Research Paper

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The induction of cyclooxygenase-2 in IL-1β-treated endothelial cells is inhibited by prostaglandin E_2 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 E_2 (PGE_2) exerts a variety of biological activities for the maintenance of local homeostasis in the body. Elucidation of PGE_2 involvement in the signalling molecules such as COX could lead to potential therapeutic interventions. Here, we have investigated the effects of PGE_2 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-$PGF_{1\alpha}$, PGE_2, $PGF_{2\alpha}$ and thromboxane B_2 (TXB_2) 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. PGE_2 (3 μM for 24 h) did not affect on COX activity and protein in untreated HUVEC. Interestingly, PGE_2 (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 coincubation with forskolin (100 μM). The increased COX activity in HUVEC treated with IL-1β was also inhibited by PGE_2 (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 PGE_2 on increased COX activity in IL-1β treated HUVEC. The results suggested that (i) PGE_2 can initiate negative feedback regulation in the induction of COX-2 elicited by IL-1β in endothelial cells, (ii) the inhibition of PGE_2 on COX-2 protein and activity in IL-1β treated HUVEC is mediated by cAMP and (iii) the therapeutic use of PGE_2 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 cytokines[7,8] during pathological states such as inflammation.

The main PGs produced in the body are prostacyclin (PGI_2), PGE_2, $PGF_{2\alpha}$, Thromboxane A_2 (TXA_2) and PGD_2. Each PGs has different characters and functions. Among the PGs, PGE_2 is a potent lipid molecule with complex proinflammatory and immunoregulatory properties.[9] PGE_2 is considered a major contributor to the production and maintenance of immunosuppression after overwhelming injury.[10] PGE_2 is believed to modulate biochemical and immunological events leading to parturition.[11] PGE_2 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 PGE_1 and 13,14-dihydro PGE_1.[13] Elucidation of the effects of PGE_2 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 PGE_2 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[14] 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 PGE_2 (0.03, 0.3 or 3 µM) or PGE_2 (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-$PGF_{1\alpha}$ (a stable metabolite of PGI_2), PGE_2, Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and thromoboxane B_2 (TXB_2; a stable metabolite of TXA_2) 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 (Clayman; 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 PGE_2 (0.03, 0.3 and 3 µM) or PGE_2 (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.[15]

The other experiment was performed to study the signalling molecule in the effects of PGE_2 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 PGE_2 (3 µM), IL-1β (1 ng/ml) plus PGE_2 (3 µM) with forskolin (10, 50 and 100 µM), IL-1β (1 ng/ml) plus forskolin (100 µM), PGE_2 (3 µM) plus forskolin (100 µM), forskolin (100 µM) alone or PGE_2 (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-$PGF_{1\alpha}$ 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.[16] 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 (OD_{650}) 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, phenylmethylsulphonyl 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-$PGF_{1\alpha}$, PGE_2, $PGF_{2\alpha}$ and TXB_2) 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_2 on COX activity as measured by the production of 6-keto-$PGF_{1\alpha}$, PGE_2, $PGF_{2\alpha}$ and TXB_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-$PGF_{1\alpha}$ (3.36 ± 0.1 ng/ml), PGE_2 (0.4 ± 0.04 ng/ml), $PGF_{2\alpha}$ (0.78 ± 0.01 ng/ml) and TXB_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-$PGF_{1\alpha}$, PGE_2 and $PGF_{2\alpha}$ was increased but not TXB_2 (Fig. 1). The production of 6-keto-$PGF_{1\alpha}$ 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_2 but not $PGF_{2\alpha}$, was only increased significantly in HUVEC treated with IL-1β 1 ng/ml (Fig. 1B). In HUVEC treated with PGE_2 (3 μM) alone, COX metabolites did not change significantly when compared to untreated HUVEC (Fig. 2). Interestingly, the increased 6-keto-$PGF_{1\alpha}$ and PGE_2 in IL-1β (1 ng/ml) treated HUVEC was significantly inhibited by PGE_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_2.

IL-1β alone, PGE_2 alone and IL-1β plus PGE_2 did not affect on cells viability (97 ± 2, 98 ± 1 and 98 ± 1%, respectively) when compare to the control untreated cells over a 24-h incubation period.

