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Prostanoids regulate angiogenesis acting primarily on IP and EP_4 receptors

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ABSTRACT

Angiogenesis is regulated by numerous activators and inhibitors, including prostanoids. Although many studies have identified their roles in inflammation, regulatory functions of prostanoids in angiogenesis are poorly understood. Here, we compared the activation of angiogenesis *in vitro* by two prostanoids with important vascular roles: prostaglandin E_2 (PGE_2) – thought to be the most important prostanoid activator of angiogenesis – and prostaglandin I_2 (prostacyclin or PGI_2), whose receptors are predominantly expressed in endothelial cells. Both of these prostanoids activate G-protein coupled receptors: EP1, EP2, EP3 and EP4 by PGE_2 and IP by prostacyclin. Human umbilical vein endothelial cells (HUVECs) were used to characterize two pivotal pro-angiogenic processes *in vitro*: cell migration (using the matrigel droplet assay developed in our laboratory) and "tube formation" (a widely accepted method of assessing formation of blood vessel precursors). The suppression of cell migration and tube formation by the IP-specific antagonist CAY10441 was more extensive (~80%) than by the EP4-specific antagonist L-161,982 (~20%). AH6809, an antagonist of EP1, EP2 and EP3 receptors did not significantly suppress angiogenesis. Expression of the pro-angiogenic receptors KDR and Tie-2 in HUVECs was preferentially suppressed by antagonism of IP and EP4 receptors, respectively. EP4 and IP receptor agonists elicited biphasic actions on angiogenic processes in which there was activation at low concentration, and rapid desensitization at high concentrations — a characteristic common to many G-protein coupled receptors. Together these findings suggest that the prostacyclin-IP pathway plays a major role in the regulation of pro-angiogenic processes in HUVECs.

Introduction

In adult organisms the formation of new blood vessels from the pre-existing vasculature, or angiogenesis, is regulated by both activating and inhibiting factors, including prostanoids (Folkman, 1995; Liekens et al., 2001). Prostanoids are synthesized from the polyunsaturated fatty acid arachidonic acid by a series of metabolic pathways, including cyclooxygenases 1 or 2 (COX-1 or COX-2) and specific prostacyclin synthases, and are important in the regulation of a number of vascular processes, including angiogenesis (Alfranca et al., 2006). Vascular effects are mediated primarily by three prostanoids: prostaglandin E_2 (PGE_2), prostaglandin I_2 (prostacyclin or PGI_2) and thromboxane A_2. In the vascular endothelium prostacyclin and PGE_2 are the key prostanoids released during angiogenesis (Gately, 2000; Gately and Li, 2004) and PGE_2 has also been reported to be an activator of angiogenesis (Nakanishi and Rosenberg, 2013; Wang and DuBois, 2004). PGE_2 functions include increase in proliferation, angiogenesis, invasion and motility and the suppression of apoptosis of both tumor and endothelial cells (Wang and Dubois, 2006; Finetti et al., 2008; Salcedo et al., 2003). In addition, PGI_2 functions in vasculature have also been studied extensively since its discovery in 1976, when it was identified as the major product of local arachidonic acid metabolism in vascular tissues (Moncada et al., 1976). Prostacyclin has many important functions including vasodilation (similar to endothelium-derived relaxing factor) and suppression of platelet aggregation (Shepherd and Katusic, 1991). Recently, prostacyclin – which is produced almost exclusively in the vascular endothelium – has been recognized for its participation in regenerative processes in the cardiovascular system, including angiogenesis and the repair of injured endothelium (Kawabe et al., 2010). Several studies have suggested that prostacyclin is important in angiogenesis. For example, vascular endothelial growth factor (VEGF) is a major activator of angiogenesis and also stimulates prostacyclin synthesis (He et al., 1999), and over-expression of the prostacyclin synthase gene induces angiogenesis in the mouse hind limb ischemia model (Hiraoka et al., 2003). Furthermore, the stable prostacyclin analogs SM-10902 and carbaprostacyclin induce angiogenesis and promote wound healing in animal models (Yamamoto et al., 1996; Liu et al., 2013).

