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Modulation of Cyclooxygenase in Endothelial Cells by Fibronectin: Relevance to Angiogenesis

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ABSTRACT

Cyclooxygenases (COX), which catalyze the formation of prostaglandins (PGs), have been implicated in angiogenesis. Adhesion of endothelial cells (ECs) to extracellular matrix (ECM) induces the expression of COX-2 and PG production. The present study was carried out to analyze the influence of the adhesive ECM protein, fibronectin (FN), in modulating COX expression and its implications to angiogenesis using in vitro cultures of human umbilical vein ECs. RT-PCR analysis showed that the level of COX-2 mRNA was significantly high while that of COX-1 decreased in ECs maintained on FN. On treatment with p38 MAPK inhibitor and anti-$\alpha_5\beta_1$ integrin antibody, FN dependent effect on COX expression was not observed. Analysis by ELISA and immunoblotting confirmed FN-dependent upregulation of COX-2 protein. The ratio of PG E_2:PG D_2 was significantly high in cells maintained on FN and on treatment with p38 MAPK inhibitor, the relative level of PG D_2 increased and that of PG E_2 decreased. Concomitant with the modulation of COX-2 and changes in PGs, ECs maintained on FN showed angiogenic response in an $\alpha_5\beta_1$ integrin/p38 MAPK dependent manner as evidenced by the expression of angiogenic markers, CD 31 and E-selectin. These results suggest a FN-$\alpha_5\beta_1$/FAK/p38 MAPK dependent upregulation of COX-2 causing a shift in the relative levels of PGs in HUVECs which contributes to the angiogenic effect of FN. J. Cell. Biochem. 105: 158–166, 2008. © 2008 Wiley-Liss, Inc.

KEY WORDS: HUVECs; FIBRONECTIN; COX-2; PROSTAGLANDINS; VEGF

Angiogenesis is the process by which new microvessels develop and grow from existing, quiescent vascular endothelium. Different cell types, extracellular matrix (ECM), growth factors and several small molecules interact in a complex manner and temporal sequence in the process of angiogenesis. Many molecules influencing angiogenesis have been identified [Yancopoulos et al., 2000], of which, inflammatory mediators are reported to contribute to the angiogenic process associated with pathological conditions such as metastatic cancer, rheumatoid arthritis, and diabetic retinopathy. Some of the inflammatory prostaglandins (PGs) are implicated in altered vascular permeability and angiogenesis [Ziche et al., 1982; Form and Auerbach, 1983].

PGs are synthesized from arachidonic acid (AA) by the action of cyclooxygenases (COX), which are the products of two distinct genes, referred to as COX-1 and COX-2. These isoforms catalyze the first two steps in prostanoid biosynthesis [Marnett and Kalgutkar, 1999]. They share about 60% homology at the amino acid level and have similar enzymatic activities, but have distinct biological functions [Tazawa et al., 1994; Williams and DuBois, 1996]. COX-1 expression is constitutive in many tissues whereas COX-2 expression is induced by inflammatory cytokines [Diaz et al., 1998]. The COX-2 of endothelial cells (ECs) is important in angiogenesis [Hull et al., 1999; Williams et al., 2000]. Over-expression of COX-2 appears to promote tumorigenesis, whereas non-steroidal anti-inflammatory drugs and COX-2 specific inhibitors suppress tumorigenesis and tumor progression [Dubois et al., 1998; Ruegg et al., 2003]. COX-2 is highly expressed in the tumor cells, activated stromal fibroblasts, infiltrating leukocytes, and angiogenic ECs. Hypoxia [Schmedtje et al., 1997], inflammatory cytokines [Nakagawa et al., 1999; Uracz et al., 2002], and angiogenic growth factors such as VEGF [Tamura et al., 2002] induce expression of COX-2 in ECs.

ECM is a key component involved in regulating the process of angiogenesis. For ECs to enter avascular tissue they must first detach themselves from basement membrane through limited, focal proteolysis and during the later invasive and proliferative phases of angiogenesis, the ECs undergo extensive interactions with a fibronectin (FN) rich matrix [Risau and Lemmon, 1988]. ECs interact

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with the individual components of ECM through specific cell surface receptors particularly transmembrane signal transducing integrin receptors [Hynes, 1999]. Integrin-mediated cell adhesion to various ECM proteins is suggested to be critical in maintaining steady state levels of COX-2 in ECs by the combined prevention of lysosome-dependent degradation and the stimulation of mRNA synthesis involving multiple signaling pathways [Zaric and Ruegg, 2005]. Moreover, adhesion dependent upregulation of COX-2 has been shown to be associated with increase in the product, PG E_2 [Zaric and Ruegg, 2005] which promotes angiogenesis [Ben-Av et al., 1995; Tsuji et al., 1998]. However, the effect of adhesion of EC to FN in modulating the expression of COX-2 and the levels of PG and its role in angiogenesis is not fully understood. In this manuscript, we report the upregulation of COX-2 causing an increase in the ratio of PG E_2:PG D_2 by FN in a p38 MAPK dependent manner, contributing to angiogenesis.

