Prostaglandin E2 promotes integrin αVβ3-dependent endothelial cell adhesion, Rac-activation and spreading through cAMP/PKA-dependent signaling

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SUMMARY

We have recently reported that inhibition of endothelial cell COX-2 by non steroidal anti-inflammatory drugs suppress αVβ3- (but not α5β1-) dependent Rac activation, endothelial cell spreading, migration and angiogenesis (Dormond et al., (2001) Nature Med. 7, 1041-1047). Here we investigated the role of the COX-2 metabolites PGE2 and TXA2 in regulating human umbilical vein endothelial cell (HUVEC) adhesion and spreading. We report that PGE2 accelerated αVβ3-mediated HUVEC adhesion and promoted Rac activation and cell spreading while the TXA2 agonist U46619 retarded adhesion and inhibited spreading. We show that the cAMP level and the cAMP-regulated protein kinase A (PKA) activity are critical mediators of these PGE2 effects. αVβ3-mediated adhesion induced a transient COX-2-dependent rise in cAMP levels, while the cell permeable cAMP analogue 8-brcAMP accelerated adhesion, promoted Rac activation and cell spreading in the presence of the COX-2 inhibitor NS-398. Pharmacological inhibition of PKA completely blocked αVβ3-mediated adhesion. A constitutively active Rac mutant (L61Rac ) rescued αVβ3-dependent spreading in the presence of NS398 or U46691, but did not accelerate adhesion, while a dominant negative Rac mutant (N17Rac) suppressed spreading without affecting adhesion. α5β1-mediated HUVEC adhesion, Rac activation and spreading were not affected by PGE2, U46691, 8-brcAMP or by the inhibition of PKA. In conclusion, these results demonstrate that PGE2 accelerates αVβ3-mediated endothelial cell adhesion through the cAMP-dependent PKA activation and induces αVβ3-dependent spreading via cAMP- and PKA-dependent Rac activation and may further contribute to the further understanding of the regulation of vascular integrins αVβ3 by COX-2/PGE2 during tumor angiogenesis and inflammation.
INTRODUCTION

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 involved in mediating or regulating angiogenesis have been identified (2). They include growth factors (i.e. Vascular Endothelial Growth Factors, VEGF\(^1\)) and their cell surface receptors, matrix-degrading enzymes (e.g. matrix metalloproteinases), vascular remodeling ligands and receptors (i.e. angiopoietins and Ties) and adhesion receptors of the integrin and cadherin families. Integrins are the main receptors for extracellular matrix (ECM) proteins and consist of two non-covalently associated \(\alpha\) and \(\beta\) subunits (3). Integrin ligand-binding affinity and adhesion-promoting activity are regulated by intracellular events (‘inside out’ signaling) (4). Upon ligand binding, integrins rapidly cluster and recruit structural (e.g. \(\alpha\)-actinin, talin, vinculin) and signaling (e.g. focal adhesion kinase, paxillin, c-src) proteins to form characteristic structures named focal contacts or focal adhesions (5). Integrins and focal adhesions propagate tensional forces between the ECM and the cytoskeleton necessary to stabilize cell adhesion and initiate signaling events essential to cell survival, proliferation and differentiation (‘outside in’ signaling) (6). Integrin \(\alpha V\beta 3\) is highly expressed in angiogenic endothelial cells but not, or to a much lower extent in quiescent endothelial cells (7-9). Several studies have demonstrated that \(\alpha V\beta 3\) antagonists effectively inhibit angiogenesis, including tumor angiogenesis. An anti-\(\alpha V\beta 3\) function-blocking mAb or an antagonistic RGD-based cyclic peptide suppressed cornea vascularization (10), retinal neovascularization (11) and tumor angiogenesis (12,13). Tumstatin, an endogenous degradation fragment of collagen IV, suppresses tumor angiogenesis by interacting with \(\alpha V\beta 3\) and inhibiting

\(^1\) The abbreviations used are: AC, adenylcyclase; COX, cyclooxygenase; ECM, extracellular matrix; EP, E-prostanoid; FGF, fibroblast growth factor; HUVEC, human umbilical vein endothelial cells; NSAIDs, non steroidal anti-inflammatory drugs; PAK, p21 activated kinase; PG, prostaglandin, PKA, protein kinase A; PLA2, phospholipase A 2; PMSF, phenyl methy sulfone fluorid; TXA2, thromboxane A 2; VEGF, Vascular endothelial growth factor
protein synthesis in endothelial cells (14). Furthermore, disruption of tumor vessels by high doses of TNF and IFNγ is associated with the inhibition of αVβ3 function in endothelial cells (15).