The stability of PGE_2 (3 μM) in cultured medium upto 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_2.

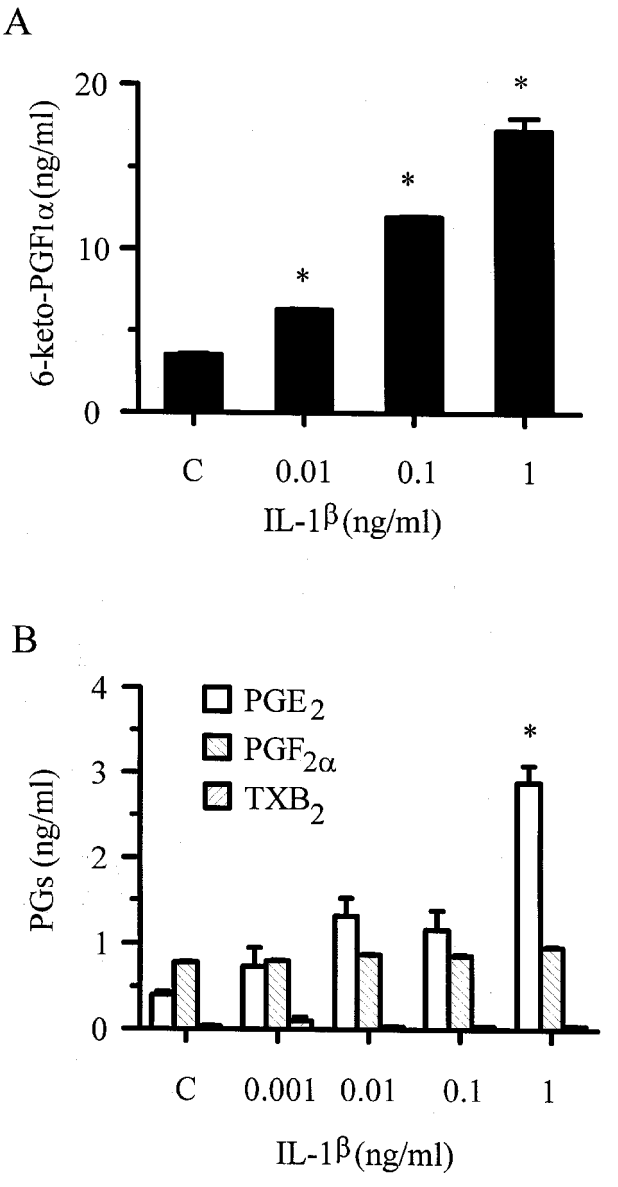

FIG. 1. Dose-dependent effects of IL-1β (1 ng/ml) on COX activity in HUVEC. COX activity was measured by the formation of 6-keto-$PGF_{1\alpha}$ (panel A), PGE_2 (panel B), $PGF_{2\alpha}$ (panel B) and TXB_2 (panel B) in the presence of exogenous arachidonic acid (10 μM; 10 min). Data are expressed as mean±SEM of 12 determinations from at least four separate experimental days. *P<0.05 when compared to untreated HUVEC at 24 h (C).

The effect of PGE_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_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_2 (3 μM) or PGE_2 (3 μM) alone was not changed when compared to untreated HUVEC (Fig. 4).

The effect of forskolin on 6-keto-$PGF_{1\alpha}$ production in HUVEC treated with IL-1β plus PGE_2

The COX activity (as measured by 6-keto-$PGF_{1\alpha}$ production) in HUVEC treated with forskolin (100 μM) plus PGE_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_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).

