Integrin-mediated Adhesion and Soluble Ligand Binding Stabilize COX-2 Protein Levels in Endothelial Cells by Inducing Expression and Preventing Degradation*

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Cyclooxygenase-2 (COX-2), a key enzyme in prostaglandin synthesis, is highly expressed during inflammation and cellular transformation and promotes tumor progression and angiogenesis. We have previously demonstrated that endothelial cell COX-2 is required for integrin αβ₁-dependent activation of Rac-1 and Cdc-42 and for endothelial cell spreading, migration, and angiogenesis (Dormond, O., Foletti, A., Paroz, C., and Ruegg, C. (2001) J. Biol. Chem. 276, 10441–10447; Dormond, O., Bezzi, M., Mariotti, A., and Ruegg, C. (2002) J. Biol. Chem. 277, 45838–45846). In this study, we addressed the question of whether integrin-mediated cell adhesion may regulate COX-2 expression in endothelial cells. We report that cell detachment from the substrate caused rapid degradation of COX-2 protein in human umbilical vein endothelial cells (HUVEC) independent of serum stimulation. This effect was prevented by broad inhibition of cellular proteinases and by neutralizing lysosomal activity but not by inhibiting the proteasome. HUVEC adhesion to laminin, collagen I, fibronectin, or vitronectin induced rapid COX-2 protein expression with peak levels reached within 2 h and increased COX-2-dependent prostaglandin E₂ production. In contrast, nonspecific adhesion to poly-L-lysine was ineffective in inducing COX-2 expression. Furthermore, the addition of matrix proteins in solution promoted COX-2 protein expression in suspended or poly-L-lysine-attached HUVEC. Adhesion-induced COX-2 expression was strongly suppressed by pharmacological inhibition of c-Src, phosphatidylinositol 3-kinase, p38, extracellular-regulated kinase 1/2, and, to a lesser extent, protein kinase C and by the inhibition of mRNA or protein synthesis. In conclusion, this work demonstrates that integrin-mediated cell adhesion and soluble integrin ligands contribute to maintaining COX-2 steady-state levels in endothelial cells by the combined prevention of lysosomal-degradation and the stimulation of mRNA synthesis involving multiple signaling pathways.

Tumor angiogenesis, i.e. the formation of new blood vessels in response to angiogenic stimuli, promotes tumor progression by stimulating tumor cell survival, tumor invasion, and metastasis formation (1). Many molecules mediating or regulating angiogenesis have been identified (2). They include vascular endothelial growth factors and their cell surface receptors, matrix-degrading enzymes, vascular remodeling ligands (i.e. angiopoietins), and their receptors and adhesion molecules of the integrin and cadherin families. Integrins are the main receptors for extracellular matrix (ECM)¹ proteins, consisting of two non-covalently associated α and β subunits (3, 4). Besides promoting physical adhesion, integrins also transduce signaling events (“outside-in” signaling), which are essential for cell spreading, migration, survival, proliferation, and differentiation. Signaling molecules downstream of integrins include Src family kinases, integrin-linked kinase, focal adhesion kinase, protein kinase C (PKC), extracellular-regulated kinase (ERK), and protein kinase B (PKB/Akt) (5, 6). These signaling pathways are also activated by growth factor receptors and the cross-talk between integrins, and growth factor receptors provide enhanced signaling efficacy, specificity, and control (6–8).

The cyclooxygenase (COX) isoenzymes COX-1 and -2 catalyze the first two steps in prostanoid biosynthesis (9). COX-1 expression is constitutive in many tissues, whereas COX-2 expression is induced by inflammatory cytokines, such as tumor necrosis factor (TNF), transforming growth factor β, or interleukin (IL)-1 (10) and by oncogenic mutations in several proto-oncogenes including c-Src (11), Ras (12), ErbB2 (13), or tumor suppressor genes such as p53 (14) or APC (15) (for review see Refs. 16 and 17). High levels of COX-2 expression are often observed in many human cancers including colon, breast, lung, and skin. Experimental studies have demonstrated that COX-2 overexpression promotes tumor progression, whereas non-steroidal anti-inflammatory drugs and COX-2-specific inhibitors suppress tumor progression (reviewed in Refs. 17 and 18). For example, in a murine model of human familial adenomatous polyposis coli, genetic inactivation of COX-2 dramatically reduced the number and size of intestinal polyps (19). Transgenic overexpression of COX-2 in the skin and in the breast promoted tumor progression and tumor progression (20, 21). Celecoxib was shown to reduce the risk of developing polyps and colon cancer in patients with

