Colocalization of Prostacyclin Synthase with Prostaglandin H Synthase-1 (PGHS-1) but Not Phorbol Ester-induced PGHS-2 in Cultured Endothelial Cells*

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The subcellular colocalization of prostacyclin synthase (PGIS) with prostaglandin H synthase (PGHS) has not been delineated. To test the hypothesis that its colocalization with PGHS is crucial for prostacyclin synthesis, we determined subcellular locations of PGIS, PGHS-1, and PGHS-2 in bovine aortic endothelial cells by immunofluorescent confocal microscopy. PGIS and PGHS-1 were colocalized to nuclear envelope (NE) and endoplasmic reticulum (ER) in resting and adenovirus-infected bovine aortic endothelial cells. PGIS and PGHS-2 were also colocalized to ER in serum-treated or adenovirus-cyclooxygenase-2-infected cells. By contrast, PGIS was not colocalized with PGHS-2 in cells induced with phorbol 12-myristate 13-acetate where PGHS-2 was visualized primarily in vesicle-like structures. The lack of colocalization was accompanied by failed prostacyclin production. Resting ECV304 cells did not produce prostacyclin and had no detectable PGHS-1 and PGIS proteins. Confocal analysis showed abnormal colocalization of PGIS and PGHS-1 to a filamentous structure. Interestingly, the abundant PGIS and PGHS-1 expressed in adenovirus-infected ECV304 cells were colocalized to NE and ER, which synthesized a large quantity of prostacyclin. These findings underscore the importance of colocalization of PGIS and PGHS to NE and ER in prostacyclin synthesis.

Prostaglandin biosynthesis is catalyzed by a series of enzymes. It is initiated by activation and translocation of cytosolic phospholipase A2, which catalyzes the release of arachidonic acid (AA) from membrane phospholipids. The free AA enters the substrate channel of prostaglandin H synthase-1 (PGHS-1, also known as cyclooxygenase-1) or PGHS-2, where it is converted to prostaglandin G2 by the cyclooxygenase activity, and PGH2 is further converted to prostaglandin H2 by the peroxidase activity of PGHS enzymes (1). PGH2 is the common precursor for prostaglandins, thromboxane, and prostacyclin (PGFL). PGJ2 is synthesized from PGH2 by a specific enzyme, PGJ2 synthase (PGIS) (2). Biosynthesis of PGFL and other prostanoids is regulated at each enzymatic step by multiple mechanisms including enzyme autoinactivation, induction of enzyme transcription, and post-translational modification. Recent studies suggest that their synthesis could be further influenced by subcellular localization of these synthetic enzymes. The subcellular locations of a few of these enzymes have been determined. Cytosolic phospholipase A2 has been reported to localize to the perinuclear region and endoplasmic reticulum (ER) (3). PGHS-1 and PGHS-2 have been reported also to be localized to perinuclear area and ER, suggesting a possible colocalization of cytosolic phospholipase A2 with PGHS-1 and PGHS-2 (4). PGIS was previously reported to be localized to plasma and nuclear membranes (5). We have shown that PGI synthase, like its related cytochrome P450 proteins, is localized to ER (6). However, it is unclear whether it is colocalized with PGHS-1 and/or PGHS-2. Since PGJ2 is a key molecule in vasoprotection and vascular tone control and PGIS occupies a pivotal position in its synthesis, it is crucial to know its subcellular localization and important to determine whether it is colocalized with its upstream enzymes. To this end, we have determined colocalization of PGIS with PGHS-1 and PGHS-2 in bovine aortic endothelial cells (BAECs) and a human endothelial cell line, ECV304, by immunofluorescent confocal microscopy. The influence of colocalization or lack of it on prostacyclin synthesis has also been evaluated by HPLC. The results indicate that colocalization of PGIS with PGHS-1 or PGHS-2 to ER is crucial for prostacyclin synthesis. Lack of colocalization leads to failed prostacyclin production.

EXPERIMENTAL PROCEDURES

Cell Culture and Materials—BAECs with passage numbers ranging from 15 to 18 were provided by Danny Wang. ECV304 cells (ATCC CRL-1988) and 293 cells (ATCC CRL-1573) were from the American Type Culture Collection (ATCC, Manassas, VA). All cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 units/ml penicillin-streptomycin at 37 °C in a humidified 5% CO2 atmosphere. Cell culture media and antibiotics were from Life Technologies, Inc. (1-14C)arachidonic acid (1-14C)AA; 55 mCi/mmole) was from Amersham Pharmacia Biotech. To induce PGHS-2 expression for immunofluorescence studies, BAECs were pretreated with 100 nM of phorbol 12-myristate 13-acetate (PMA) (Sigma) for 4 h before fixation.

Construction of PGHS-GFP Fusion Gene for Transient Transfection—Full-length human PGIS cDNA was cloned and sequenced as previously reported (7). pPGHS-GFP was constructed by cloning the PGHS PCR product into the BamHI site of the N-terminal of GFP cDNA in pEGFP-N1 vector (CLONTECH). Effectene transfection reagent (Qiagen) was used to transfect pPGHS-GFP into BAECs for transient expression.