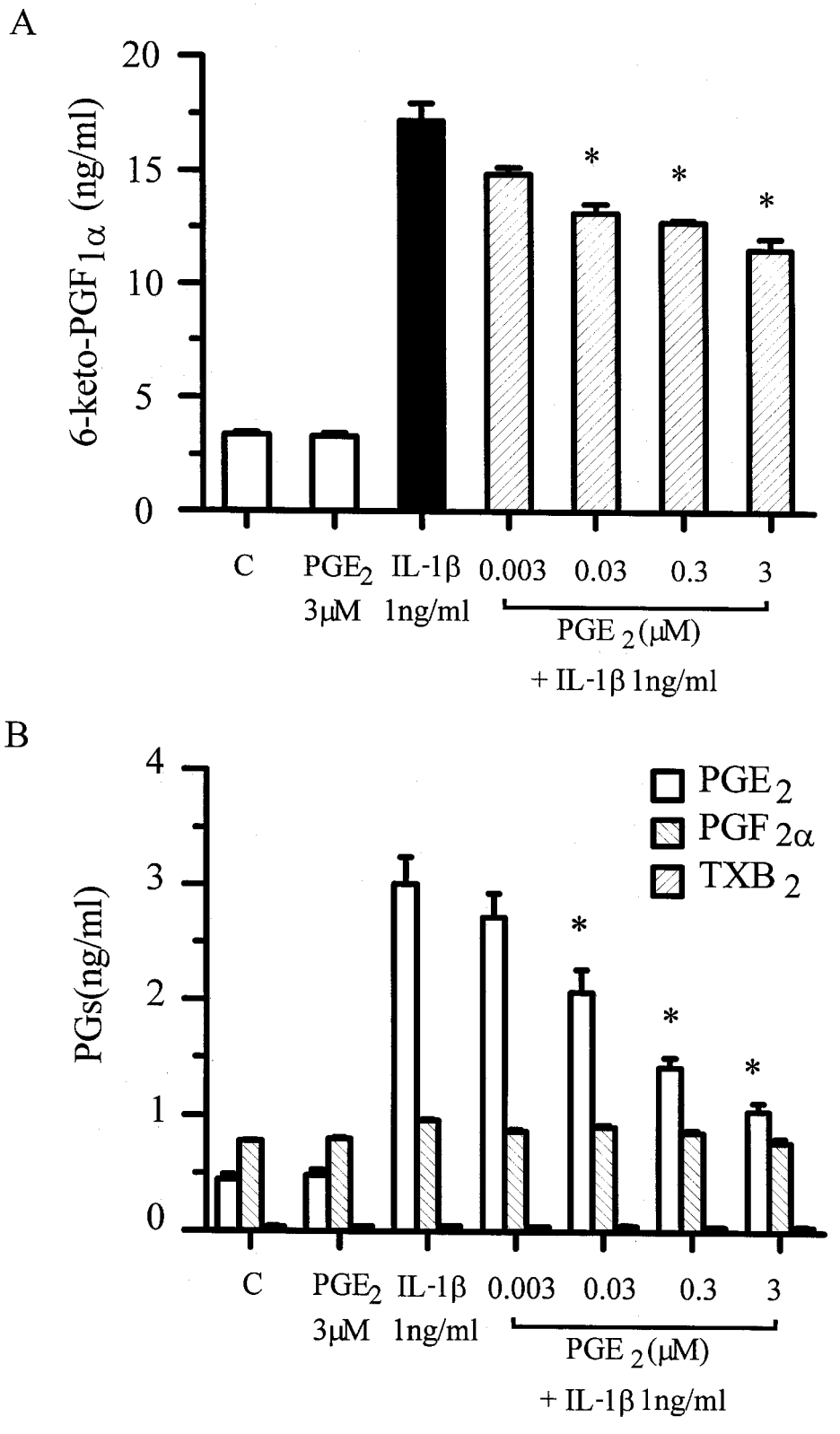

FIG. 2. The effects of PGE_2 (0.003, 0.03, 0.3 or 3 μM) on COX activity in IL-1β (1 ng/ml) treated HUVEC. COX activity was measured by the formation of 6-keto-$PGF_{1\alpha}$ (panel A), PGE_2 (panel B), $PGF_{2\alpha}$ (panel B) and TXB_2 (panel B) in the presence of exogenous arachidonic acid (10 μM; 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.