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¹ The abbreviations used are: ECM, extracellular matrix; COX, cyclooxygenase; HUVEC, human umbilical vein endothelial cell(s); PLL, poly-L-lysine; PKC, protein kinase C; ERK, extracellular-regulated kinase; P-I3-K, phosphatidylinositol 3-kinase; PKB, protein kinase B; JNK, c-Jun N-terminal kinase; TNF, tumor necrosis factor; PGE₂, prostaglandin E₂; BSA, bovine serum albumin; mAb, monoclonal antibody; PBS, phosphate-buffered saline; FCS, fetal calf serum; Act D, actinomycin D; CHX, cyclohexamide; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; IL, interleukin.
familial adenomatous polyposis coli (22). COX-2 and its main metabolite prostaglandin (PG) \(E_2\) promote tumor progression through two distinct but complementary mechanisms. First, they stimulate tumor cell survival, proliferation, migration, and invasion, and second, they induce tumor angiogenesis, which in turn favors local tumor progression and metastatic spreading. COX-2 stimulates angiogenesis by inducing vascular endothelial growth factor expression in tumor and stromal cells (23, 24), by enhancing the mitogenic activity of vascular endothelial growth factor on endothelial cells (25), and by promoting integrin \(\alpha_5\beta_3\) and cAMP/PKA-dependent activation of the small GTPases, Rac-1 and Cdc42 (26, 27), thereby resulting in increased endothelial cell proliferation, survival, and migration (reviewed in Refs 28 and 29). COX-2 expression is largely controlled at the transcriptional and post-transcriptional level (mRNA stability and translation) (30, 31). Although much is known regarding the role of growth factors and cytokines in the induction of COX-2 expression, little is known regarding the contribution of cell adhesion.

In this study, we investigated the role of cell adhesion in modulating COX-2 protein level in human umbilical vein endothelial cells (HUVEC). Here we report that COX-2 protein is rapidly degraded through a lysosomal-dependent pathway upon HUVEC detachment from the substrate and that integrin-mediated adhesion to ECM proteins induced de novo COX-2 synthesis involving signaling through multiple pathways (i.e. p38, mitogen-activated protein kinase, PI3-K, PKC, and c-Src).

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—Bovine gelatin, bovine plasma fibronectin, human plasma vitronectin, rat collagen I, BSA, murine laminin, poly-L-lysine (PLL), ammonium chloride, and LYS24002 were purchased from Sigma. CGP77675, RAD001, PKC412, and STI571 were obtained from Novartis AG (Basel, Switzerland). SB203580, PD98059, NS-398, and SC-560 were purchased from Biomol (Plymouth Meeting, PA). The DNA molecular weight marker was from Roche Applied Science. The anti-human COX-2 antibody was purchased from Cayman Chemical (Ann Arbor, MI). The anti-COX-1 rabbit polyclonal antibody (H-62) was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). The anti-human actin antibody was from Sigma. Function-blocking mAbs: FB12 (anti-\(\alpha_5\)), P1E6 (anti-\(\alpha_5\)), P1B5 (anti-\(\alpha_5\)), and LM609 (anti-\(\alpha_1\), \(\alpha_5\)) were from Chemicon (Temecula, CA); mAbs L1a/2 (anti-\(\beta_3\)), mAb G19 (anti-\(\alpha_1\)), and Sam-1 (anti-\(\alpha_5\)) and GoH3 (anti-\(\alpha_5\)) were from Beckman Coulter (Nyon, Switzerland). Colecokine was kindly provided by Pfizer AG (Großhansdorf, Switzerland). Human recombinant TNF (5 \(\times\) 10^9 units/ml) was a gift of Dr. G. Adolf (Boehringer Ingelheim, Vienna, Austria). Protease inhibitor mixture containing 4-(2-aminoethyl)-benzenesulfonyl fluoride, aprotinin, leupeptin, bestatin, pepstatin A, and E-64 were from Sigma. Lactacycin was a generous gift of Dr. F. Levy (Ludwig Institute for Cancer Research, Epalinges s/Lausanne, Switzerland).

