

CYCLOOXYGENASE (COX), existing as the COX-1 and COX-2 isoforms, converts arachidonic acid to prostaglandin H_2 , which is then further metabolized to various prostaglandins. Vascular endothelial growth factor (VEGF) has been shown to play important roles in inflammation and is upregulated by the prostaglandin E series through COX-2 in several cell types. Here, we have investigated the effects of VEGF on the COX isoform expressed in human umbilical vein endothelial cells (HUVEC). The signalling mechanism of the COX isoform expressed in endothelial cells activated with VEGF will be also investigated using the tyrosine kinase inhibitor, genistein, and protein kinase C inhibitor, staurosporine. The activity of COX-2 was assessed by measuring the production of 6-keto-prostaglandin $F_{1\alpha}$ in the presence of exogenous arachidonic acids ($10 \mu\text{M}$, 10 min) by enzyme immunoassay. The expression of COX isoform protein was detected by immunoblot using specific antibodies. Untreated HUVEC contained no COX-2 protein. In HUVEC treated with VEGF (0.01–50 ng/ml), COX-2 protein, but not COX-1, and COX activity were increased in a dose-dependent manner. Interestingly, the increased COX-2 protein and activity in response to VEGF (10 ng/ml) was inhibited by the tyrosine kinase inhibitor, genistein (0.05–5 $\mu\text{g}/\text{ml}$), but not by the protein kinase C inhibitor, staurosporine (0.1–10 ng/ml). Thus, the induction of COX-2 by VEGF in endothelial cells was mediated through protein tyrosine kinase, and the uses of specific COX-2 inhibitors in these conditions, in which VEGF was involved, might have a role.

Key words: Cyclooxygenase, Endothelium, VEGF, Tyrosine kinase, Protein kinase C

The expression of COX-2 in VEGF-treated endothelial cells is mediated through protein tyrosine kinase

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Introduction

Cyclooxygenase (COX) converts arachidonic acid to prostaglandin H_2 ,¹ which is then further metabolized to various prostaglandins, prostacyclin and thromboxane A_2 .² COX exists in at least two isoforms.³ COX-1 is expressed constitutively in endothelial cells⁴ and is probably responsible for the production of prostaglandins under physiological conditions.⁵ COX-2 is induced by pro-inflammatory stimuli, including mitogens,⁶ cytokines,^{7,8} and bacterial lipopolysaccharide,⁴ in cells *in vitro* and in inflamed sites *in vivo*.⁹ However, COX-2 has been shown to be constitutively expressed in some cell types and tissues such as brain, spinal cord, kidneys, reproductive organs, some vascular endothelial cell types and species¹⁰ including human platelets.¹¹ COX-2 has also been shown to be upregulated in some conditions such as atherosclerosis and gastric ulcer associated with *Helicobacter pylori*.^{12,13} These are thought to be physiological stress or pathological defense functions of

COX-2, and raised the hypothesis of whether another isoform of COX (COX-3) may exist.¹⁴

Vascular endothelial growth factor (VEGF)¹⁵ is a homodimeric 34–42 kDa heparin-binding glycoprotein with potent angiogenic, mitogenic and vascular permeability-enhancing activities specific for endothelial cells. VEGF is expected to play important roles in inflammation and during normal and pathological angiogenesis, a process that is associated with wound healing, embryonic development, and growth and metastasis of solid tumors.¹⁶ Elevated levels of VEGF have been reported in synovial fluids of rheumatoid arthritis patients and in sera from cancer patients.^{17–19}

Recently, COX-2 has been shown to enhance basic fibroblast growth factor-induced angiogenesis through induction of VEGF in rat sponge implants.²⁰ We have therefore investigated whether VEGF can activate COX-2 expressed in endothelial cells. The signalling mechanism by which the COX isoform is expressed in endothelial cells activated with VEGF

will be also investigated using the tyrosine kinase inhibitor, genistein, and protein kinase C inhibitor, staurosporine.

Materials and methods

Cell culture

Human umbilical vein endothelial cells (HUVEC) were obtained from babies born to normal pregnant women as previously described.²¹ All patients gave written informed consent. HUVEC were cultured in 96-well plates with human endothelial-SFM basal growth medium (Gib Thai, Bangkok, Thailand) containing 10% foetal calf serum (Gib Thai), 100 U/ml of penicillin G sodium and 100 µg/ml of streptomycin. Cells were incubated at 37°C in a humidified incubator and grown to confluence before use.