Non steroidal anti-inflammatory drugs (NSAIDs) are widely used therapeutics for the treatment of pain and inflammation. NSAIDs act by inhibiting cyclooxygenase (COX) activity and synthesis of prostaglandins and thromboxans (16). There are two known COX isoforms: COX-1, which is ubiquitously expressed and contributes to tissue homeostasis, and COX-2, which is expressed in activated leukocytes and cancer cells and promotes inflammation and cancer progression (17). Prolonged intake of NSAIDs, including COX-2 inhibitors, significantly decreases the risk of developing colon cancer and suppresses progression of pre-malignant lesions (polyps) (18-20). Moreover, NSAIDs suppress progression of established experimental tumors in mice (21,22). Recent reports indicate that the anti-tumor activity of NSAID involves inhibition of tumor angiogenesis (23,24). COX-2 inhibitors decrease VEGF production in fibroblasts and tumor cells and prevent VEGF-induced MAPK activation in endothelial cells (23,25), block αVβ3-mediated endothelial cell spreading and migration in vitro and suppress FGF-2-induced angiogenesis in vivo (26). This latter effect was not associated with any detectable changes in integrin cell surface expression nor in integrin affinity. We identified suppression of αVβ3-dependent activation of the small GTPases Cdc42 and Rac as the mechanism by which NSAID suppress spreading. Exogenous administration of PGE2 or PGI2, but not TXA2, rescued this NSAID effect, thus demonstrating that prostaglandins are critical regulators of αVβ3-mediated endothelial cell spreading and migration.

Here we extend these observations, by reporting that prostaglandin E2 accelerated αVβ3-mediated endothelial cell adhesion through the cAMP-dependent activation of PKA and induced spreading via cAMP- and PKA-dependent activation of Rac. In contrast, α5β1-mediated endothelial cell adhesion was not regulated by PGE2, intracellular cAMP levels or PKA activity.

**EXPERIMENTAL PROCEDURES**

*Reagents and antibodies*. Bovine gelatin, bovine plasma fibronectin, human plasma vitronectin, leupeptin, aprotinin, phenyl methyl sulfone fluoride (PMSF) were purchased from...
Sigma Chemie (Buchs, Switzerland). Butaprost, PGE₁ alcohol, and sulprostone where form purchased from Cayman Chemical (Ann Arbor, MI). NS-398, PGE₂, 8-brcAMP, Forskolin and H-89 were obtained from Biomol (Plymouth Meeting, PA). U46619 was from Calbiochem (LaJolla, CA). The anti-Rac mAb clone 102 was from Becton Dickinson (Basel, Switzerland). The DNA molecular weight marker was from Roche Diagnostics (Rotkreuz, Switzerland).

Cell culture and electroporation. HUVEC were prepared and cultured as previously described (15) except for the use of M199 (Life Technologies, Basel Switzerland) as basal medium. For electroporation, sub-confluent HUVEC were collected and incubated on ice for 5 minutes with 25 µg of L61Rac-, N17Rac-encoding plasmids or empty plasmid and 5 µg of pEGFP-C1 plasmid (Clontech, San Diego, California) in M199 medium without FCS and electroporated with a Gene Pulser (Biorad, Glattbrugg, Switzerland). HUVEC were resuspended in complete medium and cultured 48 hours before use in the experiments. Electroporation efficiency (routinely approx. 80%) was assessed by the analysis of EGFP fluorescence by flow cytometry.

Cell adhesion assay and spreading assays. Maxisorp II Nunc ELISA plates (Roskilde, Denmark) were coated with fibronectin (5 µg/ml), gelatin (0.5%) or vitronectin (0.5 µg/ml) in PBS overnight at 4 °C and assays were done as previously described (15). Briefly, HUVEC were resuspended in serum-free M199 medium and plated at 3x10⁴ cells/well and incubated at 37 °C. At given times, unattached cells were removed by rinsing the wells with warm PBS. Attached cells were fixed in 2% paraformaldehyde (Fluka Chemie, Buchs, Switzerland), stained with 0.5% crystal violet (Sigma Chemie) and quantified by OD reading at 620 nm (Packard Spectra Count, Meriden, CT). Results are given as OD values and represent the mean of duplicate wells ± s.d. of specific adhesion (= adhesion on ECM protein minus adhesion on BSA). If not stated otherwise, pharmacological agents were added at the time of plating and used at the following concentrations: NS-398, 100 µM; PGE₂, 100 ng/ml; 8-brcAMP, 1 mM; H-89, 5 µM; U46619, 50 µM; butaprost, 20 µM; PGE₁ alcohol, 10 ng/ml; sulprostone, 25 µM; forskolin, 10 µM. For spreading determination, the percentage of spread cells was counted in three representative high power fields.
at different times after plating. Non-spread cells were defined as small round cells with little or no
membrane protrusions, whereas spread cells were defined as large cells with extensive visible
lamellipodia (26). Results represent the percentage of spread cells in three high power fields ± s.d..