**Cell Culture and Treatments**—HUVEC were prepared and cultured as previously described (32) except for the use of M199 (Invitrogen) as a basal medium. Basal medium was supplemented with 12 \(\mu\)g/ml bovine brain extract (Clonetics, Walkersville, MA), 10 ng/ml human recombinant epidermal growth factor (Genzyme, Cambridge, MA), 25 units/ml heparin, and 1 \(\mu\)g/ml hydrocortisone (Sigma). For de novo cell attachment experiments, HUVEC were collected by trypsin treatment (Invitrogen), resuspended in serum-free M199 medium, incubated for 2 h at 37 °C in suspension, and subsequently plated at 2 \(\times\) 10^5 cells/well on 12-well plates (Evergreen Scientific, Los Angeles, CA) pre-coated with fibronectin (3 \(\mu\)g/ml), gelatin (0.5%), collagen I (10 \(\mu\)g/ml), laminin (10 \(\mu\)g/ml), or vitronectin (1 \(\mu\)g/ml) in PBS for 1 h at 37 °C. For EDTA-mediated release, cells cultured for 12 h on gelatin-coated plates were washed twice with PBS and incubated with 3 mM EDTA for various periods of time. In experiments where ECM proteins were added in solution, cells were collected by tryptase treatment, washed with PBS, and resuspended in serum-free M199 medium and incubated for 2 h at 37 °C. Laminin, collagen I, fibronectin, or vitronectin (10 \(\mu\)g each) were then added, and cells were further incubated in suspension for another 30 and 60 min. Cells were observed and photographed using an inverted microscope (Axiovert 40 CFL, Carl Zeiss AG, Zürich, Switzerland) equipped with a PowerShot G5 digital camera (Canon, Dietlikon, Swit-
expression, HUVEC were maintained in complete medium (i.e. medium supplemented with FCS and growth factors) before detachment and during cultures in suspension. FCS and growth factors did not prevent the drop in COX-2 protein level observed after cell detachment (Fig. 1B). To obtain additional evidence that adhesion was required for maintaining COX-2 protein expression, we induced HUVEC detachment by adding 3 mM EDTA to confluent HUVEC cultures. Consistently, we observed a decrease in COX-2 protein level but with a slower kinetic compared with trypsin treatment (Fig. 1C).

COX-2 protein is mostly localized on the luminal side of the rough endoplasmic reticulum and Golgi complex (37) through asymmetric insertion into the membrane via a monotopic membrane binding domain (38, 39). This localization suggested the possibilities that the decrease in COX-2 protein induced by cell detachment could be either due to cell surface transport and extracellular secretion or to intracellular proteolytic degradation. To test the first possibility, we separately monitored COX-2 protein content in cells and conditioned culture supernatant following EDTA-induced cell detachment. Whereas cell-associated COX-2 protein levels progressively decreased, there was no detectable COX-2 protein accumulation in the culture supernatant (Fig. 2A). To address the second possibility, HU-
VEC were detached by trypsin and cultured in suspension in the presence or absence of a mixture of protease inhibitors (see “Experimental Procedures”). Broad inhibition of protease activity suppressed COX-2 degradation in HUVEC cultured in suspension (Fig. 2B). We then repeated the experiment in the presence of ammonium chloride, an inhibitor of lysosomal proteolytic activity (34), or lactacystin, a drug that inhibits trypsin-like, chymotrypsin-like, and peptidylglutamyl peptide-hydrolyzing activities of the proteasome (35). Ammonium chloride, but not lactacystin treatment, prevented the decrease in COX-2 protein observed upon cell detachment (Fig. 2, C and D).