Measurement of the COX activity

Confluent HUVEC were gently washed twice with phosphate-buffered saline (PBS) and incubated with human endothelial-SFM basal growth medium (200 µl/well) with no addition, VEGF (0.01, 0.1, 1 or 10 ng/ml) alone, genistein (a protein tyrosine kinase inhibitor; 5 µg/ml) alone, staurosporine (a protein kinase C inhibitor; 10 ng/ml) alone, or VEGF (10 ng/ml) + genistein (0.05, 0.5 or 5 µg/ml) or staurosporin (0.1, 1 or 10 ng/ml) for 24 h. After this, the medium was removed and washed twice with PBS. COX activity was measured by the production of 6-keto-prostaglandin (PG) F_{1α} (a stable metabolite of PGI₂ that is the major COX metabolite in endothelial cells) in the replaced fresh medium containing exogenous arachidonic acid (10 µM for 10 min) using an enzyme immunoassay (EIA kit, Amersham, Buckingham, UK).

Immunoblot (Western blot) analysis

HUVEC that were untreated (control), treated with genistein (5 µg/ml) alone, staurosporine (10 ng/ml) alone, VEGF (0.01–50 ng/ml) alone, VEGF (10 ng/ml) + genistein (5 µg/ml) or VEGF (10 ng/ml) + staurosporine (10 ng/ml) were cultured in six-well plates (37°C, for 24 h). After incubation, cells were extracted and a specific monoclonal COX-1 or COX-2 antibody was used to detect the expression of COX isoform as previously described.²²

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.²³ 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 in each well). The extent of reduction of MTT to formazan within cells was quantitated by the measurement of optical density at 650 nm using a microplate reader (BIORAD, California, USA).

Statistical analysis

Results are shown as mean ± standard error of the mean from triplicate determinations (wells) from at least three separate experimental days ($n = 9$). Student's paired or unpaired *t*-tests were used as appropriate to determine the significance of differences between means, and $p < 0.05$ was taken as statistically significant.

Materials

Staurosporine, genistein, dimethyl sulfoxide (DMSO), PBS (pH 7.4), Trisma base, ethylenediamine tetraacetic acid, triton X-100, phenylmethylsulphonyl fluoride, pepstatin A, leupeptin, glycerol, bromphenol blue, 2-mercaptoethanol, sodium dodecyl sulphate, anti-rabbit IgG antibody, goat IgG, premixed BCIP/NBT solution, MTT, penicillin G sodium and streptomycin were supplied by Sigma Chemical Company (St. Louis, MO, USA). 6-keto-PGF_{1α} assay kits were purchased from Amersham (USA). Human endothelial-SFM basal growth medium and foetal calf serum were obtained from Gib Thai. A specific monoclonal COX-1 (human) or COX-2 (human) antibody was obtained from Cayman Chemical Company (Sapphire, Australia). Recombinant human VEGF was purchased from R&D (Minneapolis, MN, USA). Pure nitrocellulose membrane (0.45 µm) and filter paper were purchased from BIO-RAD.

Results

The effects of VEGF on the COX isoform expressed in HUVEC

Control untreated cells contained COX-1 protein but not COX-2 protein (Figs. 1 and 2, lane 1). The amount of COX-1 protein expressed in HUVEC treated with VEGF (0.01, 0.1, 1, 10 or 50 ng/ml) did not change when compared with control untreated cells at 24 h (Fig. 2). Interestingly, COX-2 protein was induced in HUVEC treated with VEGF (0.01, 0.1, 1, 10 or 50 ng/ml) in a dose-dependent manner (Fig. 1).

The effects of VEGF on COX activity in HUVEC

In HUVEC treated with VEGF (0.01, 0.1, 1 or 10 ng/ml), COX activity as measured by the production of 6-keto-PGF_{1α} in the presence of exogenous arachidonic acids (10 µM for 10 min) was also increased in a dose-dependent manner. The increased COX activity in VEGF-treated HUVEC was significant at 0.1 ng/ml of VEGF ($p < 0.05$, $n = 12$; Fig. 3).

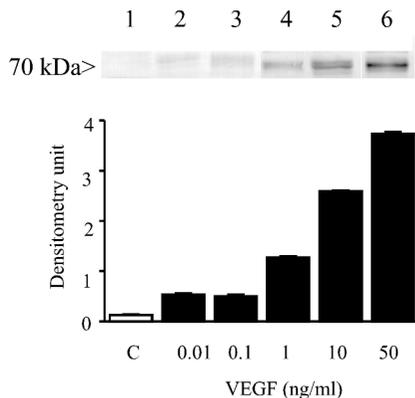


FIG. 1. The effects of VEGF on COX-2 expressed in HUVEC. COX-2 was detected by Western blots using specific antibodies to COX-2 in cell extracts of HUVEC treated with no addition (lane 1) or with VEGF (0.01, 0.1, 1, 10 or 50 ng/ml; lanes 2–6) for 24 h. Equal amounts of protein (20 μ g/lane) were loaded 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 an image one-dimensional program (densitometry unit).