**GTPases assays.** HUVEC were plated on gelatin (0.5%) or fibronectin (5 µg/ml) -coated
wells (Evergreen Scientific, Los Angeles, CA) in M199 medium with 1% FCS. After 30 minutes (or
as indicated) cells were washed once with ice-cold PBS and immediately lysed in buffer containing
1% NP-40, 50 mM Tris pH 7.4, 150 mM NaCl, 10% glycerol, 1 mM PMSF, 1 µg/ml aprotinin and
1 µg/ml leupeptin. Cleared extracts were mixed with 20 µg PAK-GST in the presence of
 glutathione agarose beads (Sigma Chemie). After 1 hour incubation at 4 °C, beads were pelleted by
centrifugation, washed three times in lysis buffer and the proteins eluted in SDS-PAGE sample
buffer and analyzed by Western blotting using a monoclonal antibody to Rac. Total Rac was
determined in cell lysate. The ECL system was used for detection (Amersham-Pharmacia Biotech,
Zürich, Switzerland).

**Determination of intracellular cAMP concentrations.** HUVEC were plated on fibronectin or
gelatin-coated wells (Evergreen Scientific) for 45 minutes. HUVEC were washed twice in PBS and
cellular cAMP was extracted with 0.1 M HCl, and quantified using an enzyme immunoassay
(Biomol, Plymouth Meeting, Pennsylvania) according to the manufacturer’s protocol. Values were
normalized to the protein concentration using Bio-Rad protein assay (Bio-Rad, Hercules, CA), and
expressed as fmol/µg protein.

**Protein Kinase A assay.** HUVEC were plated in serum-free medium in gelatin or fibronectin-
coated wells (Evergreen Scientific). At the indicated time cells were washed once in cold PBS and
lysed with cold hypotonic extraction buffer (20 mM Tris, pH 7.5, 5 mM EDTA, 1 mM PMSF, 10
µg/ml aprotinin). PKA activity was determined by the incorporation of phosphate in Kemptide using
the non radioactive Peptag system (Promega, Madison, WI). PKA activity was normalized to
protein concentration and expressed as pmol incorporated phosphate/min/µg protein.
Reverse Transcription polymerase chain reaction (RT-PCR). Total RNA was prepared from HUVEC using the RNeasy system (Qiagen, Basel, Switzerland). 2 µg of total RNA were reversed transcribed (Superscript II, Life Technologies, Basel, Switzerland) and cDNA was subjected to PCR amplifications using primer pairs specific for EP1, EP2, EP3 and EP4 cDNAs as described by Sheng et al. (27). PCR primer sequences: EP1F, ACCGACCTGGCGGGCCACCTGA; EP1R, CGCTGAGCGTGTTGCACACCAG; EP2F, TCCAATGACTCCCAGTCTGAGG; EP2R, TGCATAGATGACAGGCAGCACG; EP3F, GATCACCATGCTGCTCACTG; EP3R, AGTTATGCGAAGAGCTAGTCC; EP4F, GGGCTGGCTGTCACCGACCTG; EP4R, GGTGCGGCGCATGAAGCTGGCG (Microsynth, Balgach, Switzerland). Amplification conditions: 40 cycles of 30 seconds at 94°C, 1 minute at 62°C and 1 min at 72 °C.