Taken together these data demonstrate that the HUVEC detachment from the substrate causes a rapid decrease in COX-2 protein level, independently of serum stimulation, and that this decrease is due to lysosomal-mediated degradation.

**HUVEC Adhesion to Multiple Extracellular Matrix Proteins Induces COX-2 Protein Synthesis**—We next tested whether integrin-mediated HUVEC adhesion to ECM proteins did induce COX-2 protein expression. To this purpose, HUVEC were detached and maintained in suspension for 2 h to deplete COX-2 protein and then plated on laminin, collagen I, fibronectin, vitronectin, PLL, a substrate that promotes nonspecific integrin-dependent cell attachment to the substrate (40), or on BSA (a non-adhesive substrate). The integrin specificity of the adhesion was confirmed in short-term cell adhesion experiments by adding function blocking anti-integrin mAbs to HUVEC at the time of plating on the different substrates. Adhesion to laminin was mostly mediated by α5β1. Adhesion to collagen I was largely mediated by α2β1, with a minor contribution by α1β1 and α3β1. Adhesion to fibronectin was largely mediated by α5β1, with a minor contribution of α2β1, whereas adhesion to vitronectin was mediated by integrin α5β2 (Fig. 3). HUVEC adhesion to each of the four ECM proteins induced a time-dependent increase in COX-2 protein level, whereas integrin-independent adhesion to PLL or lack of adhesion (i.e. HUVEC on BSA) did not increase the level (Fig. 4A). Increase in COX-2 protein levels were observed as soon as 30 min after plating, whereas peak levels were observed at 2 h. At this time point, we observed some differences in the relative induction of COX-2 by with collagen I and laminin being the most potent inducers (Fig. 4B).

To demonstrate that the increase in COX-2 protein level upon adhesion translated into increased enzymatic activity, we monitored the generation of PGE_{20}, one of the main products of COX-2 enzymatic activity, in the culture supernatant of HUVEC plated on different ECMs. An increase of 2–3-fold was observed 4 h after plating on laminin, collagen I, fibronectin, and vitronectin compared with cells seeded on PLL- or BSA-coated wells (Fig. 5A). To confirm that PGE_{20} was the product of COX-2, HUVEC were plated on gelatin, another ligand for integrin α2β1 (32), and incubated for 24 h in the absence or presence of the COX-2-specific inhibitors celecoxib or NS-398. Both COX-2-specific inhibitors completely inhibited PGE_{20} production at doses of 10 μM. Celecoxib or NS-398 also inhibited PGE_{20} production induced by TNF, a potent inducer of COX-2 expression in endothelial cells (Fig. 5B). In contrast, the COX-1 inhibitor SC-560 did not suppress PGE_{20} production (Fig. 5C), consistent with previous observations (26).

From these results, we concluded that integrin-mediated HUVEC adhesion to multiple ECM proteins induced COX-2 protein expression, which translated into increased COX-2-dependent PGE_{20} production.

**Soluble Integrin Ligands Promote COX-2 Expression**—Whereas binding to substrate-immobilized ligands is the most effective way for integrins to transduce outside-in signaling and elicit cellular responses (6), there is evidence that the binding of soluble ligands is sufficient to initiate some outside-in signaling events and cellular responses (see “Discussion”). To test whether integrin ligation by soluble ligands was able to induce COX-2 expression, we performed two different experiments. First, we added soluble collagen I, fibronectin, or gelatin to HUVEC kept in suspension in the absence of FCS and measured COX-2 protein 30 and 60 min later. Each of these ECM proteins induced a marked increase in COX-2 protein expression (Fig. 6A). Second, we plated HUVEC on PLL and cultured them serum-free for 2 h before adding gelatin in solution. As shown in Fig. 6B, the addition of gelatin induced a time-dependent expression of COX-2. These data indicate that integrin ligation by soluble ligands is sufficient to induce COX-2 expression in HUVEC.