VEGF (0.01, 0.1, 1 or 10 ng/ml) did not affect cell viability when compared with the control untreated cells over a 24 h incubation period ($p > 0.05$, $n = 12$).

The effects of genistein (tyrosine kinase inhibitor) and staurosporine (protein kinase C inhibitor) on COX activity in VEGF-treated HUVEC

Interestingly, the increased COX activity as measured by the production of 6-keto-PGF_{1 α} in the presence of

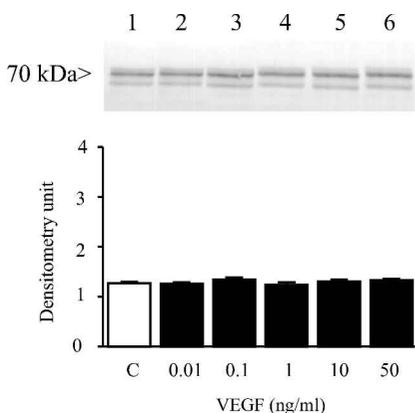


FIG. 2. The effects of VEGF on COX-1 expressed in HUVEC. COX-1 was detected by Western blots using specific antibodies to COX-2 in cell extracts of HUVEC treated with no addition (lane 1) or with VEGF (0.01, 0.1, 1, 10 or 50 ng/ml; lanes 2–6) for 24 h. Equal amounts of protein (20 μ g/lane) were loaded 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 an image one-dimensional program (densitometry unit).

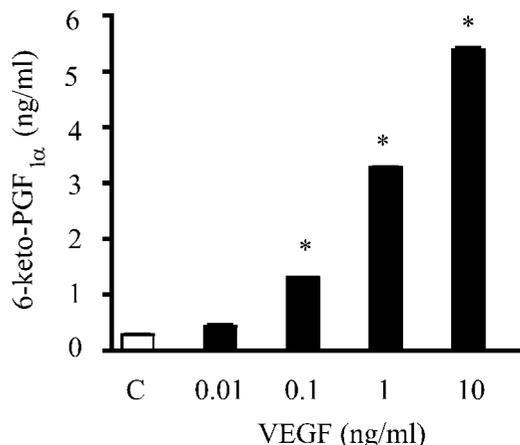


FIG. 3. Dose-dependent effects of VEGF (0.01–10 ng/ml) on COX activity in HUVEC. COX activity was measured by the formation of 6-keto-PGF_{1 α} in the presence of exogenous arachidonic acid (10 μ M, 10 min). Data are expressed as mean \pm standard error of the mean of 12 determinations from at least four separate experimental days. * $p < 0.05$ when compared with untreated HUVEC at 24 h (C).

exogenous arachidonic acids (10 μ M for 10 min) in HUVEC activated with VEGF (10 ng/ml) was inhibited by genistein (0.05, 0.5, or 5 μ g/ml, at 24 h) in a dose-dependent manner (Fig. 4A) but not by staurosporine (0.1, 1, or 10 ng/ml, at 24 h; Fig. 4B). The inhibition of increased COX activity in VEGF-treated HUVEC was significant at 0.5 μ g/ml of genistein ($p < 0.05$, $n = 12$; Fig. 4A). Moreover, the production of 6-keto-PGF_{1 α} in untreated HUVEC was not affected when cells were co-incubated with genistein alone (5 μ g/ml; Fig. 4A) or staurosporine alone (10 ng/ml; Fig. 4B) for 24 h.

Genistein (0.05–5 μ g/ml) and staurosporine (0.1–10 ng/ml) did not affect cell viability when compared with the control untreated cells over a 24 h incubation period (all $> 90\%$ of untreated cells).

The effects of genistein (tyrosine kinase inhibitor) and staurosporine (protein kinase C inhibitor) on the COX isoform expressed in VEGF-treated HUVEC

The induction of COX-2 protein by VEGF (10 ng/ml) in HUVEC was inhibited by genistein (5 μ g/ml, at 24 h) but not by staurosporine (10 ng/ml, at 24 h) (Fig. 5). The amount of COX-1 protein expressed in HUVEC treated with genistein (5 μ g/ml) alone, staurosporine (10 ng/ml) alone, VEGF + genistein (5 μ g/ml) or VEGF + staurosporine (10 ng/ml) at 24 h was not changed when compared with untreated control cells (Fig. 6).