RESULTS

PGE2 accelerates αVβ3-mediated cell spreading and adhesion. We have previously reported that NSAIDs suppressed αVβ3-dependent endothelial cell spreading and migration and that this effect was prevented by exogenous addition of prostaglandins (i.e. PGE2 or PGI2). These results identified COX-2 and derived prostaglandins as critical regulators of vascular integrin αVβ3 function (26). To investigate more in detail the role of prostaglandins in the regulation of αVβ3 function, we first characterized the effects of PGE2 and TXA2, two prostanoids implicated in the modulation of angiogenesis (28,29), on the integrin-mediated adhesion and spreading kinetics of human umbilical vein endothelial cells (HUVEC). HUVEC attach on gelatin and vitronectin through integrin αVβ3, while they predominantly use integrin α5β1 to attach to fibronectin (15). On vitronectin and gelatin half maximal and maximal adhesion and spreading were observed at 30-45 and 60 minutes after plating, respectively (Fig. 1A and 2, top and middle panels). On fibronectin, adhesion and spreading proceeded with a faster kinetics: half maximal and maximal levels were observed at 15 minutes and 30 minutes after plating, respectively (Fig. 1A and 2, bottom panels). Addition of PGE2 during adhesion on vitronectin or gelatin accelerated HUVEC cell attachment in a dose-dependent manner to reach a kinetics similar to adhesion on fibronectin (Fig. 1A, top and
Sixty minutes after plating maximal adhesion was observed on both substrates regardless of the presence or absence of PGE2. The acceleration of HUVEC adhesion on gelatin and vitronectin induced by PGE2 were paralleled by an acceleration in the spreading kinetics (Fig. 2). HUVEC spreading on fibronectin was not further accelerated by PGE2. The TXA2 analogue U46619 caused a mild but consistent retardation of HUVEC adhesion and inhibition of spreading on vitronectin and gelatin, resulting in a approx. 30% suppression of cell adhesion and 80% inhibition of spreading at 60 minutes, at a dose of 50 µM (Fig. 1B and 2). U46619 had no effect on HUVEC adhesion or spreading to fibronectin (Fig. 1B and 2).

From these results we concluded that PGE2 accelerated initial αVβ3-dependent HUVEC adhesion and spreading kinetics on gelatin and vitronectin, while U46619 delayed adhesion and suppressed spreading. α5β1-mediated HUVEC adhesion and spreading on fibronectin were not affected.

**HUVEC express functional PGE2 receptors EP2 and EP4.** PGE2 binds to and activates four different E-prostanoid (EP) receptor subtypes: EP1, EP2, EP3 and EP4 (30). To determine which EP receptor was involved in mediating the PGE2 effect, we first determined the EP receptor subtype mRNA expression by RT-PCR. Amplification products for EP2 and EP4, but not EP1 and EP3 were obtained (Fig. 3A). To demonstrate functionality of EP2 and EP4 receptors, we performed adhesion assays in the absence or presence of butaprost, a selective EP2 receptor agonist, and PGE1 alcohol, a selective EP4 receptor agonist. Both agonists accelerated HUVEC adhesion to gelatin with similar kinetics as observed for PGE2 (Fig. 3B) while they had no effect on HUVEC adhesion to fibronectin (data not shown). The EP3 agonist sulprostone had no effect (data not shown). Taken together these results identify EP2 and EP4 prostanoid receptors as functional PGE2 receptors on HUVEC.

**αVβ3-mediated adhesion results in COX-2-dependent increase in cAMP levels.** PGE2 and TXA2 exert many of their biological effects through G-protein-dependent adenylcyclase (AC) (31). To test whether the PGE2 and U46619 effects on αVβ3-mediated HUVEC adhesion involved
modulation of cAMP levels, we measured cAMP concentrations in HUVEC in response to adhesion to gelatin or fibronectin. Adhesion on gelatin caused a transient two-fold rise in cAMP levels (Fig. 4A, left panel), while adhesion on fibronectin caused a transient, four-fold rise which was more prolonged compared to the one on gelatin (Fig. 4A, right panel). Peak cAMP concentrations were reached 15 minutes after plating on both substrates and returned to pre-adhesion levels within 30 minutes on gelatin and within 60 minutes on fibronectin. Adhesion in the presence of U46619 completely prevented the rise in cAMP level on gelatin, but only marginally suppressed the cAMP increase on fibronectin (Fig. 4A). HUVEC adhesion on gelatin or fibronectin in the presence of exogenous PGE2 resulted in identical maximal cAMP levels and kinetics (Fig. 4A). The increase in cAMP levels observed in response to HUVEC adhesion to gelatin was completely abolished by NS-398 and this effect was reversed by the addition of exogenous PGE2 while the rise in cAMP induced by adhesion to fibronectin was insensitive to NS-398 treatment (Fig. 4B). To collect direct evidence for a role of cAMP in accelerating αVβ3-dependent adhesion and promoting spreading, we plated HUVEC on gelatin in the absence or presence of the cell permeable cAMP analog 8-brcAMP and of NS-398 or U46619. 8-brcAMP accelerated HUVEC adhesion and this effect was insensitive to NS-398 or U46619 (Fig. 4C). NS-398 slightly delayed the initial kinetics of attachment, and after one hour incubation, control and NS-398-treated HUVEC attached to identical extents. Addition of 8-brcAMP induced strong HUVEC spreading in the presence of NS-398 or U46619 (Fig. 4D). Similar results were obtained by elevating cAMP levels with forskolin (data not shown).