**Multiple Signaling Pathways Mediate Adhesion-dependent COX-2 Induction in Endothelial Cells**—Integrin ligation can activate multiple signaling pathways including the mitogen-activated protein kinase and the PI3-K-PKB/Akt pathways.
Adhesion-dependent COX-2 Expression Requires de Novo mRNA and Protein Synthesis—Cytokine-induced COX-2 expression is controlled transcriptionally (i.e. mRNA synthesis) and post-transcriptionally (i.e. mRNA stability and translation) (30, 31). To test which one of these mechanisms was predominantly involved in mediating adhesion-induced COX-2 expression, we monitored mRNA levels by semi-quantitative reverse transcription-PCR and protein expression by Western blotting in the absence or presence of actinomycin D (Act D, a RNA synthesis inhibitor) or cyclohexamide (CHX, a protein synthesis inhibitor) in suspended and fibronectin-adherent HUVEC. As expected, the COX-2 protein level was low in suspended HUVEC, whereas it was high in fibronectin-attached cells. Act D and CHX were equally effective in preventing COX-2 protein expression upon HUVEC adhesion to fibronectin (Fig. 8A). COX-2 mRNA levels were high in both suspended and fibronectin-attached HUVEC. Likewise, Act D and CHX efficiently inhibited COX-2 induction in HUVEC plated on laminin, collagen, and vitronectin (Fig. 8B).

From these results, we concluded that adhesion-induced COX-2 protein expression required de novo mRNA and protein synthesis and was therefore mainly regulated at the transcriptional and translational levels.

**DISCUSSION**

COX-2, the inducible form of the COX isoenzymes, is highly expressed in the tumor environment by tumor cells, activated stromal fibroblasts, infiltrating leukocytes, and angiogenic endothelial cells. Hypoxia (41), inflammatory cytokines, (e.g. IL-1) (42, 43), and angiogenic factors such as vascular endothelial growth factor (44) are well known inducers of COX-2 expression in endothelial cells. In contrast, little is known regarding the role of ECM proteins and cell adhesion in regulating endothelial cell COX-2 expression. In this work, we have addressed this issue by testing the effect of integrin-dependent cell adhesion to different ECM proteins on COX-2 protein expression and activity in cultured HUVEC.

Here we report three main findings. First, the detachment from the substrate caused a rapid drop in COX-2 protein level through lysosomal-dependent proteolytic degradation. This conclusion is based on the observations that HUVEC detachment induced by trypsin or EDTA caused a rapid decreased in COX-2 protein levels and that proteinase inhibitors or ammonium chloride, an inhibitor of lysosomal acidic proteolytic activity, prevented this effect. In contrast, the proteasome inhibitor lactacystin had no protective effect. Furthermore, there was no evidence of release of COX-2 into the cell culture medium. Detachment-induced protein degradation could not be prevented by the addition of serum or growth factors, indicating that protein degradation following de-adhesion is dominant over serum-induced COX-2 expression (45, 46). Second, integrin-mediated adhesion to various immobilized ECM proteins as well as soluble ECM proteins induced COX-2 protein expression and COX-2-dependent PGE$_2$ production. This conclusion is based on the observations that HUVEC in which COX-2 was depleted by a 2-h culture in suspension re-expressed COX-2 when plated on laminin, collagen I, fibronectin, and vitronectin, four well characterized ECM proteins that promote integrin-dependent HUVEC adhesion, but not when plated on PLL, a substrate that promotes cell adhesions through electrostatic attraction (i.e. independently of integrin engagement), or on BSA, a non-adhesive protein. Adhesion-induced COX-2 expression was functionally relevant, because it correlated with an increased production of PGE$_2$. The specific COX-2 inhibitors, NS-398 and celecoxib, but not the COX-1-specific inhibitor SC-560 fully blocked this PGE$_2$ production, thereby demonstrating that COX-2 was solely responsible for this effect.

(6). To determine which signaling molecules and pathways were mostly involved in mediating adhesion-induced COX-2 expression, serum-starved HUVEC were kept in suspension for 2 h and then plated on laminin, collagen I, fibronectin, and vitronectin in the absence or presence of pharmacological inhibitors of different signaling molecules. The ERK1/2 inhibitor PD98059, the p38 inhibitor SB203580, and the c-Src inhibitor CGP77675 efficiently suppressed COX-2 expression in HUVEC plated on all four ECM proteins (Fig. 7). The PI3-K inhibitor LY294002, the PKC inhibitor PKC412, and the c-Kit/platelet-derived growth factor receptor/cAb inhibitor STI571 partially inhibited COX-2 expression. In contrast, RAD001 and inhibitor of the mammalian target of rapamycin kinase, a direct substrate of PKB/Akt, had no effect (Fig. 7).