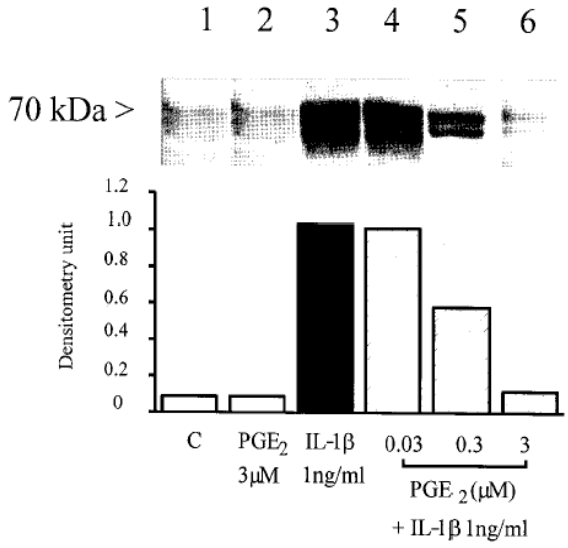

FIG. 3. The effects of PGE_2 on COX-2 protein expressed in IL-1β (1 ng/ml) treated HUVEC. COX-2 protein was detected by Western blots using polyclonal antibodies to COX-2 in cell extracts of HUVEC treated with no addition (lane 1), PGE_2 (3 μM) alone (lane 2), IL-1β (1 ng/ml) alone (lane 3) or IL-1β (1 ng/ml) plus PGE_2 (0.03, 0.3 or 3 μM; lane 4 to 6) 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).

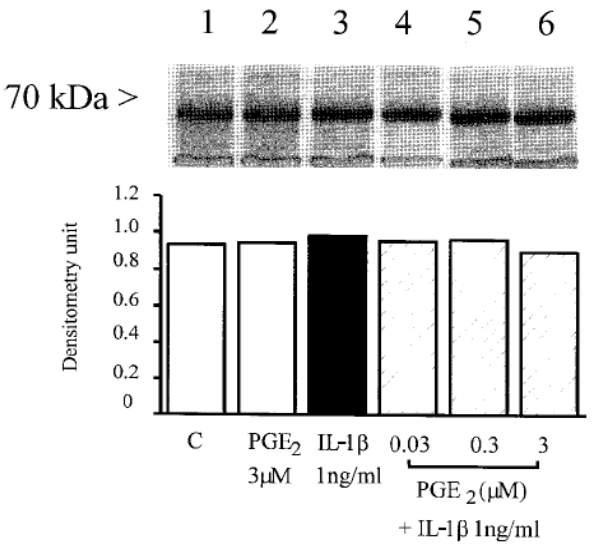

FIG. 4. The effects of PGE_2 on COX-1 protein expressed in IL-1β (1 ng/ml) treated HUVEC. COX-1 protein was detected by Western blots using polyclonal antibodies to COX-2 in cell extracts of HUVEC treated with no addition (lane 1), PGE_2 (3 μM) alone (lane 2), IL-1β (1 ng/ml) alone (lane 3) or IL-1β (1 ng/ml) plus PGE_2 (0.03, 0.3 or 3 μM; lanes 4–6) 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).

Forskolin alone, forskolin plus IL-1β, forskolin plus PGE_2 and forskolin plus IL-1β with PGE_2 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 PGE_2

HUVEC treated with forskolin (100 μM) alone or forskolin (100 μM) plus PGE_2 (3 μM) contain no COX-2