Taken together these data demonstrate that αVβ3-mediated HUVEC adhesion results in a transient and COX-2-dependent increase in cAMP levels, while α5β1-mediated adhesion induces a robust and COX-2 independent cAMP rise. Exogenous addition of 8-brcAMP accelerates αVβ3-dependent HUVEC adhesion also in the presence of a COX-2 inhibitor.

**Rac mediates cAMP-induced spreading but not adhesion.** The small GTP-binding protein Rac is a critical regulator of cell spreading (32). COX-2 activity and prostaglandin production are essential for αVβ3-dependent Rac activation in HUVEC (26). In light of the ability of 8-brcAMP to promote cell spreading in the presence of NS-398, we asked the question whether 8-brcAMP
was able to reverse the inhibition of αVβ3-mediated Rac activation caused by NS-398. First, we analyzed the activation kinetics of Rac in HUVEC plated on gelatin and fibronectin using a PAK pull-down assay. HUVEC adhesion on gelatin and fibronectin resulted in rapid activation of Rac with peak activities at 15 and 30 minutes, respectively (Fig. 5A). Next we measured Rac activity in HUVEC at 30 minutes after plating on gelatin in the absence or presence of NS-398, 8-brcAMP or a combination thereof. Addition of NS-398 during adhesion completely inhibited Rac activation, and this effect was nearly completely reversed by the concomitant addition of 8-brcAMP (Fig. 5B). Addition of 8-brcAMP during adhesion resulted in a slight increase in Rac activity compared to adhesion on gelatin alone.

Next we asked the question whether Rac activation was involved in mediating the acceleration of αVβ3-dependent HUVEC adhesion and in mediating HUVEC spreading in response to 8-brcAMP. To address these questions we electroporated HUVEC with an expression vector encoding for a constitutive active (L61Rac) or for a dominant negative form (N17Rac) of Rac (26), and then tested the adhesive and spreading properties of these cells. L61Rac did not accelerate HUVEC adhesion to gelatin and N17Rac did not delay it. Also, N17 Rac did not prevent the acceleration of HUVEC adhesion induced by 8-brcAMP and L61Rac did not prevent the adhesion delay caused by U46691 (Fig. 5C). In contrast, L61Rac fully reversed the inhibition of HUVEC spreading caused by NS-398 and U46691, while N17Rac suppressed HUVEC spreading and this effect was not reversed by 8-brcAMP (Fig. 5D).

From these results we concluded that αVβ3-dependent HUVEC spreading in response to cAMP elevation requires Rac activation, while cAMP-induced acceleration of αVβ3-dependent HUVEC adhesion does not.

**αVβ3-dependent HUVEC adhesion and Rac activation depend on PKA.** Protein kinase A (PKA) was reported to regulate αVβ3-dependent angiogenesis (33) and Rac-dependent migration of carcinoma cells (34). Since elevated cAMP levels promote PKA activation, we next tested whether HUVEC adhesion to gelatin or fibronectin promoted PKA activation. A basal PKA activity was observed at time of plating, and adhesion on gelatin induced a transient increase in PKA activity.
activity (Fig. 6A). NS-398 strongly suppressed adhesion-induced PKA activation but did not inhibit basal PKA activity, while the pharmacological PKA inhibitor H-89 fully suppressed both basal and adhesion-induced PKA activity (Fig. 6A). PGE2 induced a robust PKA activation even in the presence of NS-398 (Fig. 6A). HUVEC adhesion to fibronectin induced a strong increase in PKA activity, which was completely insensitive to NS-398 (Fig. 6B). To test whether PKA activity was required for αVβ3- and α5β1-dependent HUVEC adhesion and spreading we plated cells on gelatin and fibronectin in the absence or presence of H-89. H-89 strongly suppressed HUVEC adhesion to gelatin while it had no effect on HUVEC adhesion on fibronectin (Fig. 6C and D). Furthermore, addition of 8-brcAMP did not reverse H-89-induced inhibition of cell adhesion on gelatin (Fig. 5C). In PAK pull down assays we investigated the requirement of PKA in αVβ3- and α5β1-dependent Rac activation. H-89 completely suppressed αVβ3-dependent Rac activation, while it did not affect α5β1-mediated Rac activation (Fig. 6E). Addition of PGE2 or 8-brcAMP did not rescue inhibition of αVβ3-dependent Rac activation caused by H-89 (Fig. 6E). We next tested whether constitutively active Rac could rescue the suppression of αVβ3-dependent HUVEC adhesion caused by H-89. The result of this experiment demonstrated that expression of L61Rac was not sufficient to rescue suppression of αVβ3-dependent HUVEC adhesion caused by H-89. HUVEC plated on fibronectin in the presence of H-89 spread normally (Fig. 6G), consistent with the full activation of Rac in the presence of H-89 observed during HUVEC adhesion to fibronectin (Fig. 6E, right panel).