Prostanoids typically act in an autocrine and paracrine fashion by binding to specific receptors in target cells and there is evidence that both PGE_2 and prostacyclin may be required for the optimal activation

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of angiogenesis (Hata and Breyer, 2004). PGE_2 and prostacyclin bind to their specific 7-membrane-spanning G-protein-coupled receptors in target cells. Currently, there are four identified receptors for PGE_2 (EP1–4) and one for prostacyclin (IP) although the number of prostacyclin receptors is somewhat controversial. In the brain, at least two distinct prostacyclin receptors, designated IP1 (identical to the one expressed by endothelium) and IP2 (found only in the central nervous system), have been identified (Takechi et al., 1996). Considering the lack of general consensus about IP2 existence and the fact that endothelial cells have not been found to express a putative second receptor, what we refer to as IP receptor here should be considered the same as IP1, a name used in some reports. EP receptors differentially activate intracellular signaling mechanisms (Birukova et al., 2007). Thus, EP1 is coupled to the Gq alpha G-protein subunit and mediates the protein kinase C (PKC)-dependent increase in IP3 (inositol 1,4,5-trisphosphate) and Ca^{++}. In contrast, EP2 and EP4 activate Gs, which increases cyclic adenosine monophosphate (cAMP) levels, and EP3 activates Gi, which decreases cAMP concentrations (reviewed in Bos et al., 2004). The IP receptor activates both cAMP synthesis (through Gs subunit activation) and the PKC pathway in a Gq-dependent manner (Bos et al., 2004). EP4 has been found to be most important in the regulation of angiogenesis by PGE_2 (Rao et al., 2007; Yanni et al., 2009), whereas pro-angiogenic processes in endothelial cells may be suppressed by IP antagonism (Osawa et al., 2012).

Although PGE_2 and prostacyclin and their corresponding EP4 and IP receptors clearly exhibit the capacity to regulate angiogenesis, information on their relative contributions is deficient. In the present work we hypothesize that prostacyclin acting through IP receptors is the main activator of pro-angiogenic processes in vascular endothelial cells. To test this hypothesis we have comparatively evaluated the roles of EP and IP receptors on the pro-angiogenic processes (cell migration and blood vessel precursor formation) in primary human endothelial cells (HUVECs).

Materials and methods

Chemicals

CAY10441, 16,16-dimethyl prostaglandin E_2 (16,16-dimethyl-PGE_2), taprostene, AH6809 and L-161,982 were obtained from Cayman Chemical (Ann Arbor, MI USA). Other chemicals were obtained from the suppliers indicated below.

Human umbilical vein endothelial cell culture and treatment

HUVECs were isolated from mixed donors to minimize potential effects of genetic variability (Cambrex BioScience, Mt. Waverley, VIC, Australia), and were cultured in EGM-2 medium (Cambrex BioScience) that was supplemented with 2% fetal bovine serum (FBS), epidermal growth factor (5.0 ng/mL), hydrocortisone (0.2 μg/mL), VEGF (0.5 ng/mL), basic fibroblast growth factor (10 ng/mL), insulin-like growth factor-1 (20 ng/mL), ascorbic acid (1 μg/mL), and heparin (22.5 μg/mL). Prior to treatments confluent HUVECs were washed with PBS and removed from culture dishes by trypsin-EDTA Solution (Sigma-Aldrich, Castle Hill, NSW, Australia) followed by washing in Dulbecco's minimal essential medium supplemented with 10% FBS and resuspension in serum-free EGM-2 medium. Cell number in resulting suspensions were determined using a Countess automated cell counter (Invitrogen Australia, Mount Waverley, VIC) in a modified trypan blue exclusion assay according to the manufacturer's suggestions. All treatments were conducted for 20 h in cells cultured at 5% CO_2, 37 °C and 100% humidity. None of the treatments produced toxicity in HUVECs, as judged by trypan blue exclusion assay (data not shown).