MATERIALS AND METHODS

MATERIALS

MCDB131 medium, antibiotic–antimycotic solution, *o*-phenylene diamine dihydrochloride, DEPC, diamino benzidine, Tris, glycine, protease inhibitor cocktail, bovine serum albumin, human plasma FN, polylysine, monoclonal antibodies against VEGF, E-Selectin, COX-2, and HRP-conjugated secondary antibody were purchased from M/s Sigma Aldrich Co (USA). Antibody against $\alpha_5\beta_1$ integrin was a product of GIBCO BRL Products (USA). Perfect RNA Mini isolation kits and C-Master RT Plus PCR kits were purchased from Eppendorf (Germany). NC membrane was from BIORAD (USA). Tissue culture plastic wares were purchased from NUNC (Denmark). All other reagents used were of extra pure quality from Merck (India).

METHODS

Isolation and culture of HUVECs. ECs were isolated by collagenase perfusion of the umbilical vein as described before [Jaffe et al., 1973; Kumar et al., 2007]. The viability of isolated HUVECs was determined by Trypan blue exclusion. Cells (1.5×10^6 cells/ml) in serum free MCDB131 medium [Kiran et al., 2006] were seeded in tissue culture plates (60 mm, NUNC) passively coated with FN, Col I or polylysine (50 μg/ml), allowed to attach overnight, unattached cells were removed and attached cells were maintained in culture in serum-free MCDB131 medium in a Sanyo carbon dioxide incubator at 37°C and 95% air/5% CO_2 atmosphere. Morphological changes were examined micro photographically. The cultures were immunostained for the expression of EC specific markers such as CD 31 and vWF.

Cyclooxygenase assay. COX (EC 1.14.99.1) was assayed by oxygraphic method [Fritsche et al., 2001]. The enzyme extract was incubated in 0.1 M Tris–HCl (pH 8.0) containing 5 mM EDTA, 2 mM phenol, and 1 mM hematin at 37°C. The reaction was started by the addition of 100 μM AA and monitored by measuring the amount of oxygen used, using a Hansa Tech, England Oxygraph.

Estimation of prostaglandins, PG E_2 and PG D_2. The level of PGs in the culture supernatants were determined by HPLC by the method of Holtmann et al. [1990]. Briefly, the culture medium was subjected to solid-phase extraction on 1 ml Sep Pak C18 columns (Waters, MA). The eluates containing the eicosanoids were concentrated under a stream of nitrogen. Initial calibration of the C18 column was done using PG E_2 and PG D_2 standards by isocratic reversed-phase high-pressure liquid chromatography at a flow rate of 2 ml/min in a Shimadzu LC-10AT HPLC and the mobile phase consisted of acetonitrile (31.9% v/v), acetic acid (0.1% v/v), and water (68% v/v) adjusted to pH 4.5 with sodium acetate. Each sample was analyzed for the separation and determination of PG E_2 and PG D_2 in a single run. Peak areas corresponding to different concentrations of PG E_2 and PG D_2 were determined and a calibration curve was constructed from which the concentrations of PG E_2 and PG D_2 in the samples were calculated.

ELISA. Amount of E-Selectin, CD 31 (PECAM1), and COX-2 were quantitated by ELISA [Engvall and Perlman, 1971] using HRP conjugated secondary antibody. *o*-phenylene diamine dihydrochloride was used as substrate. Color intensity at 495 nm was read in a multiwell microplate reader (Thermo Multiskan Spectrum) and normalized with cell protein in each sample as determined by the method of Lowry et al. [1951].

Semi quantitative reverse transcription PCR (RT-PCR). Total RNA from HUVECs maintained in culture was isolated using Perfect RNA Mini isolation kit (Eppendorf) according to manufacturer's instruction. The nucleotide sequence of primer pairs used to determine the levels of human COX-1, COX-2, and GAPDH mRNA were as follows:

COX-1 (299 bp) forward primer-5′-CAAACGCTCCCATTTTTACA-CTC-3′ and reverse primer-5′-TGGCATGTAGTAGTCTCTTGGCA-3′, COX-2 (452 bp) forward primer-5′-AATTCCTCATCCAACTAT-GTTCC-3′ and reverse primer-5′-ATACTGTTCTCCGTACCTTCACC-3′ GAPDH (680 bp) forward primer-5′-CGGAGTCAACGGATTTG-GTCGTAT-3′ and reverse primer-5′-GCAGGTCAGGTCCACCACT-GAC-3′.

The primer sequences were selected from NCBI nucleotide database and custom synthesized by Sigma-Aldrich Chemicals Co, Bangalore, India. Twenty microliters (2 μg) of the isolated RNA was used as template for reverse transcription and amplification and the reaction was performed in an Eppendorf thermocycler in a single step, as described before [Kumar et al., 2007]. Briefly, the reaction mixture was incubated at 53°C for 60 min for reverse transcription and 94°C for 2 min for initial denaturation and cycled 30 times each at 94°C for 15 s (template denaturation), 58°C for 30 s (primer annealing), and 68°C for 45 s (primer elongation). Appropriate negative controls were used without reverse transcriptase. PCR products were resolved in a 1.75% agarose gel containing ethidium bromide and the bands were visualized by UV transillumination in a BioRad gel doc. The amplification was not in the saturating range for the primer sets employed. The relative intensity of the bands was quantitated using BioRad Quantity One version 4.5 software in a BioRad gel doc.