From these experiments, we concluded that PKA activity mediates basal and PGE2/8-brcAMP-stimulated αVβ3-dependent HUVEC adhesion and Rac activation, and that active Rac does not reconstitute cell adhesion in the absence of PKA activity. In contrast, PKA is not required for α5β1-dependent cell adhesion and spreading.

**DISCUSSION**

COX-2 expression in tumor cells and in the tumor microenvironment promotes tumor progression and this effect involves the induction of tumor angiogenesis. Mice lacking COX-2 have
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deficient VEGF expression, reduced tumor angiogenesis and suppressed tumor growth (35). COX-2 inhibitors decrease VEGF production in fibroblasts and tumor cells and prevent MAPK activation in response to VEGF (23,25,35). Pharmacological inhibition of COX-2 in endothelial cells blocks αVβ3-mediated Rac activation resulting in reduced cell spreading and migration in vitro and suppressed angiogenesis in vivo (26). Two of the major COX-2-derived prostanoids, PGE2 and TXA2, have been shown to promote angiogenesis (28,29), but the mechanisms involved are only partially characterized. In this study we have investigated the effect of PGE2 and TXA2 on αVβ3- and α5β1-mediated HUVEC adhesion and spreading. Here we report: first, PGE2 accelerated HUVEC adhesion, induced Rac-activation and stimulated Rac-dependent spreading mediated by integrin αVβ3, while the TXA2 agonist U46691 delayed adhesion and inhibited spreading mediated by αVβ3. PGE2 signaled to HUVEC through EP receptors 2 and 4. Second, αVβ3-mediated HUVEC adhesion resulted in a COX-2/PGE2-dependent transient rise in cAMP concentration and activation of the cAMP-dependent PKA. Third, αVβ3-mediated HUVEC adhesion required PKA, but not Rac activity, while αVβ3-mediated spreading required both PKA and Rac activities. Fourth, integrin α5β1-dependent HUVEC adhesion, Rac activation and spreading were not regulated by COX-2/PGE2, or TXA2 and did not depend on PKA activity. Taken together, these observations demonstrate that αVβ3-dependent HUVEC adhesion and Rac-dependent spreading are positively regulated by COX-2-derived PGE2 through cAMP/PKA-dependent signaling, while α5β1-mediated adhesion and spreading occur independently of this pathway.

There is increasing evidence indicating that cAMP levels can be regulated by integrin ligation and, on the other hand, that cAMP contributes to the modulation of integrin function. For example, activating anti-β1 integrin antibodies induce MCF-7 breast carcinoma cell migration by stimulating a rise in intracellular cAMP concentration (36). The mechanism by which integrin ligation leads to an increase in cAMP level is not completely known. Our results suggest the existence of two distinct and integrin-specific mechanisms. The first one involves αVβ3-mediated and COX-2-dependent production of PGE2, stimulation of EP2/EP4 receptors and activation of AC. This pathway is consistent with the requirement of COX-2 activity to induce a cAMP rise in response to αVβ3 ligation (Fig. 4A), cell spreading (Fig. 4D) and migration (26), and with the classical signaling
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pathway of prostaglandins (20). Stimulation of COX-2-dependent PGE2 production upon αVβ3-dependent adhesion could result from αVβ3-mediated activation of phospholipase A2 (PLA2) and production of arachidonic acid, the substrate of COX. In this respect, it has been recently reported that αVβ3 ligation induces membrane translocation and activation of PLA2 with subsequent release of arachidonic acid in bovine pulmonary artery endothelial cells (37). PLA2 appears to be stimulated by integrin ligation in several cell types, and in some cases its activation has been linked to production of arachidonic acid, activation of PKC and cell spreading (38,39). The second mechanism involves α5β1-mediated, COX-2/PGE2-independent activation of AC. This is supported by the observation that NS-398 does not inhibit the increase in cAMP concentration observed in HUVEC plated on fibronectin (Fig. 4B). β1 integrin ligation with the RGD cell-binding sequence of fibronectin or with β1-activating antibodies followed by mechanical stress, has been reported to caused a rapid increase in intracellular cAMP levels and PKA activity in endothelial cells (40). G protein α subunit inhibitors suppressed this effect, suggesting that integrin ligation and mechanical stress may stimulate AC through the activation of integrin-coupled heterotrimeric G proteins (40).

