The induction of cyclooxygenase-2 in IL-1β-treated endothelial cells is inhibited by prostaglandin E2 through cAMP

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Prostaglandins (PGs) have numerous cardiovascular and inflammatory effects. Cyclooxygenase (COX), which exists as COX-1 and COX-2 isoforms, is the first enzyme in the pathway in which arachidonic acid is converted to PGs. Prostaglandin E2 (PGE2) exerts a variety of biological activities for the maintenance of local homeostasis in the body. Elucidation of PGE2 involvement in the signalling molecules such as COX could lead to potential therapeutic interventions. Here, we have investigated the effects of PGE2 on the induction of COX-2 in human umbilical vein endothelial cells (HUVEC) treated with interleukin-1β (IL-1β 1 ng/ml). COX activity was measured by the production of 6-keto-PGF1α, PGE2, PGF2α and thromboxane B2 (TXB2) in the presence of exogenous arachidonic acids (10 μM for 10 min) using enzyme immunoassay (EIA). COX-1 and COX-2 protein was measured by immunoblotting using specific antibody. Untreated HUVEC contained only COX-1 protein while IL-1β treated HUVEC contained COX-1 and COX-2 protein. PGE2 (3 μM for 24 h) did not affect on COX activity and protein in untreated HUVEC. Interestingly, PGE2 (3 μM for 24 h) can inhibit COX-2 protein, but not COX-1 protein, expressed in HUVEC treated with IL-1β. This inhibition was reversed by coinubcation with forskolin (100 μM). The increased COX activity in HUVEC treated with IL-1β was also inhibited by PGE2 (0.03, 0.3 and 3 μM for 24 h) in a dose-dependent manner. Similarly, forskolin (10, 50 or 100 μM) can also reverse the inhibition of PGE2 on increased COX activity in IL-1β treated HUVEC. The results suggested that (i) PGE2 can initiate negative feedback regulation in the induction of COX-2 elicited by IL-1β in endothelial cells, (ii) the inhibition of PGE2 on COX-2 protein and activity in IL-1β treated HUVEC is mediated by cAMP and (iii) the therapeutic use of PGE2 in the condition which COX-2 has been involved may have different roles.

Key words: COX-2, PGs, IL-1β, cAMP, Signalling pathway, Endothelium

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 cytokines7,8 during pathological states such as inflammation.

The main PGs produced in the body are prostacyclin (PG1), PGE2, PGF2α, Thromboxane A2 (TXA2) and PGD2. Each PGs has different characters and functions. Among the PGs, PGE2 is a potent lipid molecule with complex proinflammatory and immunoregulatory properties.9 PGE2 is considered a major contributor to the production and maintenance of immunosuppression after overwhelming injury.10 PGE2 is believed to modulate biochemical and immunological events leading to parturition.11 PGE2 also exerts a variety of biological activities for the maintenance of local homeostasis in the body.12 Interestingly, we have shown in previous studies that the induction of COX-2 elicited by endotoxin (lipopolysaccharide, LPS) in endothelial cells is inhibited by PGE2 and 13,14-dihydro PGE2.13 Elucidation of the effects of PGE2 on the signalling molecule such as COX could lead to potential therapeutic interventions and understanding of the feedback regulation of COX in endothelial cells. Here, we have investigated the effects of PGE2 on the induction of COX-2 in human umbilical vein endothelial cells (HUVEC) treated with interleukin-1β (IL-1β) (1 ng/ml).
Material and methods

Cell culture

Human umbilical vein endothelial cells (HUVEC) were obtained from babies born to normal pregnant women as previously described and cultured in 96-well plates with Human Endothelial-SFM Basal Growth Medium (Gibco) containing 10% fetal calf serum (Gibco), 100 units/ml penicillin G sodium and 100 μg/ml streptomycin. Cells were incubated at 37°C in a humidified incubator and grown to confluence before use.

Measurement of COX activity

Confluent HUVEC were gently washed two times with phosphate-buffered saline (PBS) and replaced with fresh medium (200 μl/well) before use. Cells were treated with no addition, IL-1β (1 ng/ml), IL-1β (1 ng/ml) plus PGE₂ (0.03, 0.3 or 3 μM) or PGE₂ (3 μM) alone for 24 h, after which time the medium was removed and washed twice with PBS. COX activity was measured by the production of four COX metabolites, e.g. 6-keto-PGF₁α (a stable metabolite of PGI₂), PGE₂, Prostaglandin F₂α (PGF₂α) and thromboxane B₂ (TXB₂; a stable metabolite of TXA₂) in the replaced fresh medium containing exogenous arachidonic acid (10 μM for 10 min) using enzyme immunoassay (EIA). Briefly, 50 μl of standard PGs or samples were added to pre-coated mouse anti-rabbit IgG microtitre plates (96-well). Then, PGs acetylcholinesterase tracer (Clay-man; 50 μl) and rabbit antiserum of PGs were added. The plate was covered with plastic film and incubated for 18 h at 4°C, after which time the wells were emptied and rinsed five times with wash buffer (PBS containing 0.05% Tween). Ellman’s reagent (Cayman; 200 μl) was added to each well and the plates were shaken on a microtitre plate shaker. The duration of the reaction was about 90 min. A yellow colour develops which can be read using a microplate reader (BIORAD; OD 415 nm).