STATISTICAL ANALYSIS

All the data are expressed as mean with standard error of mean. The statistical significance of difference was analyzed by Duncan's One-way Analysis of Variance (ANOVA) using SPSS 11.0 Software. A value of $P < 0.05$ was considered significant.

RESULTS

ACTIVITY OF CYCLOOXYGENASE IN HUVECs MAINTAINED ON FN SUBSTRATUM

To study the effect of FN on the activity of COX in ECs, HUVECs were maintained in culture on FN, Col I, or polylysine coated substrata and the total activity of COX in terms of oxygen consumption was assayed and the results are shown in Figure 1. The activity of COX was significantly high in cells maintained on Col I and FN as compared to polylysine, but the activity was almost similar in the case of cells on Col I and FN.

mRNA AND PROTEIN EXPRESSION OF COX-2 IN CELLS MAINTAINED ON FN SUBSTRATUM

To examine whether the increase in the activity of COX in ECs adhered on matrix protein was due to difference in the expression of genes of both COX-1 and COX-2, the expression of these mRNAs was studied by RT-PCR analysis and the results are shown in Figure 2A. The expression of COX-1 was significantly low while that of the inducible COX-2 increased significantly in cells maintained on FN as compared to the cells maintained on Col I or polylysine. The FN effect on mRNA expression was apparently more pronounced in the case of COX-2 than COX-1. ELISA showed that the levels of COX-2 protein were significantly high in cells maintained on FN as compared to those maintained on Col I and polylysine substrata (Fig. 2B) indicating upregulation of COX-2 gene.

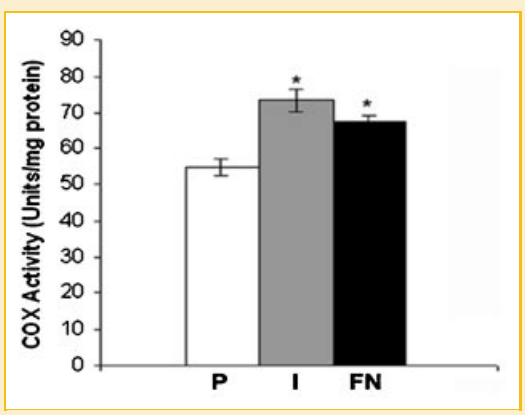

Fig. 1. Activity of cyclooxygenase in cells maintained on different matrix protein substrata. Isolated HUVECs were maintained in culture on fibronectin (FN), Collagen I (Col I), and polylysine (P) coated plates for 48 h in MCDB131 medium in a carbon dioxide incubator. At the end of the experiment, activity of COX was assayed in cell lysates as described in the text and expressed as specific activity (nanomoles of oxygen used/min/mg protein). The values given are the average of quadruplicate experiments ± SEM. *Statistically significant compared to P ($P < 0.05$).