cAMP modulates integrin-dependent cell adhesion and migration, but depending on the cell type and context, it can exert stimulatory or inhibitory effects. For example, increase in cAMP levels was shown to promote adhesion of immature thymocytes to fibronectin (41), to inhibit adhesion of mature T lymphocytes to fibronectin (42), and to suppresses leukocyte adhesion and migration in response to chemoattractants (43). Our findings indicate that in endothelial cells, an elevation of cAMP accelerates αVβ3-mediated adhesion and promotes Rac-dependent spreading via activation of PKA. Recently, cAMP-dependent PKA activation was reported to induce β1-integrin mediated MDA-435 breast carcinoma cell migration in response to growth factors through activation of Rac and inhibition of RhoA (34). There is also emerging evidence that PKA can positively and negatively regulate integrin-mediated cell adhesion and migration of carcinoma and sarcoma-derived cell lines, endothelial cells and neutrophils (33,34,44-46). The precise mechanisms by which PKA regulates integrin-dependent events, and in particular Rac activity, however, remain largely unknown.
Taken together, we propose the following working model for the regulation of αVβ3-mediated endothelial cell adhesion and spreading by PGE2. Upon αVβ3-dependent adhesion there is a transient rise in cAMP levels and PKA activity dependent on COX-2-mediated production of PGE2 and on signaling through EP2/EP4 receptors. Increased PKA activity accelerates αVβ3-dependent adhesion through a Rac-independent mechanism and stimulates αVβ3-dependent spreading through a Rac-dependent mechanism. In contrast the rise in cAMP levels and PKA activity observed after α5β1-mediated adhesion do not depend on COX-2 or PGE2 and are not required for α5β1-dependent HUVEC adhesion and spreading.

This latter observation suggest that ligation of integrin α5β1 may generate a cAMP rise and PKA activation which can then promote αVβ3-dependent endothelial cell adhesion and spreading/migration. Such a cross-talk is consistent with a recent report demonstrating that ligation of integrin α5β1 stimulated αVβ3-dependent endothelial cell migration and angiogenesis (33). Optimal migration and angiogenesis is likely to require intermediate cAMP levels and PKA activity while sub-threshold or excessive PKA activity would result in static adhesion. Regulation of cAMP levels may occur through modulation of cAMP generation by AC or degradation by phosphodiesterase. Indeed, growth-factor stimulated carcinoma cell migration was shown to require both cAMP-dependent PKA activity and phosphodiesterase-mediated cAMP degradation (34,47).

In conclusion, we have demonstrated that PGE2-mediated rise in cAMP promotes αVβ3-mediated endothelial cell adhesion through the activation of PKA and induces spreading via PKA-dependent Rac activation. These results may contribute to the further understanding of the regulation of vascular integrins αVβ3 by COX-2/PGE2 during tumor angiogenesis and inflammation.

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FIGURE LEGENDS

FIG. 1. **PGE2 accelerates and U46691 delays αVβ3-mediated cell adhesion.** HUVEC were plated on vitronectin, gelatin or fibronectin in the absence or presence of PGE2 (A) or U46619 (B), at the indicated concentrations. Attached cells were fixed at the indicated times and revealed by crystal violet staining. Results are given as O.D., and values represent the mean of duplicate determination ± s.d. (n=3).

FIG. 2. **PGE2 promotes and U46691 suppresses αVβ3-mediated cell spreading.** HUVEC were plated on vitronectin, gelatin or fibronectin in the absence or presence of PGE2 (100 ng/ml) or U46619 (50 µM), as indicated. Attached cells were fixed at 15, 30, 45 and 60 minutes after plating, and stained by crystal violet. Results represent the percentage of spread cells over total adherent cells in three high power fields ± s.d. (n=2).