Immunoblot (Western blot) analysis

HUVEC which were untreated, treated with IL-1β (1 ng/ml), IL-1β (1 ng/ml) plus PGE₂ (0.03, 0.3 and 3 μM) or PGE₂ (3 μM) alone were cultured in six-well culture plates (37°C; for 24 h). After 24 h incubation, cells were extracted and analysed by immunoblotting using specific antibodies for COX-1 and COX-2 protein (a generous gift from Dr Gary O’Neill, Merck Frosst, Canada) as previously described.

The other experiment was performed to study the signalling molecule in the effects of PGE₂ on COX expression by using forskolin (cAMP activator). HUVEC were treated with no addition, IL-1β (1 ng/ml), IL-1β (1 ng/ml) plus PGE₂ (3 μM), IL-1β (1 ng/ml) plus PGE₂ (3 μM) with forskolin (10, 50 and 100 μM), IL-1β (1 ng/ml) plus forskolin (100 μM), PGE₂ (3 μM) plus forskolin (100 μM), forskolin (100 μM) alone or PGE₂ (3 μM) alone for 24 h, after which time, the medium was removed and replaced with fresh medium containing exogenous arachidonic acid (10 μM for 10 min). The medium was then removed to measure COX activity by 6-keto-PGF₁α production. The remained cells were extracted and analysed by immunoblotting using specific antibodies for COX-1 and COX-2 protein.

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. At the end of each experiment, cells in 96-well plates were incubated (37°C; 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 each well). The extent of reduction of MTT to formazan within cells was quantitated by the measurement of optical density at 650 nm (OD₆₅₀) using a microplate reader (BIORAD, USA).

Statistical analysis

The results are shown as mean standard error of the mean (SEM) of triplicate determinations (wells) from at least four separate experimental days (n=12). 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.

Materials

DMSO, phosphate buffered saline (PBS; pH 7.4), Trizma base, EDTA, triton X-100, phenylmethylsulfonyl fluoride (PMSF), pepstatin A, leupeptin, glycerol, bromphenol blue, 2-mercaptoethanol, sodium dodecyl sulphate (SDS), forskolin, anti-rabbit IgG antibody, goat IgG, premixed BCIP/NBT solution, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), penicillin G sodium and streptomycin were supplied by Sigma Chemical Company (St Louis, MO, USA). PGs (6-keto-PGF₁α, PGE₂, PGF₂α and TXB₂) and their respective acetylcholinesterase tracer and rabbit antiserum, pre-coated mouse anti-rabbit IgG microtitre plates (96-well) and Ellman’s reagent were purchased from Cayman (Sapphire Bioscience, Australia). Human Endothelial-SFM Basal Growth Medium and fetal calf serum was obtained from GibThai (Thailand). Recombinant human IL-1β, were purchased from Genzyme (USA). Pure nitrocellulose membrane (0.45 micron) and filter paper were purchased from BIO-RAD (USA).
Results

The effect of PGE\textsubscript{2} on COX activity as measured by the production of 6-keto-PGF\textsubscript{1α}, PGE\textsubscript{2}, PGF\textsubscript{2α} and TXB\textsubscript{2} in HUVEC treated with IL-1β (1 ng/ml)

Untreated HUVEC in the presence of arachidonic acid (10 μM for 10 min) release lower amounts of 6-keto-PGF\textsubscript{1α} (3.36 ± 0.1 ng/ml), PGE\textsubscript{2} (0.4 ± 0.04 ng/ml), PGF\textsubscript{2α} (0.78 ± 0.01 ng/ml) and TXB\textsubscript{2} (0.04 ± 0.01 ng/ml). In IL-1β (0.01, 0.1 and 1 ng/ml) treated HUVEC; the production of 6-keto-PGF\textsubscript{1α}, PGE\textsubscript{2} and PGF\textsubscript{2α} was increased but not TXB\textsubscript{2} (Fig. 1). The production of 6-keto-PGF\textsubscript{1α} in HUVEC treated with IL-1β (0.01, 0.1 and 1 ng/ml) was increased significantly in a dose-dependent manner (Fig. 1A). This increase was significantly at 0.01 ng/ml of IL-1β. The others, PGE\textsubscript{2} but not PGF\textsubscript{2α}, was only increased significantly in HUVEC treated with IL-1β 1 ng/ml (Fig. 1B). In HUVEC treated with PGE\textsubscript{2} (3 μM) alone, COX metabolites did not change significantly when compared to untreated HUVEC (Fig. 2). Interestingly, the increased 6-keto-PGF\textsubscript{1α} and PGE\textsubscript{2} in IL-1β (1 ng/ml) treated HUVEC was significantly inhibited by PGE\textsubscript{2} (0.03, 0.3 or 3 μM) in a dose-dependent manner (Fig. 2). This inhibition was significant at 0.03 μM of PGE\textsubscript{2}.