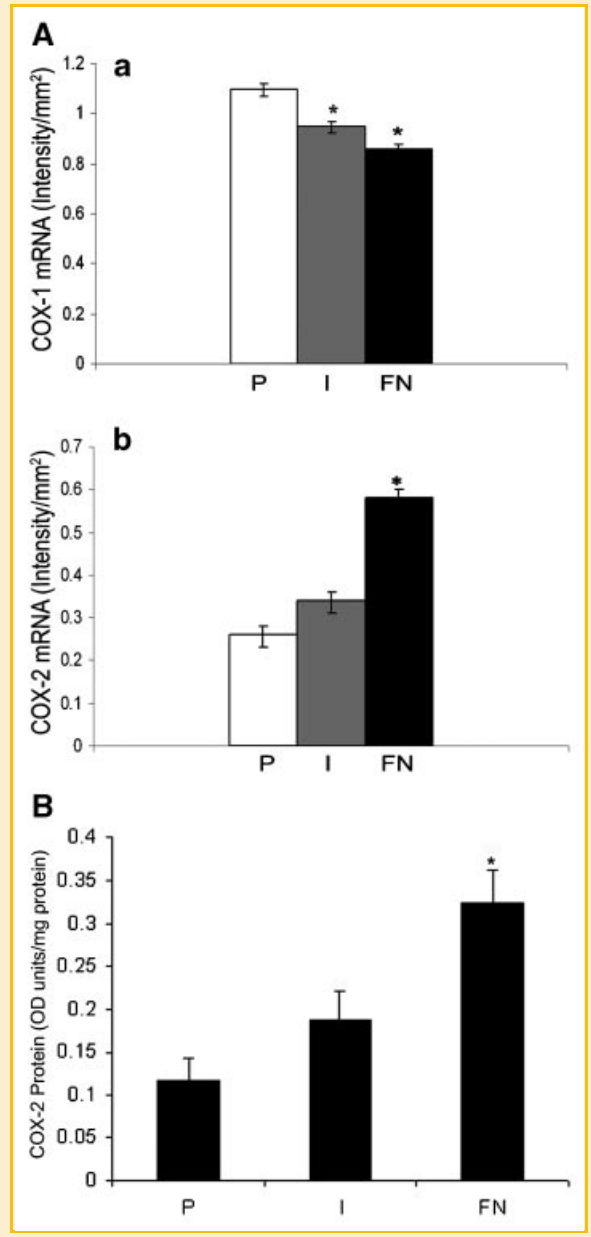

Fig. 2. Changes in the levels of COX-2 mRNA and protein in cells maintained on different substrata. A: HUVECs were maintained in culture for 48 h on polylysine, FN and Col I coated culture dishes as described in legends to Figure 1. Cells were harvested, total RNA isolated and mRNA levels of COX-2 (a) and COX-2 (b) were analyzed by RT-PCR. The PCR products were separated on 1.75% agarose gel and bands visualized by ethidium bromide staining. Relative intensity of the bands were quantitated by BioRad Quantity One 4.5 software and normalized with that for internal control (GAPDH). Values given are the average of quadruplicate experiments ± SEM. *Statistically significant compared to P ($P < 0.05$). B: Parallel cultures were maintained as above and analyzed for the protein expression of COX-2 using ELISA. The values given are the average of quadruplicate experiments ± SEM. *Statistically significant compared to P ($P < 0.05$).

LEVELS OF PG E_2 AND PG D_2 IN CELLS MAINTAINED ON FN SUBSTRATUM

In order to examine whether changes in the expression and activity of COX affect the production of different PGs, the major products of AA metabolism by COX viz., PG E_2 and PG D_2 were measured in culture supernatants using HPLC. The level of PG E_2 in cells maintained on FN was similar to that on polylysine. But the level of PG D_2 was significantly low in cells maintained on FN when compared to non-matrix substratum, polylysine. The ratio of PG E_2:PG D_2 was found to be significantly high in cells maintained on FN substratum as compared to Col I and polylysine (Table I).

MODULATION OF COX IN ECs BY FN—MECHANISM OF ACTION

Activity of COX. To study the mechanism of FN mediated upregulation of COX-2, the effect of inhibitors of various intracellular signaling pathways on COX-2 in ECs maintained on FN was examined (Fig. 3A). The activity of total COX was significantly high in cells maintained on FN and treated with SB202190, a p38 MAPK inhibitor, as compared to the control cells maintained on FN without the inhibitor. The inhibition of p38 MAPK caused an increase in the activity of total COX in cells maintained on polylysine also but the increase was not as significant as that observed in the cells maintained on FN and treated with SB202190. However, the activity was significantly decreased in cells maintained on FN and treated with PD98059, an ERK inhibitor whereas there was no significant difference in cells treated with wortmannin, a PI3K inhibitor. The COX activity was significantly lowered on blocking the cells on FN matrix substratum with anti-$\alpha_5\beta_1$ antibody.

Expression of COX. As the activity of COX was found to be high in cells treated with p38 MAPK inhibitor, the mRNA and protein expression patterns of the COX in cells maintained on FN were analyzed further. On inhibition of p38 MAPK, both the mRNA (Fig. 3B) and the protein (Fig. 3C) levels of COX-2 were down-regulated in cells maintained on FN. However, the mRNA expression of COX-1 was increased more than two fold in cells treated with SB202190 as compared to the untreated control cells maintained on FN without the inhibitor (Fig. 3B). On the contrary, the inhibition of p38 MAPK caused a significant increase in COX-2 mRNA and a marginal increase in COX-1 mRNA levels in the case of cells maintained on polylysine.

On treatment of ECs maintained on FN with anti-$\alpha_5\beta_1$ integrin antibody, the mRNA and protein levels of COX-2 decreased significantly whereas the COX-1 mRNA expression was upregulated when compared to the control cells maintained on FN without the antibody (Fig. 3B,C).

Changes in PG E_2 and PG D_2. The level of PG E_2 was significantly decreased while that of PG D_2 significantly increased when the cells maintained on FN were treated with SB202190 (Table II); the ratio of PG E_2:PG D_2 was significantly low in cells treated with SB202190.

TABLE I. Changes in the Prostaglandin Levels in Cells Maintained on Different Matrix Protein Substrata

	PG E_2 (nM)	PG D_2 (nM)	PG E_2:PG D_2
P	52.8 ± 1.32	36.05 ± 1.30	1:0.7
I	42.7 ± 1.09	114.14 ± 1.94	1:2.7
FN	53.90 ± 1.17	26.43 ± 1.12	1:0.5*

HUVECs were maintained in culture on polylysine (P), collagen I (Col I), and fibronectin (FN) coated plates for 48 h and the prostanoids secreted into the medium were quantitated by HPLC. The values given are the average of quadruplicate experiments ± SEM.
*Statistically significant compared to polylysine, $P < 0.05$.

LEVELS OF ANGIOGENIC MARKERS IN HUVECs MAINTAINED ON FN SUBSTRATUM

PG E_2 appears to promote angiogenic phenotype in ECs while PG D_2 has an opposite effect. In order to examine whether FN dependent modulation of COX in ECs was related to angiogenesis, the expression of markers of angiogenesis in ECs maintained on FN was studied and the results are shown in Figure 4. The EC specific angiogenic markers, CD 31 and E-selectin in cells maintained on FN with and without p38 MAPK inhibitor, SB202190 were studied using ELISA. The angiogenic markers, E-selectin (Fig. 4A) and CD 31 (Fig. 4B) were found to be significantly low in cells treated with SB202190 as compared to the untreated control cells maintained on FN without the inhibitor.

