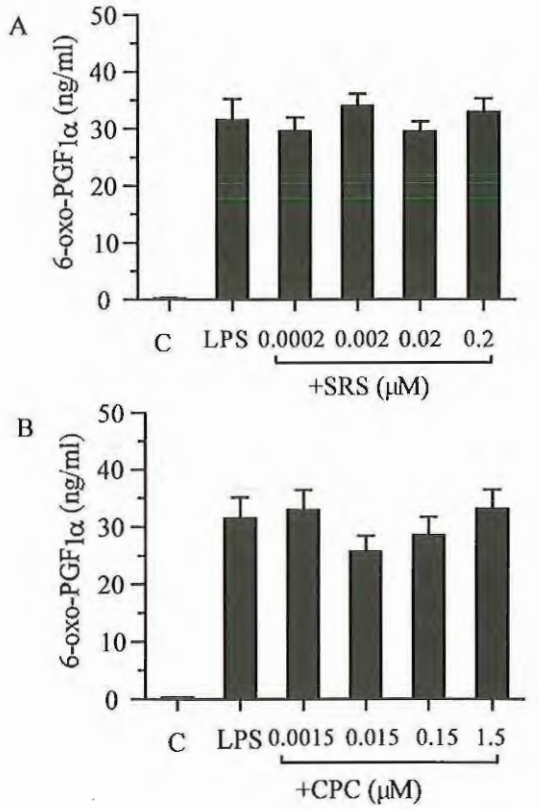

Figure 5 The effect of protein kinase C inhibitors, staurosporine (SRS; panel A) and calphostin C (CPC; panel B) on the accumulation of 6-oxo-$PGF_{1\alpha}$ in BAEC activated with LPS (1 μg/ml for 24 h). Data are expressed as mean ± SEM of 9 determinations from at least 3 separate experimen-tal days. $^{*}p < 0.05$ when compared to treated cells at 24 h (LPS).

Effect of protein kinase C inhibitors on COX activity

In BAEC, the increase in COX activity following LPS was inhibited by calphostin C only at the highest con-centration (1.5 μM; Figure 6), but not by staurosporine (up to 0.2 μM; Figure 6). The addition of either calphostin C (1.5 μM) or staurosporin (0.2 μM) to cells during the 15 min incubation with exogenous AA did not affect the formation of 6-oxo $PGF_{1\alpha}$.

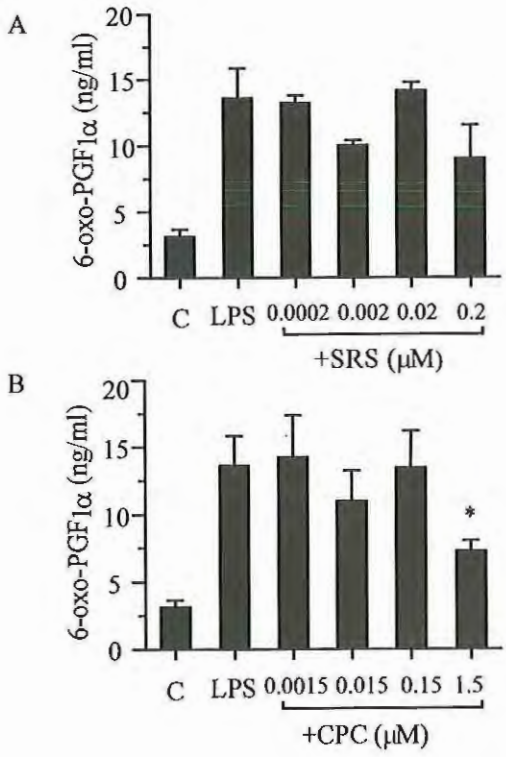

Figure 6 The effect of protein kinase C inhibitors, staurosporine (SRS; panel A) and calphostin C (CPC; panel B) on COX activity in BAEC activated with LPS (1 μg/ml for 24 h). Data are expressed as mean ± SEM of 9 determinations from at least 3 separate experimental days. $^{*}p < 0.05$ when compared to treated cells at 24 h (LPS).

DISCUSSION

The results show that the tyrosine kinase inhibitors, erbstatin or genistein, but not the protein kinase C inhibitors, calphostin C or staurosporine, concentration dependently in-hibit the expression by LPS of COX-2 activity and protein in endothelial cells. These findings clearly demonstrate that the signal transduction pathways leading to induction of COX-2 by LPS involve the phosphorylation of protein tyrosine kinase.