IL-1β alone, PGE\textsubscript{2} alone and IL-1β plus PGE\textsubscript{2} did not affect on cells viability (97 ± 2, 98 ± 1 and 98 ± 1%, respectively) when compared to the control untreated cells over a 24-h incubation period.

The stability of PGE\textsubscript{2} (3 μM) in cultured medium up to 24 h was also tested and has not changed significantly between 3 (2.97 ± 0.2), 6 (2.98 ± 0.1), 12 (2.95 ± 0.2) and 24 (2.97 ± 0.2) hours incubation of PGE\textsubscript{2}.

The effect of PGE\textsubscript{2} on COX isoform expressed in HUVEC treated with IL-1β

Untreated HUVEC contained no COX-2 protein (Fig. 3). COX-2 protein was expressed in HUVEC treated with IL-1β (1 ng/ml; Fig. 3) for 24 h. Interestingly, this induction of COX-2 in HUVEC treated by IL-1β (1 ng/ml) was inhibited by PGE\textsubscript{2} (0.03, 0.3 or 3 μM) in a dose-dependent manner (Fig. 3). The amount of COX-1 protein expressed in HUVEC treated with IL-1β (1 ng/ml), IL-1β (1 ng/ml) plus PGE\textsubscript{2} (3 μM) or PGE\textsubscript{2} (3 μM) alone was not changed when compared to untreated HUVEC (Fig. 4).

The effect of forskolin on 6-keto-PGF\textsubscript{1α} production in HUVEC treated with IL-1β plus PGE\textsubscript{2}

The COX activity (as measured by 6-keto-PGF\textsubscript{1α} production) in HUVEC treated with forskolin (100 μM) plus PGE\textsubscript{2} (3 μM) or forskolin (100 μM) alone was not changed in comparison with untreated HUVEC (Fig. 5; white bar). Interestingly, the inhibition of increased COX activity in IL-1β (1 ng/ml) treated HUVEC by PGE\textsubscript{2} was reversed in a dose-dependent manner when cells were coincubated with forskolin (10, 50 or 100 μM; Fig. 5; black and hatch bar). Moreover, the increased COX activity in IL-1β (1 ng/ml) treated HUVEC was synergised when cells were coincubated with forskolin (100 μM; Fig. 5; black bar).
FIG. 2. The effects of PGE$_2$ (0.003, 0.03, 0.3 or 3 μM) on COX activity in IL-1β (1 ng/ml) treated HUVEC. COX activity was measured by the formation of 6-keto-PGF$_{1α}$ (panel A), PGE$_2$ (panel B), PGF$_{2α}$ (panel B) and TXB$_2$ (panel B) in the presence of exogenous arachidonic acid (10 μM; 10 min). Data are expressed as mean±SEM of 12 determinations from at least four separate experimental days. *P<0.05 when compared to IL-1β treated HUVEC at 24 h.
Forskolin alone, forskolin plus IL-1β, forskolin plus PGE2 and forskolin plus IL-1β with PGE2 did not affect on cells viability (98 ± 2, 95 ± 1, 96 ± 3 and 94 ± 3%, respectively) when compared to the control untreated cells over a 24-h incubation period.

The effect of forskolin on COX isoform expressed in HUVEC treated with IL-1β plus PGE2

HUVEC treated with forskolin (100 μM) alone or forskolin (100 μM) plus PGE2 (3 μM) contain no COX-2
FIG. 6. The effects of forskolin on COX-2 protein expressed in IL-1β (1 ng/ml) plus PGE$_2$ (3 μM) treated HUVEC. COX-2 protein was detected by Western blots using polyclonal antibodies to COX-2 in cell extracts of HUVEC treated with no addition (lane 1), PGE$_2$ (3 μM; lane 2) alone, forskolin (100 μM; lane 3) alone, IL-1β (1 ng/ml; lane 4) alone, IL-1β (1 ng/ml) plus PGE$_2$ (3 μM; lane 5), IL-1β (1 ng/ml) plus PGE$_2$ (3 μM) with forskolin (100 μM; lane 6), IL-1β (1 ng/ml) plus forskolin (100 μM; lane 7) or PGE$_2$ (3 μM) plus forskolin (100 μM; lane 8) for 24 h. Equal amounts of protein (20 μg/lane) were loaded in each lane. Similar results were obtained with cell extracts from three separate batches of cells. The significant differences between each band were compared by scanner densitometry using image 1D program (densitometry unit).