As VEGF has been reported to upregulate COX-2 levels and the VEGF level was high in cells maintained on FN, cells were treated with anti-VEGF antibody to examine the effect of VEGF on COX-2 levels in cells maintained on FN. On blocking the VEGF produced by the cells on FN, both the mRNA and protein levels of COX-2 were significantly reduced (Fig. 5A,B).

DISCUSSION

The results presented here show that FN modulates COX in ECs particularly COX-2 by regulating the expression of the gene in an $\alpha_5\beta_1$ integrin mediated downstream signaling involving p38 MAPK pathway. The evidence in support of this are (a) the mRNA and protein levels of COX-2 were high in cells cultured on FN, (b) on p38 MAPK inhibition, the mRNA and protein expression of COX-2 were lowered, and (c) blocking of the FN specific integrin receptor, $\alpha_5\beta_1$ decreased the levels of COX-2 protein and mRNA.

Recent reports have shown that the adhesion to purified ECM proteins such as vitronectin [Murphy et al., 2003], FN [Han et al., 2004], collagen I [Cho et al., 2004], or a cell-derived matrix [Khan et al., 2004] promote COX-2 expression in various cell types. Cells over-expressing COX-2 appear to undergo phenotypic changes, including increased adhesion to ECM proteins and resistance to apoptosis [Tsujii and DuBois, 1995]. There appears to be an essential role for adhesion-induced COX-2 expression in EC function [Ruegg and Mariotti, 2003] and multiple integrins binding to immobilized or soluble integrin ligands induce de novo mRNA and protein expression of COX-2 in ECs which is mediated by multiple signaling molecules including c-Src, PI3-K, MEK 1/2, p38 MAPK, and PKC [Zaric and Ruegg, 2005]. The effect of FN on COX-2 expression in ECs appears to involve $\alpha_5\beta_1$ integrin activation followed by downstream signaling. This was evidenced by blocking the effect of FN by anti-$\alpha_5\beta_1$ integrin and activation of FAK/Src followed by p38 MAPK activation (data not shown). Further, a reciprocal relation