FIG. 3. **HUVEC express functional PGE2 receptors EP2 and EP4.** (A) expression of EP1, EP2, EP3, and EP4 receptor mRNA in HUVEC was analyzed by RT-PCR. The amplified products were visualized by agarose gel electrophoresis and ethidium bromide staining. M, DNA molecular weight marker. (B) HUVEC were plated on gelatin in the absence or presence of 20 µM of butaprost, a EP2 agonist, 10 ng/ml PGE1 alcohol, a EP4 agonist and 100 ng/ml PGE2. Attached cells were fixed at 0, 15, 30, 45 and 60 minutes after plating and revealed by crystal violet staining. Results are given as O.D. and values represent the mean of duplicate determination ± s.d. (n=3).

FIG. 4. **Cell adhesion induces a transient cAMP rise and 8-brcAMP promotes adhesion and spreading.** HUVEC were plated on gelatin or fibronectin in the absence or presence of (A) 100 ng/ml PGE2 or 50 µM U46619 or (B) 100 µM NS-398, 100 ng/ml PGE2, or a combination thereof, as indicated. HUVEC were lysed before (t=0) and at 15, 30, 45 and 60 minutes after
plating, and the cAMP concentration in the lysates was determined using an enzyme immunoassay. Results are given as fmol/µg protein and values represent the mean of duplicate determinations ± s.d. (n=3). (C) HUVEC were plated on gelatin in the absence or presence of 100 µM NS-398, 1 mM 8-brcAMP, 50 µM U46619 and 1 mM 8-brcAMP, or combinations thereof, as indicated. Attached cells were fixed at the indicated times and revealed by crystal violet staining. Results are given as O.D. values and represent the mean of duplicate determinations ± s.d. (n=3). (D) HUVEC plated under the same conditions as in (C) were fixed, stained and photographed 60 minutes after plating (n=3).

**FIG. 5. 8-brcAMP promotes Rac activation and Rac-dependent αVβ3-mediated spreading.** (A) HUVEC were plated on gelatin or fibronectin and after 13, 30 and 45 minutes, cells were lysed and total and active Rac determined. (B) HUVEC were plated on gelatin in the absence or presence of 100 µM NS-398, 1 mM 8-brcAMP and combination thereof. After 30 minutes, cells were lysed and total and active Rac determined. (n=2). The bars under the blots give the active Rac/total Rac ratio determined from the scans of the blots. (C) Control HUVEC and HUVEC overexpressing constitutive active (L61Rac) or dominant negative (N17Rac) mutants were plated on gelatin in the presence of 8-brcAMP (1 mM) or U46691 (50 µM) as indicated. Attached cells were fixed at the indicated times and revealed by crystal violet staining. Results are given as O.D. values and represent the mean of duplicate determinations ± s.d. (n=2). (D) Control HUVEC and HUVEC overexpressing L61Rac or N17Rac mutants were plated on gelatin in the presence of NS-398 (100 µM), U46691 (50 µM) and 8-brcAMP (1 mM) as indicated. Attached cells were fixed 60 minutes after plating. Results represent the percentage of spread cells over total adherent cells in three high power fields ± s.d. (n=2).

**FIG. 6. αVβ3-dependent HUVEC adhesion requires PKA activation.** (A, B) HUVEC were plated on gelatin (A) or fibronectin (B) in the presence of 100 µM NS-398 ± 100 ng/ml PGE2, or 5 µM H-89 as indicated and PKA activity was measured in the cell lysate at the indicated times. Results are given as incorporated pmol PO₄ / minute /µg protein and values represent the mean of
duplicate determinations ± s.d. (n=2). (C, D) HUVEC were plated on gelatin (C) or fibronectin (D) in the presence of the PKA inhibitor H-89 (5 µM), 1 mM of 8-brcAMP, or a combination thereof, as indicated. Attached cells were fixed at the indicated times and revealed by crystal violet staining. Results are given as O.D. values and represent the mean of duplicate determination ± s.d. (n=3). (E) HUVEC were plated on gelatin (left panel) or on fibronectin (right panel) in the absence or presence of 100 nM PGE2, 1 mM 8-brcAMP and 5 µM H-89 as indicated. After 30 minutes, cells were lysed and active and total Rac were determined. (n=2). The bars under the blots give the active Rac/total Rac ratio determined from the scans of the blots. (F) Control HUVEC and HUVEC expressing L61Rac were plated on gelatin in the absence or presence of 5 µM H-89. Attached cells were fixed at the indicated times and revealed by crystal violet staining. Results are given as O.D. values and represent the mean of duplicate determination ± s.d. (n=3). (G) HUVEC plated on fibronectin in the absence or presence of the PKA inhibitor H-89 (5 µM) were scored for spreading. Results are given as % spreading and represent the mean of triplicate determination ± s.d. (n=3).
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