Discussion

Here, we showed that the induction of COX-2 elicited by IL-1β in HUVEC can be inhibited by PGE$_2$ in a dose-dependent manner. Moreover, PGE$_2$ had no affect on either COX-1 protein or activity. Interestingly, forskolin (cAMP activator) can reverse this inhibition of PGE$_2$ on COX-2 protein and activity in IL-1β treated HUVEC. The results suggested that (i) PGE$_2$ is a negative feedback regulator through cAMP in the induction of COX-2 elicited by IL-1β in endothelial cells and (ii) the uses of PGE$_2$ in the condition in which COX-2 has been involved may be therapeutic.

PGs induce a wide range of biological actions that are mediated by specific membrane-bound receptors. Among the PGs, PGE$_2$ is considered to exert a variety of biological activities such as the maintenance of local homeostasis in the body, it is a major contributor to the production and maintenance of immunosuppression after overwhelming injury and an important factor for implantation and decidualization. Therefore, PGE$_2$ is a lipid molecule with complex inflammatory modulation and immunoregulatory properties. Our results have been supported that PGE$_2$ can act as anti-inflammation and immunosuppression in the induction of COX-2 in endothelial cells by IL-1β.

The exact mechanisms by which PGE$_2$ inhibited COX-2 induction in endothelial cells activated with IL-1β are not known. These may involve binding to specific cell surface receptors and influencing second messenger systems through G-proteins. Indeed, these should be complex because the effects of PGE$_2$ are exerted by a variety of PGE receptors which are
different in their signal transduction properties. There are at least four subtypes of PGE receptors. The EP1 and EP3 receptors are coupled to Ca$^{2+}$ mobilization and the inhibition of adenylate cyclase, respectively, and the EP2 and EP4 receptors are coupled to the same signal transduction pathway, stimulation of adenylate cyclase.

However, our studies showed that forskolin (cAMP activator) can reverse the inhibition of PGE$_2$ on COX-2 induced in IL-1$\beta$ treated HUVEC suggesting PGE$_2$ may inhibit COX-2 expressed in IL-1$\beta$ treated HUVEC through cAMP inhibition via EP3 receptors. PGE$_2$ is one of the PGs or COX metabolites, such as PGI$_2$, PGE$_2$, PGD$_2$, PGF$_{2\alpha}$ and TXA$_2$, synthesized by COX-1 and COX-2 which are involved in physiology and pathology, respectively. Each COX isoform can produce different COX metabolites in different cell types such as PGI$_2$ is a major COX-1 and COX-2 metabolite in endothelial cells while PGE$_2$ is a major COX-2 metabolite in macrophages. These differences in COX metabolite production in different cell types may be resulted from the feedback regulation of each COX metabolite produced. Our results showed that PGE$_2$ (0.03 $\mu$M) inhibited PGE$_2$ production (30% inhibition; Fig. 2A) more than PGI$_2$ production (20% inhibition; Fig. 2B) in IL-1$\beta$ treated endothelial cells. These may explain the COX metabolites produced in IL-1$\beta$ treated endothelial cells that PGI$_2$ released in highest amounts and the lesser extent of PGE$_2$, PGF$_{2\alpha}$ and TXA$_2$, respectively. Thus, elucidation of the feedback regulation of each COX metabolite will help us to understand the variety of COX metabolites produced in different cells and may lead to potential therapeutic interventions. In our studies here, we showed that PGE$_2$ is a negative feedback regulator of the induction of COX-2, but not COX-1, in endothelial cells activated with IL-1$\beta$. These suggested that PGE$_2$ series may have negative feedback regulation of COX-2 induction in endothelial cells, since our previous study showed that PGE$_1$ and PGE$_0$ can inhibit the induction of COX-2 in endothelial cells activated with LPS. PGE$_2$ series have been used in clinical disorders such as peripheral vascular occlusive diseases, NSAIDs-induced gastric ulcer, abortion and impotence. Thus, we proposed that uses of PGE$_2$ in the condition in which COX-2 has been involved may be therapeutic and the effects of other COX metabolites such as PGI$_2$ or PGF$_{2\alpha}$ on COX-2 expressed in different cells should be elucidated.

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References


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