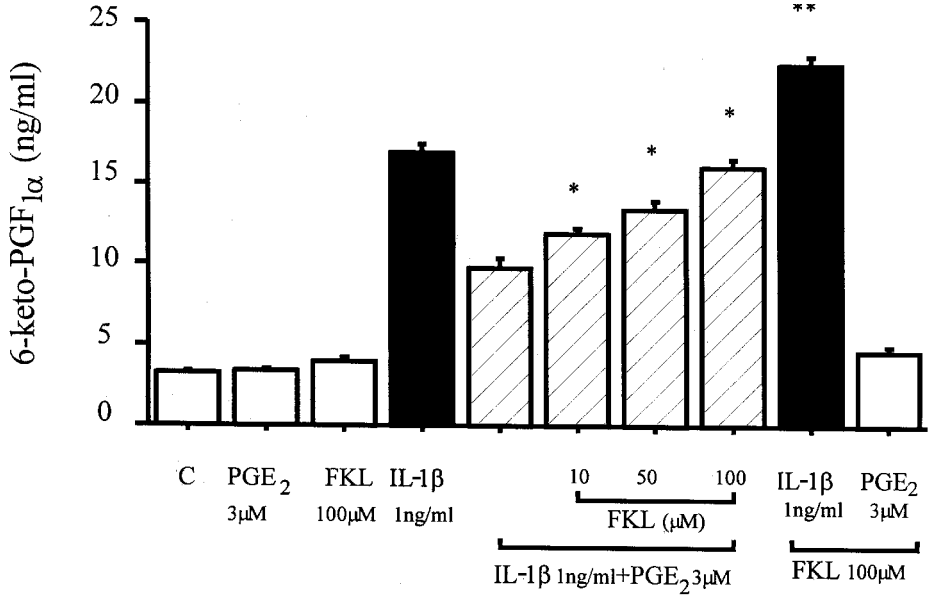

FIG. 5. The effects of forskolin (10, 50 or 100 μM) on COX activity in IL-1β (1 ng/ml) plus PGE_2 (3 μM) treated HUVEC. COX activity was measured by the formation of 6-keto-$PGF_{1\alpha}$ in the presence of exogenous arachidonic acid (10 μM; 10 min). Data are expressed as mean±SEM of 12 determinations from at least four separate experimental days. *P<0.05 when compared to IL-1β plus PGE_2 treated HUVEC at 24 h. **P<0.05 when compared to IL-1β treated HUVEC at 24 h.

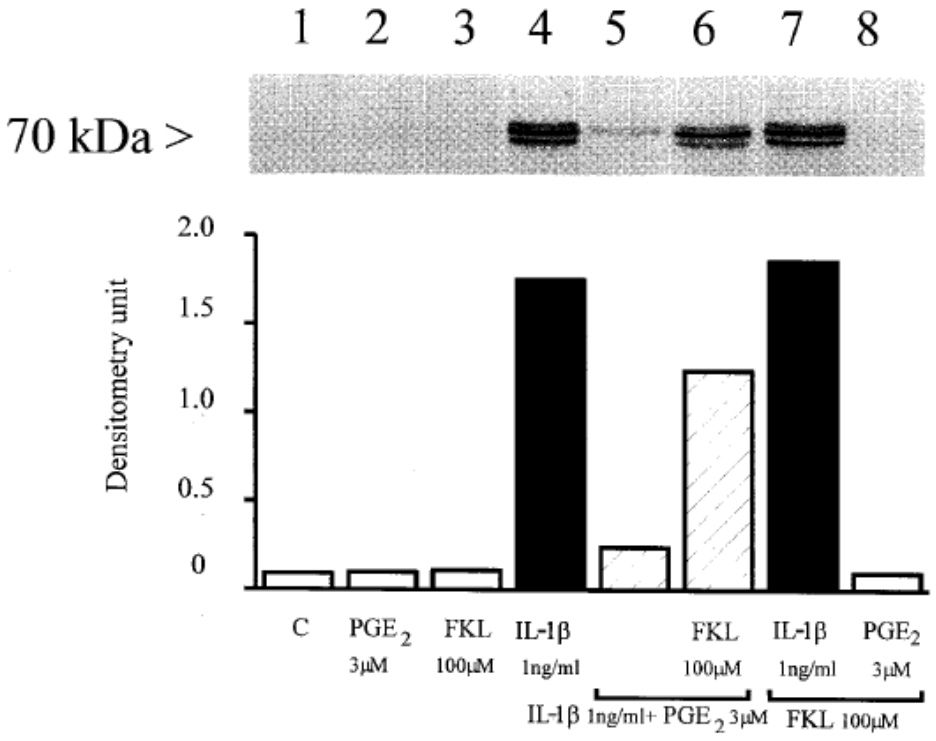

FIG. 6. The effects of forskolin on COX-2 protein expressed in IL-1β (1 ng/ml) plus PGE_2 (3 μM) treated HUVEC. COX-2 protein was detected by Western blots using polyclonal antibodies to COX-2 in cell extracts of HUVEC treated with no addition (lane 1), PGE_2 (3 μM; lane 2) alone, forskolin (100 μM; 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).