Endothelial migration assay

Matrigel droplet migration assays were performed as previously described (Szymczak et al., 2008). Briefly, HUVECs were trypsinized, washed and resuspended in serum-free EGM-2 medium (to a density of 3×10^6 cells/mL unless indicated otherwise), as described above. Cell suspensions were combined in a 1:1 ratio with Cultrex basement membrane extracts (Type 2, PathClear; Trevigen, Gaithersburg, MD USA). From that suspension, 20 μL (containing 3×10^4 cells) were layered onto the surface of 6-well tissue-culture dishes to form well-defined droplets. Dishes were placed at 37 °C for 5 min to facilitate semi-solidification, followed by the addition of 2 mL of medium (containing treatments) to each well, and incubation for a further 20 h. The number of cells that migrated out of the droplets was determined by phase-contrast microscopy (Olympus CKX41 inverted microscope fitted with an sc100 camera, Notting Hill, VIC, Australia). Data are presented as relative average number ± SD of migrated cells, compared to controls, and were obtained from three independent droplets.

Endothelial tube formation assay

Endothelial tube formation assays were performed as previously described (Szymczak et al., 2008). Briefly, Cultrex basement membrane extract, Type 2, PathClear solution (0.3 mL) was applied to each of the wells in 24-well tissue-culture plates and was allowed to solidify for 0.5 h at 37 °C, followed by the addition of 1 mL of medium containing treatments. HUVECs were removed from cell culture dishes by trypsinization and washed as described above, and then resuspended in EGM-2 serum-free medium. From that suspension, 10^5 cells (unless indicated otherwise) were applied to each well and incubated for 20 h, as described above. Endothelial tubes were photographed using phase-contrast microscopy and digital photography (Olympus CKX41 microscope/sc100 camera). The relative lengths of the tubes were determined by digital image analysis (AnalySIS getIT software; Olympus). Data are presented as relative average ± SD of total tube lengths, compared to controls, obtained from three independent wells.

Real-time reverse transcription polymerase chain reaction (real-time RT-PCR) analysis

HUVEC cells (~90% confluence) were seeded onto matrigel-coated 6-well plates. After 24 h cells were treated with CAY10441 (30 μM), L-161,982 (30 μM) or dimethyl sulfoxide, (DMSO; 0.3%, vehicle control) and were incubated for a further 24 h. Total RNA was extracted using the Purelink RNA mini kit (Ambion; Mulgrave, VIC, Australia) and quantified spectrophotometrically (NanoDrop Technologies, BioLab Pty Ltd; Scoresby, VIC Australia). RNA samples were treated with RQ1 DNase (Promega; Alexandria, NSW, Australia) prior to real-time RT-PCR. Real-time RT-PCR was conducted in a Rotor-Gene 6000 thermal cycler (Corbett Life Science, Mortlake, NSW, Australia) using express one-step SYBR GreenERTM Universal qPCR supermix (Life Technologies Australia, Mulgrave VIC) according to the manufacturer's instructions. The suitability of the chosen RT-PCR conditions for each gene was assessed by melting curve and agarose gel analysis.

RT-PCR primers used in this study were described previously (Figueiredo et al., 2011): Tie-2 (Angiopoietin-1 receptor): upper — TACTAATGAAGAAATGACCCTGG; lower — GGAGTGTGTAATGTTGGAAA TCT (generating a fragment of 826 bp); KDR (vascular endothelial growth factor receptor-2): upper — TGCCTACCTCACCTGTTTC; lower — GGCTCTTTCGCTTACTGTTC (generating a fragment of 114 bp); and glyceraldehyde 3-phosphate dehydrogenase (GAPDH): upper — GGTGAA GGTCGGAGTCAACG; lower — CAAAGTTGTCATGGATGACC (generating a fragment of 496 bp). Relative gene expression was calculated using the formula, $2^{\Delta\Delta CT}$ where $\Delta CT = CT_{GAPDH}\text{-}CT_{target}$ and $\Delta\Delta CT = \Delta CT_{treatment}\text{-}\Delta CT_{control}$ (Livak and Schmittgen, 2001). mRNA levels were quantified relative to the level of GAPDH mRNA in HUVECs treated