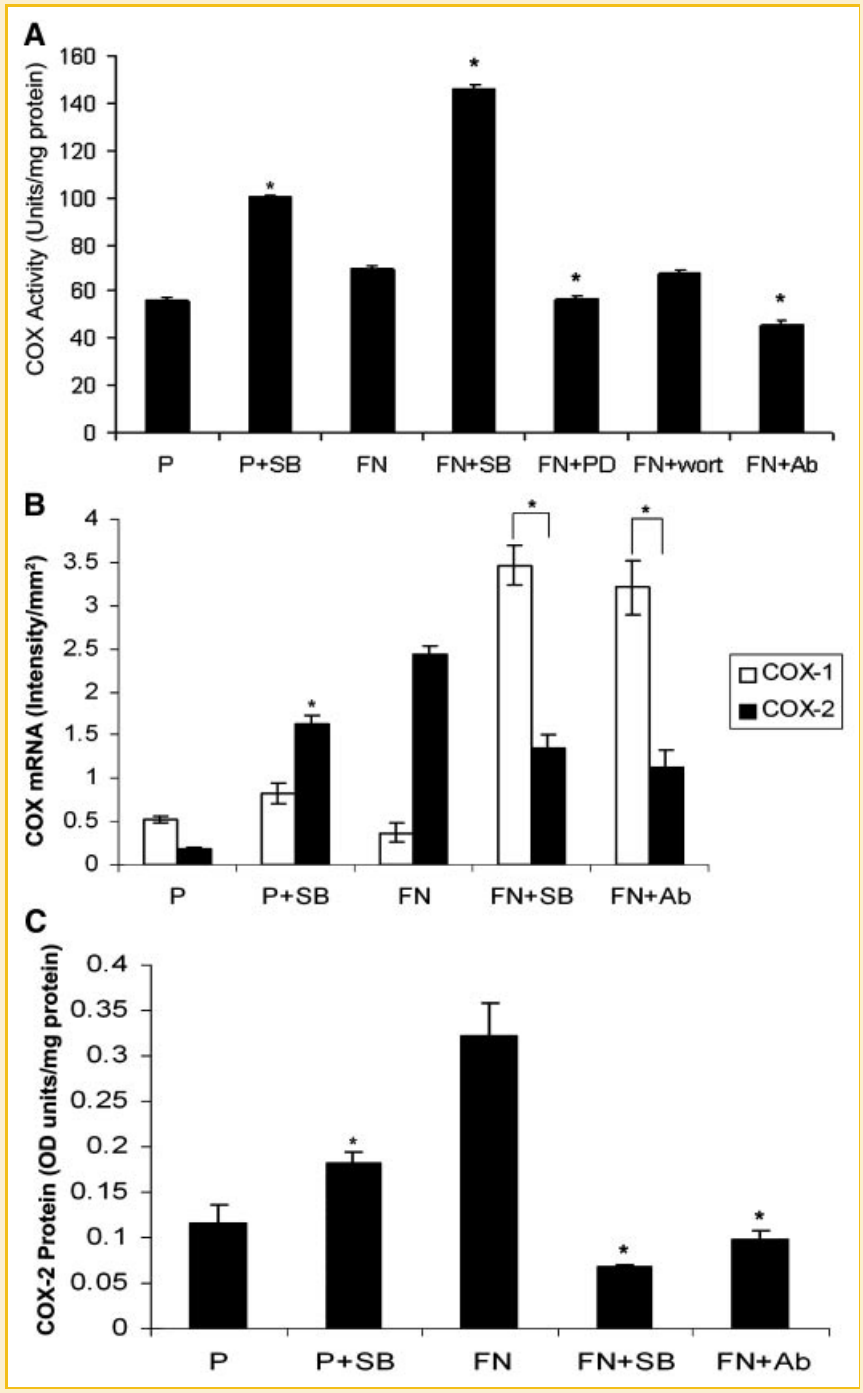

Fig. 3. Changes in the activity and level of mRNA and protein of COX-2 in cells maintained on FN substratum—effect of inhibitors of signaling pathways. A: Isolated HUVECs were maintained in culture on different substrata for 48 h in the presence and absence of pharmacological inhibitors of various signaling pathways and the activity of COX was assayed as described in legends to Figure 1. P, polylysine; FN, fibronectin; P + SB, polylysine + SB202190; FN + SB, fibronectin + SB202190; FN + PD, fibronectin + PD98059; FN + wort, fibronectin + wortmannin; and FN + Ab, fibronectin + blocking anti-$\alpha_5\beta_1$ antibody. The values given are the average of quadruplicate experiments ± SEM. *Statistically significant compared to FN ($P < 0.05$). B: HUVECs were maintained in culture on polylysine and fibronectin coated culture dishes for 48 h as described in legends to Figure 1 with (P + SB, FN + SB) or without (P,FN) p38 MAPK inhibitor, SB202190. A parallel culture was also maintained on FN and treated with anti-$\alpha_5\beta_1$ antibody (FN + Ab). Cells were harvested, total RNA isolated and mRNA levels of COX-1 and COX-2 were analyzed by RT-PCR. The PCR products were separated on 1.75% agarose gel and bands visualized by ethidium bromide staining. Relative intensity of the bands were quantitated by BioRad Quantity One 4.5 software and normalized with that for internal control (GAPDH). Values given are the average of quadruplicate experiments ± SEM. *Statistically significant when compared to respective controls without inhibitors ($P < 0.05$). C: Parallel cultures were maintained as above and analyzed for the protein expression of COX-2 using ELISA. The values given are average of quadruplicate experiments ± SEM. *Statistically significant when compared to respective controls without inhibitors ($P < 0.05$).

TABLE II. Effect of p38 MAPK Inhibition on the Levels of Prostaglandins in Cells Maintained on FN Substratum

	PG E_2 (nM)	PG D_2 (nM)	PG E_2:PG D_2
P	52.8 ± 1.32	36.05 ± 1.30	1:0.7
P + SB	57.77 ± 1.22	26.7 ± 1.84	1:0.5
FN	53.90 ± 1.17	26.43 ± 1.12	1:0.5
FN + SB	28.2 ± 0.69	65.36 ± 1.59	1:2.3*

HUVECs were maintained in culture on polylysine and fibronectin coated plates for 48 h with (P + SB, FN + SB) or without p38 MAPK inhibitor, SB202190 (P,FN). The prostanoids secreted into the medium were quantitated by HPLC. The values given are the average of quadruplicate experiments ± SEM.
*Statistically significant compared to FN, $P < 0.05$.

between the expression of COX-2 and COX-1 appears to exist, as FN dependent upregulation of COX-2 is associated with downregulation of COX-1 and inhibition of p38 MAPK reversed these effects. Our results confirm the report that COX-2 expression is adhesion-dependent in ECs [Zaric and Ruegg, 2005] and further