Discussion

In the present paper, we have shown that VEGF could induce COX-2 protein expressed in HUVEC

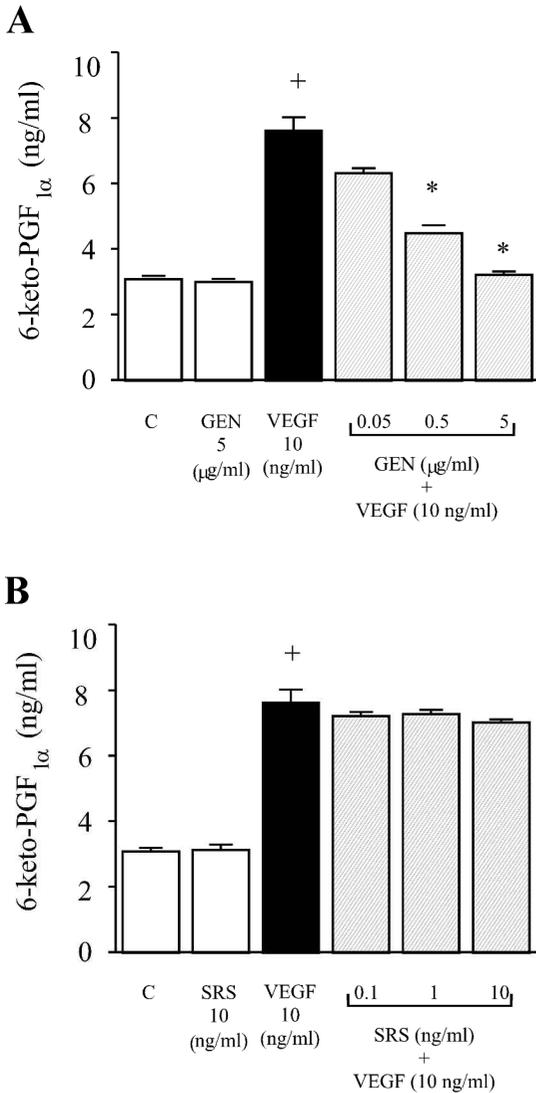


FIG. 4. The effects of (A) genistein (GEN) (0.05–5 µg/ml) and (B) staurosporine (SRS) (0.1–10 ng/ml) on COX activity in HUVEC treated with VEGF (10 ng/ml) for 24 h. COX activity was measured by the formation of 6-keto-PGF_{1α} in the presence of exogenous arachidonic acid (10 µM, 10 min). Data are expressed as mean ± standard error of the mean of 12 determinations from at least four separate experimental days. * *p* < 0.05 when compared with VEGF-treated HUVEC at 24 h; ⁺ *p* < 0.05 when compared with untreated HUVEC at 24 h (C).

while the amount of COX-1 protein expression was not affected. VEGF also increased COX activity as measured by the production of 6-keto-PGF_{1α} (a stable metabolite of PGI₂) in a dose-dependent manner. This increased COX activity was dependent on COX-2; Fig. 3 clearly shows that VEGF increased 6-keto-PGF_{1α} production in the presence of exogenous arachidonic acid in a dose-dependent manner, while COX-2 protein only (not COX-1 protein) was induced (Figs. 1 and 2). Moreover, the increased COX activity and COX-2 protein in VEGF-treated HUVEC were decreased when cells were co-incubated with genistein (tyrosine kinase inhibitor; Figs.

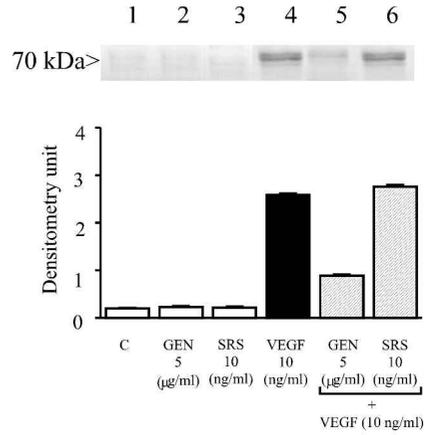


FIG. 5. The effects of genistein (GEN) and staurosporine (SRS) on COX-2 expressed in VEGF-treated HUVEC. COX-2 was detected by Western blots using specific antibodies to COX-2 in cell extracts of HUVEC treated with no addition (lane 1), with genistein (5 µg/ml) alone (lane 2), with staurosporine (10 ng/ml) alone (lane 3), with VEGF (10 ng/ml) alone (lane 4), with VEGF (10 ng/ml) + genistein (5 µg/ml; lane 5) or with VEGF (10 ng/ml) + staurosporine (10 ng/ml; lane 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 an image one-dimensional program (densitometry unit).