These results demonstrate that the c-Src-mitogen/stress-regulated kinases (i.e. ERK1/2 and p38) and the PI3-K pathways are two main pathways mediating integrin-induced COX-2 expression.
Third, adhesion-induced COX-2 expression required de novo mRNA and protein synthesis and was depended on signaling events involving multiple pathways (c-Src, the mitogen- and stress-regulated kinases such as ERK1/2 and p38, PI3-K, and, to a lesser extent, PKC).

These findings have at least two relevant implications. First, they define lysosomal-dependent proteolytic degradation as a

**FIG. 5.** HUVEC adhesion to different ECM proteins stimulates PGE2 production. A, serum-starved HUVEC were collected and seeded at 2 x 10⁵ cells/well on 12-well plates coated with laminin (LM), collagen I (Col I), fibronectin (FN), vitronectin (VN), PLL, or BSA. After 4 h, conditioned media were collected and PGE2 concentration was determined by enzyme immunoassay. B, HUVEC were plated at 2 x 10⁵ cells/well on 12-well plates coated with gelatin in the absence or presence of TNF (100 ng/ml) and of the COX-2 inhibitors, NS-398 or celecoxib, as indicated. C, HUVEC were plated at 2 x 10⁵ cells/well on 12-well plates coated with gelatin in the presence of the indicated concentrations of the COX-2 inhibitor NS-398 and of the COX-1 inhibitor SC-560. 24 h later, conditioned media were collected and the PGE2 concentration was determined by enzyme immunoassay. Results are expressed as picogram of PGE2/ml of a 2 x 10⁵ cell culture supernatant and represent the mean ± S.D. of duplicate determinations.

**FIG. 6.** COX-2 was induced by soluble integrin ligands. A, cells were preincubated in serum-free medium in suspension for 2 h, and then gelatin (Gel), fibronectin (FN), or collagen I (Col I) (10 μg each) were added to the medium. Cells were left in suspension for another 30 and 60 min. Samples were analyzed by Western blotting for COX-2 and actin protein levels. B, HUVEC were plated on PLL and cultured for 2 h in the absence of FCS. Collagen I was added to the medium (10 μg), and cells were harvested 15, 30, and 60 min later for COX-2 and actin determination by Western blotting.

**FIG. 7.** Multiple signaling pathways were involved in adhesion-mediated COX-2 protein expression. Cells were collected and serum-starved in suspension for 2 h. Prior to plating on laminin (LM), collagen I (Col I), fibronectin (FN), and vitronectin (VN), pharmacological inhibitors of signaling molecules were added: LY 294002 (20 μM); SB203580 (20 μM); PD98059 (20 μM); RAD 001 (0.1 μM); PKC 412 (1 μM); STI571 (10 μM); and (CGP77675 (5 μM). 2 h later, total cell extracts were prepared and analyzed for COX-2 and actin levels by Western blotting. For control, no inhibitor was added.