The experiments described in this study were designed to assess the role of two intracellular signalling pathways, protein tyrosine kinase and protein kinase C (PKC), in the transduction of the effects of LPS on COX-2 activity. These results demonstrate a more important role of tyrosine kinase than PKC in the signal transduction pathway leading to expression of COX-2. Neither set of inhibitors, however, interfered directly with the enzyme activity measured, i.e. they were not inhibitors of the COX-2 activity, but of the sequential steps leading to the expression of the respective proteins.

LPS is a major component of the outer membrane of gram negative bacteria. Although it interacts with many types of cells and is linked to numerous events associated with sepsis and endotoxin shock[37-38], the mechanisms underlying these actions are poorly understood. Two distinct pathways for LPS stimulation of cells have been identified, the direct stimulation by LPS and the indirect stimulation via cytokines release from LPS-stimulated cells[38-39]. The later step after LPS activation is the signal transduction mechanisms by which LPS induces alterations in cell function or the expression of proteins such as COX-2.

It is well-documented that LPS binds to a number of different constituents on the surface of cells or in the plasma/serum (i.e. LPS-binding protein or LBP[38-39]). Recently, two different cell surface molecules, CD14 and CD18, have been proposed as LPS receptors[40-42], but their contribution to the cellular activation afforded by LPS is unclear. LBP which is soluble[43-46], binds to the lipid A component of LPS[47], and these LPS-LBP complexes are recognized by receptors such as CD14 or CD18 molecules[38] present on monocytes[42]. In macrophages, CD14 is the im-portant cell binding protein[42], but not CD18 as with the scavenger receptors[38,41]. Although

endothelial cells do not have mCD14 (cell membrane CD14), LPS-LBP complexes react with another form of CD14, soluble CD14; sCD14, to form sCD14-LPS complexes on endothelial cells[48].

What are the intracellular signaling events that mediate specific LPS responses in endothelial cells? In some cell types such as lymphocytes, macrophages or mesangial cells, a pertussis toxin-sensitive guanine nucleotide binding protein (G-protein) has been identified which couples the binding of LPS to the cell surface to the intracellular signal transduction pathways leading to the response such as altered gene expression of some cytokines e.g. IL-1[9-12]. However, some of the actions of LPS in cells are unaffected by pertussis toxin treatment[10,49], suggesting that these responses elicited by LPS do not occur through a single class of a G-protein-dependent signalling mechanism. A Ca^{2+} signalling following activation of PAF receptor has recently been shown to be involved in the activation of guinea-pig macrophages and neutrophils by LPS[50].

An increased phosphorylation of certain proteins occurs after exposure of murine peritoneal macrophages to LPS, raising the possibility that protein kinases may be involved in the signal transduction process[13]. The two major types of protein kinase are serine-/threonine and tyrosine kinase. The phospholipid/Ca^{2+} dependent protein kinases (serine/threonine protein kinase, protein kinase C) could play a crucial role in the signal transduction and altered gene expression after LPS exposure[14-15,39]. In this study, protein kinase C inhibitors, however, did not inhibit the induction of COX-2 in endothelial cells after activation with LPS. In addition to protein kinase C, which is serine/threonine protein kinase, tyrosine kinase has also been suggested to play a role in the signal transduction mechanism. Binding of different ligands (i.e. EGF or PDGF) to cell surface receptors stimulates tyrosine phosphorylation and this event is thought to be part of the signal transduction mechanism that mediates later cellular responses[51]. The phosphorylation of tyrosine residues on cellular proteins, leading to formation of AA metabolites, has been observed in many cell types. Activation of tyrosine kinase may, therefore, be an integral part of the signal transduction pathways leading to expression of COX-2 in various cell types. In our experiments, roles of tyrosine kinases were implicated through the use of the inhibitors, erbstatin and genistein which were shown to be a selective inhibitor of tyrosine (vs serine/threonine) kinases in 1987[27,30] and have been used widely since then.

The demonstration that inhibitors of tyrosine kinase suppress the induction of COX-2 provides a possible explanation for the anti-inflammatory activities of tyrosine kinase inhibitors such as herbimycin A, leflunomide or tyrphostins in a variety of disease models, i.e rheumatoid synovitis in rat[52-55], and the prevention by a tyrphostin (AG126) of the mortality caused by endotoxaemia in mouse[56]. Furthermore, the finding that inhibitors of tyrosine kinase suppress the induction of COX-2, reinforces the hypothesis that agents which prevent the induction of COX-2 may well be useful in the prevention of local (inflammation) or systemic inflammatory conditions (e.g. systemic inflammatory response syndrome, arteriosclerosis, etc.).

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