4 and 5) while COX-1 protein was unaffected (Fig. 6). However, the increased COX activity and COX-2 protein in VEGF-treated HUVEC were not changed when cells were co-incubated with staurosporine

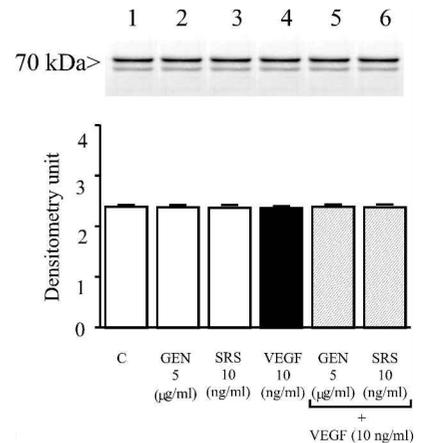


FIG. 6. The effects of genistein (GEN) and staurosporine (SRS) on COX-1 expressed in VEGF-treated HUVEC. COX-1 was detected by Western blots using specific antibodies to COX-1 in cell extracts of HUVEC treated with no addition (lane 1), with genistein (5 µg/ml) alone (lane 2), with staurosporine (10 ng/ml) alone (lane 3), with VEGF (10 ng/ml) alone (lane 4), with VEGF (10 ng/ml) + genistein (5 µg/ml; lane 5) or with VEGF (10 ng/ml) + staurosporine (10 ng/ml; lane 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 an image one-dimensional program (densitometry unit).

(protein kinase C inhibitor; Figs. 4 and 5). The COX-1 protein was also unaffected when cells were co-incubated with staurosporin (Fig. 6). Therefore, our results showed that the induction of COX-2 in HUVEC treated with VEGF was mediated through protein tyrosine kinase but not by protein kinase C.

VEGF is an endothelium-specific peptide that potently stimulates angiogenesis, vasodilation and microvascular hyperpermeability. Previous study showed that VEGF rapidly increased phosphorylation and activity of cytosolic PLA₂, and stimulated release of arachidonic acid in HUVEC resulted in prostaglandin production.²⁴ However, it was not shown whether the COX isoform mediates these effects. Therefore, the uses of exogenous arachidonic acid as substrates will help to evaluate COX enzyme directly. Recently, it has been shown that VEGF upregulates COX-1 in bovine aortic endothelial cells and HUVEC.²⁵ In contrast, here, our results showed that VEGF directly increased COX activity in HUVEC through the induction of COX-2. However, the amount of COX-1 protein expressed in HUVEC treated with VEGF did not change when compared with untreated HUVEC. A major concern of the different results was the cross-reactivity and source of the antibodies used. For COX-1 detection, Bryant *et al.*²⁵ used a 1 : 7500 dilution of anti-sheep COX-1 raised to rabbit while we used a 1 : 2000 dilution of specific COX-1 monoclonal antibody (clone CX-111). For COX-2 detection, Bryant *et al.*²⁵ used a 1 : 10,000 dilution of anti-human COX-2 raised to rabbit while we used a 1 : 2000 dilution of specific COX-2 monoclonal antibody. The other explanation was whether another COX isoform (COX-3) may exist. However, these points could be argued and await elucidation.

Moreover, we also showed that the increased COX activity and COX-2 protein in HUVEC treated with VEGF was mediated through protein tyrosine kinase, but not protein kinase C (Figs. 4 and 5). These findings were correlated with previous studies showing that VEGF receptors on endothelial cells were acted via protein tyrosine kinase²⁶⁻²⁸ and the induction of COX-2 in endothelial cells by several mitogens was mediated through protein tyrosine kinase.²⁹ Therefore, the downstream regulation of PG production in HUVEC activated with VEGF was affected at several sites such as COX-2 protein, tyrosine phosphorylation and phospholipase A₂.

In summary, our studies are the first to show that COX-2 can be induced in HUVEC treated with VEGF through protein tyrosine kinase. VEGF is known to be involved in pathological conditions such as inflammation, atherosclerosis and carcinogenesis.¹⁶ Thus, the uses of specific COX-2 inhibitors in these conditions, in which VEGF was involved, might have a role.³⁰⁻³²

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