**FIG. 5.** HUVEC adhesion to different ECM proteins stimulates PGE2 production. A, serum-starved HUVEC were collected and seeded at 2 x 10⁵ cells/well on 12-well plates coated with laminin (LM), collagen I (Col I), fibronectin (FN), vitronectin (VN), PLL, or BSA. After 4 h, conditioned media were collected and PGE2 concentration was determined by enzyme immunoassay. B, HUVEC were plated at 2 x 10⁵ cells/well on 12-well plates coated with gelatin in the absence or presence of TNF (100 ng/ml) and of the COX-2 inhibitors, NS-398 or celecoxib, as indicated. C, HUVEC were plated at 2 x 10⁵ cells/well on 12-well plates coated with gelatin in the presence of the indicated concentrations of the COX-2 inhibitor NS-398 and of the COX-1 inhibitor SC-560. 24 h later, conditioned media were collected and the PGE2 concentration was determined by enzyme immunoassay. Results are expressed as picogram of PGE2/ml of a 2 x 10⁵ cell culture supernatant and represent the mean ± S.D. of duplicate determinations.
new mechanism by which COX-2 activity is rapidly suppressed in endothelial cells and implicate integrin-mediated cell adhesion as a critical inhibitory cue of this process. COX-2 expression was shown to be largely regulated at the transcriptional (i.e., mRNA expression) and post-transcriptional levels (i.e., mRNA stability and protein translation). Many transcription factors inducing COX-2 gene expression have been identified, including CCAAT/enhancer-binding protein-β, phospho-cAMP-response element-binding protein, NF-IL-6, AP1, NF-kB, and T-cell factor-4/hyphocyte-enhancing factor 1 (30, 47). COX-2 mRNA stability is regulated through an AU-rich element within the 3′-untranslated region (48). Furthermore, the protein translation from mature COX-2 mRNA can be regulated by translational silencers such as TIA-1 or CUGBP2 (49, 50).

Recently, proteasome-dependent COX-2 degradation was reported to regulate constitutive COX-2 protein levels in tumor cell lines and neural tissue. Treatment of four tumor cell lines with the 26 S proteasome-complex inhibitor MG-132 for 24 h resulted in the accumulation of COX-2 protein (51). Assuming that the proteasome was the only pathway responsible for COX-2 degradation, the half-life of COX-2 protein was estimated between 3.5 and 8 h depending on the tumor cell line studied (51). Treatment of the neuroblastoma-derived cell line HT4 and rat embryonic brain with the proteasomal inhibitor PSI or with cadmium, a heavy metal that disrupts the ubiquitin/proteasome pathway, stimulated the expression of COX-2 mRNA, the accumulation of polyubiquitinated COX-2 protein, and the increased production of PGE2 (52, 53). Thus, based on these reports and on our data, it appears that both the proteasomal and the lysosomal pathways can degrade COX-2 and thereby regulate its level. It is also possible that different degradation pathways are used in differing physiological or pathological situations. For example, the proteasomal pathway was suggested to play a role in regulating the physiological turnover of COX-2 protein and its inhibition may contribute to the increased accumulation of COX-2 and PGE2 production in conditions of sustained inflammation (51, 54). In contrast, the lysosomal pathway could become relevant in acute situations in which COX-2 activity and its pro-survival and pro-inflammatory effects need to be rapidly suppressed. This may be the case during detachment-induced apoptosis (i.e., anoikis) (55).

The second relevant conclusion is that integrin-mediated adhesion is a critical event in regulating COX-2 protein expression. Strikingly, besides cell adhesion to ECM proteins, the addition of soluble ECM proteins to HUVEC maintained in suspension or attached to PLL was already effective in inducing COX-2 expression, indicating that integrin-ligation per se is sufficient for this effect. Dynamic integrin-ligand binding is critically involved in transducing blood shear stress occurring

![Fig. 8. Adhesion-induced COX-2 expression involved transcriptional regulation. A, HUVEC were incubated in suspension for 2 h in the presence of ActD (5 μg/ml), CHX (5 μg/ml), or in medium alone (Ctrl) and plated in fibronectin-coated wells for 2 h. Total cell extracts were isolated and analyzed for COX-2 and actin protein levels by Western blotting. Total RNA was isolated, and COX-2 mRNA level was monitored by RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript was used as a reference housekeeping transcript. Products were separated on 1% agarose gel. B, cells were treated as in panel A and plated on wells coated with fibronectin, laminin, collagen I, and vitronectin. COX-2 and actin proteins were detected by Western blotting.](image)