protein (Fig. 6; lanes 3 and 8, respectively). Similarly in COX activity, the inhibition of COX-2 induced in IL-1β (1 ng/ml) treated HUVEC by PGE_2 was also reversed when cells were coincubated with forskolin (100 μM; Fig. 6; lanes 4 to 6). However, unlike COX activity, the amounts of COX-2 protein induced in IL-1β (1 ng/ml) treated HUVEC was slightly increased when cells were coincubated with forskolin (100 μM; Fig. 6; lane 7). The amount of COX-1 protein expressed in HUVEC treated with foskolin (100 μM) alone, IL-1β (1 ng/ml) plus PGE_2 (3 μM), IL-1β (1 ng/ml) plus PGE_2 (3 μM) with forskolin (100 μM), IL-1β (1 ng/ml) plus forskolin (100 μM) or PGE_2 (3 μM) plus forskolin (100 μM) was not changed when compared to untreated HUVEC (Fig. 7).

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,[12] it is a major contributor to the production and maintenance of immunosuppression after overwhelming injury[10] and an important factor for implantation and decidualization.[17] 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

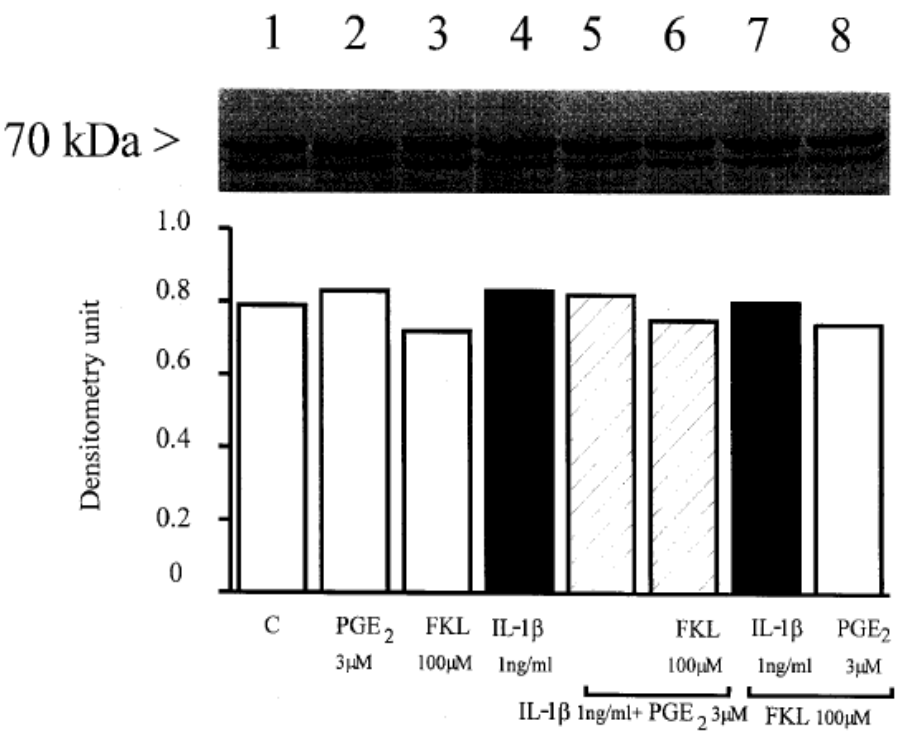

FIG. 7. The effects of forskolin on COX-1 protein expressed in IL-1β (1 ng/ml) plus PGE_2 (3 μM) treated HUVEC. COX-1 protein was detected by Western blots using polyclonal antibodies to COX-1 in cell extracts of HUVEC treated with no addition (lane 1), PGE_2 (3 μM; lane 2) alone, forskolin (100 μM; 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 lanes. 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).

different in their signal transduction properties.[18] 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.[19] However, our studies showed that forskolin (cAMP activator) can reverse the inhibiton of PGE_2 on COX-2 induced in IL-1β treated HUVEC suggesting PGE_2 may inhibit COX-2 expressed in IL-1β 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,[4–8] 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.[20] 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 μM) inhibited PGE_2 production (30% inhibition; Fig. 2A) more than PGI_2 production (20% inhibition; Fig. 2B) in IL-1β treated endothelial cells. These may explain the COX metabolites produced in IL-1β 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-1β. These suggested that PGE 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.[13] PGE series have been used in clinical disorders such as peripheral vascular occlusive diseases,[21] NSAIDs-induced gastric ulcer,[22] abortion[23] and impotence.[24] 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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