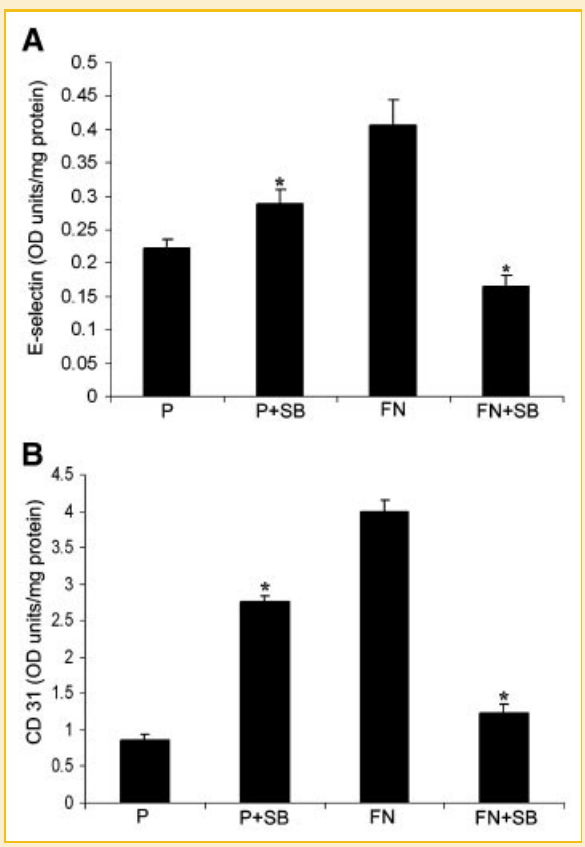

Fig. 4. Effect of p38 MAPK inhibition on the levels of angiogenic markers, E-selectin and CD 31 in cells maintained on FN substratum. HUVECs were maintained in culture on polylysine and fibronectin coated culture dishes for 48 h as described in legends to Figure 1 in the presence (P + SB, FN + SB) or absence (P,FN) of p38 MAPK inhibitor, SB202190. The levels of angiogenic marker proteins, E-selectin (A) and CD 31 (B) were quantitated by ELISA. The values given are average of quadruplicate experiments ± SEM. *Statistically significant when compared to respective controls without inhibitors ($P < 0.05$).

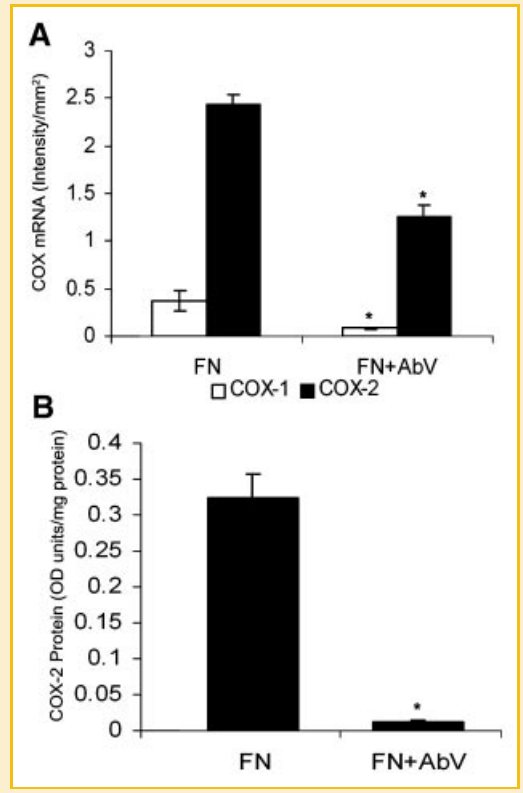

Fig. 5. Changes in the levels of mRNA and protein of COX in cells treated with anti-VEGF antibody. A: HUVECs were maintained in culture on fibronectin coated culture dishes for 48 h as described in legends to Figure 1 in the presence (FN + AbV) or absence (FN) of blocking anti-VEGF antibody. Cells were harvested, total RNA isolated and mRNA levels of COX-1 and COX-2 were analyzed by RT-PCR. The PCR products were separated on 1.75% agarose gel and bands visualized by ethidium bromide staining. Relative intensity of the bands were quantitated by BioRad Quantity One 4.5 software and normalized with that for internal control (GAPDH). Values given are average of quadruplicate experiments ± SEM. *Statistically significant when compared to FN ($P < 0.05$). B: Parallel cultures were maintained as above and analyzed for the protein expression of COX-2 using ELISA. The values given are average of quadruplicate experiments ± SEM. *Statistically significant when compared to FN ($P < 0.05$).

show an adhesive matrix protein, FN-specific regulation of COX in HUVECs involving upregulation of COX-2 and downregulation of COX-1. Results of the experiments on inhibition of p38 MAPK and blocking $\alpha_5\beta_1$ integrin by specific antibodies suggested that FN-$\alpha_5\beta_1$ integrin dependent activation of p38 MAPK causes upregulation of COX-2.

However, on treatment of cells maintained on non-matrix substratum, polylysine, with SB202190, both the mRNA and protein levels of COX-2 increased significantly, along with an upregulation of angiogenic markers viz., E-selectin and CD 31 when compared to the control cells maintained on polylysine without the inhibitor. There was only a marginal change in the level of COX-1 mRNA. Negative signaling elicited by endogenously produced matrix components or other factors to p38 MAPK in cells maintained on non-matrix substratum might modulate these effects.

The upregulation of COX-2 has important consequences in regulating the EC function as there was a change in the production of PGs by ECs maintained on FN. PG I_2, PG F_{2a}, and PG E_2, the main eicosanoids and PG D_2, a minor eicosanoid, are reported to be derived from AA by the action of COX in HUVECs [Lo'pez et al., 1993]. Our results show that the ECs when maintained on FN matrix substratum synthesized and secreted more amounts of PG E_2 and less amounts of PG D_2 increasing the ratio of PG E_2:PG D_2 in cells maintained on FN. This ratio was reversed when the cells were treated with p38 MAPK inhibitor indicating p38 MAPK dependent modulation of COX expression and PG production.