![Fig. 9. Proposed model for integrin-mediated regulation of COX-2 protein level in HUVEC. Based on the obtained results, we propose the following model for integrin-regulated COX-2 expression in endothelial cells. On the one side, integrin-mediated adhesion induces de novo COX-2 gene expression (mRNA transcription and protein translation) through c-Src, MEK1/2, p38, PI3-K, and, to a lesser extent, PKC-dependent signaling events. Soluble ligands also induce COX-2 protein expression. On the other side, stable endothelial cell adhesion prevents lysosome-dependent proteolytic degradation of COX-2 protein.](image)
at the endothelial cell luminal surface into intracellular signaling events (56, 57). The induction of COX-2 by soluble integrin ligands observed in the present study is consistent with the reported induction of COX-2 expression in endothelial cells (58–60) and osteoblasts (61, 62) in response to fluid shear stress. These observations raise the possibility that vascular integrins on tumor endothelial cells may contribute to sustain COX-2 expression in response to increased shear stress due to the pathological architecture of tumor vessels (63) or to increased levels of circulating soluble ECM protein fragment observed in cancer conditions (64, 65).

Integrin-mediated COX-2 expression requires de novo mRNA and protein synthesis and is mediated by multiple signaling molecules including c-Src, PI3-K, MEK1/2, p38, and PKC. These signaling molecules have been previously reported to mediate COX-2 expression in other models, including keratinocytes exposed to UV (66, 67), taxanes (68), TNF (69), or IL-1 (70). In view of our results, one should consider the possibility that COX-2 expression observed in these or other models may require the integration of signaling events that are originating from the ECM, which are transduced by integrins (7). Recent reports have implicated adhesion to purified ECM proteins such as vitronectin (71), fibronectin (72), collagen I (73), or a cell-derived matrix (74) in promoting COX-2 expression and prostaglandin production in various cell types. In one report, mAb LM609, an α3β1 mAb that blocks αvβ3-mediated endothelial cell adhesion and angiogenesis (75), or LIBS 6, an α2β1 mAb specific for activated αvβ3 (76), but not other anti-αv or anti-β3 mAbs were shown to stimulate prostaglandin production, induce endothelial cell proliferation, and inhibit in vitro angiogenesis in a COX-1- and COX-2-dependent and COX-independent manner (71). In this study, the putative role of other integrins (i.e. β1 family) in inducing prostaglandin production was not systematically addressed using equivalent mAbs. Therefore, the α3β1 specificity of the observed effect cannot be claimed based on the presented data. The observation that ligation of several different integrins can induce COX-2 expression suggests that the effect (i.e. COX-2 expression) is more important than the specific factor (i.e. integrins or ligands) eliciting it, suggesting an essential role for adhesion-induced COX-2 expression in endothelial cell function (6). Thus, our findings extend these reports by demonstrating that multiple integrins binding to immobilized or soluble integrin ligands induce de novo COX-2 expression in endothelial cells.

A main question raised by our study concerns the mechanisms by which cell detachment promotes COX-2 degradation and integrin-mediated adhesion prevents it. The fact that multiple integrins may be able to elicit the same effect suggests that this effect may be associated with events downstream of integrins. One of these effects could be the disruption of the cytoskeleton during cell detachment and its rearrangement during adhesion. There are indeed a number of reports demonstrating that modulation of the cytoskeleton affects COX-2 levels and prostaglandin production. For example, microtubule-interfering agents (including taxol and nocodazole) enhanced COX-2 protein and mRNA levels and PGE2 synthesis in breast cancer cells (68, 77). Disruption of the actin microfilaments with cytochalasin D in adherent HUVEC resulted in increased COX-2 protein levels, consistent with published results (data not shown). Although at first these preliminary data tend to speak against a central role of the intact cytoskeleton in preventing COX-2 protein degradation, further experiments are needed before this possibility can be formally ruled out.

In conclusion, our data demonstrate that integrins promote COX-2 expression in endothelial cells through two complementary mechanisms. On the one side, they stimulate gene expression and protein synthesis, and on the other side, they suppress lysosomal-dependent degradation (Fig. 9). These data provide further evidence for a bidirectional functional interaction between COX-2 and integrins in endothelial cells with potential therapeutic implications. Immobilized and soluble integrin ligands promote COX-2 expression, whereas COX-2-derived prostaglandins stimulate integrin-dependent responses that promote angiogenesis (i.e. α3β1 and Rac-dependent endothelial cell migration).

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