Among the products of the COX pathway, PG E_1, PG E_2, and TX A_2 are reported to promote angiogenesis [Ziche et al., 1982; Form and Auerbach, 1983; Daniel et al., 1999]. In contrast, 15-deoxy-$\Delta^{12,14}$-PG J_2, a dehydration product from PG D_2, induces EC apoptosis by activation of PPAR γ [Bishop-Bailey and Hla, 1999] and inhibits angiogenesis [Xin et al., 1999]. PGs have been known to modulate EC proliferation, migration, and capillary formation associated with angiogenesis [Graeber et al., 1990] and aspirin, an inhibitor of COX, inhibited the formation of cell–cell contact and capillary-like structures associated with angiogenic process [Kiran et al., 2006] suggesting a close association between prostanoids and angiogenesis. Moreover, the selective inhibition of COX-2 caused a decrease in the proliferative activity of HUVECs by cell cycle arrest to the G1 phase, as well as an inhibition of capillary-like tube formation [Yazawa et al., 2005]. PG E_2 by itself has been shown to influence angiogenesis in vivo and induces the expression of VEGF [Ben-Av et al., 1995; Seno et al., 2002]. PG E_2 may also contribute to angiogenesis by activating the NO/cGMP signaling pathway through PKA/PI3K/Akt-dependent increase in eNOS activity in HUVECs [Namkoong et al., 2005].

The high levels of angiogenic markers, E-selectin and CD 31 found in cells maintained on FN was significantly downregulated on inhibition of p38 MAPK which can be correlated with the COX-2 levels and PG production. The level of VEGF and VEGFR2 were more in cells maintained on FN [Viji et al., 2008], however on treatment with p38 MAPK inhibitor, their levels decreased significantly (data not shown). Further, the VEGF protein levels were very low in cells maintained on FN and treated with anti-$\alpha_5\beta_1$ integrin indicating that the increase in VEGF production by cells on FN is through the $\alpha_5\beta_1$ integrin pathway (data not shown). COX-2 has also been reported to contribute to angiogenesis through the production of PGs and VEGF [Tsujii et al., 1998; Cianchi et al., 2001] and by enhancing the mitogenic activity of VEGF on ECs [Jones et al., 1999]. Our results show that in ECs maintained on FN, the signaling pathway downstream of $\alpha_5\beta_1$ integrin upregulates COX-2, VEGF and other angiogenic markers concomitantly with the upregulation of PG E_2: PG D_2 ratio in a p38 MAPK dependent pathway. It therefore appears that the actual profile and the ratio of the downstream COX metabolites are more important in angiogenesis regulation rather than the level of COX protein or activity.

COX-2 expression is largely controlled at the transcriptional and post-transcriptional levels (mRNA stability and translation) [Ramsay et al., 2003; Dixon, 2004] and by soluble growth factors such as VEGF [Tamura et al., 2002]. It appears that apart from an effect of adhesion of ECs to FN and downstream signaling on the upregulation of COX-2 and downregulation of COX-1, the VEGF produced by the ECs also may exert an autocrine effect modulating the expression of COX-2 gene. This was evidenced by a decrease in the level of COX-2 in cells treated with anti-VEGF antibody. The levels of both VEGF and COX-2 were positively correlated with microvessel density and angiogenesis [Gallo et al., 2001; Cianchi et al., 2001]. Over-expression of COX-2 in colon cells is accompanied by upregulation of VEGF, bFGF, nitric oxide synthases, and angiogenesis [Tsuji et al., 1998]. Reports on PG E_2-induced VEGF gene expression in rat gastric microvascular ECs [Pai et al., 2001] and VEGF-induced upregulation in COX-2 mRNA, protein and its enzyme activity in human microvascular ECs [Tamura et al., 2002] suggest that there may be a positive feed back loop for continuous local production of VEGF and COX-2 dependent PG E_2 in ECs. Further, a cis-acting GATA element in the promoter region of COX-2 (as is present in the promoter region of several EC specific functions such as PECAM-1, eNOS, and vWF) that mediates VEGF-dependent induction of COX-2 gene expression has been characterized in vascular ECs [Tamura et al., 2002]. Nuclear factor-κB (NF-κB) responsive element is found in the promoter regions of VEGF and COX-2 [Rossi et al., 2000; Huang et al., 2001; Tanabe and Tohnai, 2002]. Immunoblot analysis revealed that there was nuclear translocation of NF-κB in cells maintained on FN (data not shown) indicating the involvement of this transcription factor in the upregulation of responsive genes.

It therefore appears that FN through $\alpha_5\beta_1$ integrin mediated downstream signaling may cause upregulation of COX-2 and the PGs so formed can influence VEGF production and angiogenesis. A direct effect of FN through $\alpha_5\beta_1$ integrin/p38 MAPK on VEGF production is also possible. As migrating ECs interact with provisional FN rich matrix, FN-$\alpha_5\beta_1$/FAK/Src/p38 MAPK signaling or FN-$\alpha_5\beta_1$/FAK/Src/p38 MAPK/VEGF pathway may lead to COX-2 upregulation in ECs causing a shift in favor of pro-angiogenic prostanoids that may promote the angiogenic phenotype.

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