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¶The abbreviations used are: AA, arachidonic acid; PGHS, prostaglandin H synthase; PGF2α, prostaglandin F2α; PGH2, prostaglandin H2; PGIS, prostacyclin synthase; PGI2, prostacyclin; ER, endoplasmic reticulum; NE, nuclear envelope; BAEC, bovine aortic endothelial cell; PMA, phorbol 12-myristate, 13-acetate; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; m.o.i., multiplicity of infection; HPLC, high pressure liquid chromatography; Ad, adenovirus.

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Recombinant Adenovirus—Replication-deficient recombinant adenoviruses, Ad-PGIS, Ad-PGHS-1, and Ad-PGHS-2, were generated by homologous recombination and amplified in 293 cells as described previously (8). Viruses were purified by CsCl density gradient centrifugation. Virus titers were determined by a plaque assay method using serial dilutions of the recombinant viruses to infect 293 cells in DMEM supplemented with 2% FBS. 2 ml of culture medium containing 0.8% of low melting point agarose (SeaPlaque, FMC) was overlaid after infection. Numbers of plaques were determined by counting the plaques formed within 2 weeks. For immunofluorescent microscopy, BAECs were infected with a mixture of 50 multiplicity of infection (m.o.i.) per cell of Ad-PGIS in the presence or absence of Ad-PGHS-1 or of Ad-PGHS-2 for 2 h in DMEM containing 2% FBS, and 24 h after infection, the infected cells were fixed for confocal study.

Antibodies for Immunofluorescence Staining—A rabbit polyclonal antibody against PGIS prepared in our laboratory was diluted at 1:50 for staining of endogenous PGIS in resting cells, 1:100 for PMA-induced cells, and 1:200 for PGIS-overexpressed cells. A monoclonal antibody against PGHS-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used at 1:25 dilution for immunofluorescence staining of endogenous PGHS-1 in resting cells and 1:100 for PGHS-1-overexpressed cells. A monoclonal antibody against PGHS-2 (Santa Cruz Biotechnology) was diluted at 1:50 for PMA-induced BAECs. Goat polyclonal antibodies against von Willebrand factor (Santa Cruz Biotechnology) were diluted at 1:50. Donkey anti-rabbit Ig, fluorescein-linked secondary antibody (Amersham Pharmacia Biotech), and rhodamine RedTM-X goat antimouse IgG secondary antibody (Molecular Probes, Inc., Eugene, OR) were diluted at 1:50 for immunostaining experiments.

![Figure 1](http://example.com/fig1.jpg)

**Fig. 1.** a, colocalization of endogenous PGIS with PGHS-1 in BAECs by double-staining immunofluorescent confocal microscopy. A and B, cells were stained for PGIS and PGHS-1, respectively. C, overlay of A and B. Bar, 20 μm. D, shows GFP distribution in BAECs transfected with pGFP-PGIS. b, colocalization of overexpressed PGIS and PGHS-1 by recombinant adenovirus in BAECs. A and B, cells were stained for PGIS and PGHS-1, respectively. C, overlay of A and B. Bar, 20 μm.
Fig. 2. a, colocalization of endogenous PGIS with PGHS-2 in BAECs cultured in 10% FBS. PGIS (A) and PGHS-2 (B) were visualized by double-staining immunofluorescent confocal microscopy. C, the image overlay of A and B. Bar, 20 μm. b, lack of colocalization of PGIS with PGHS-2 in BAECs induced by PMA. A and B, cells were stained for PGIS and PGHS-2, respectively. C, overlay of A and B. Bar, 20 μm. c, colocalization of PGIS (A) with transgenically overexpressed PGHS-2 (B) in BAECs. C, image overlay of A and B. Bar, 20 μm. d, colocalization of PGIS (A) with PGHS-2 (B) in cells co-infected with 50 m.o.i. each of Ad-PGIS and Ad-PGHS-2, respectively. C, image overlay. Bar, 20 μm.
Immunofluorescence Confocal Microscopy—Cells were washed with phosphate-buffered saline and fixed for 15 min at room temperature with 100% methanol. The samples were blocked with 10% FBS in phosphate-buffered saline for 30 min. Primary antibodies in the same blocking solution were added and incubated for 30 min. After five additional 5-min washes, samples were examined with a Bio-Rad MRC1000 confocal microscope, and images were processed with Adobe Photoshop software. More than 100 cells were inspected per experiment, and cells with typical morphology were presented.

Analysis of AA Metabolites by Reverse-phase High Pressure Liquid Chromatography (HPLC)—Cells were incubated in serum-free DMEM containing 10 μM[1-14C]AA at 37 °C for 10 min, the media were collected, and the eicosanoids in the media were extracted by Sep-Pak Cartridge (Waters Associates) as described previously (9). Analysis of 14C-labeled AA metabolites was achieved by reverse-phase HPLC, a solvent delivery system (Waters model 2690) equipped with an on-line radioisotope detector (Packard 150-TP). The stationary phase was Inertsil 7 ODS-3 column (4.6 × 150 mm; Vercopak, Taiwan). The mobile phase consisted of gradient elution between solvent A (acetonitrile) and B (0.1% acetic acid, pH 3.7) under the following conditions at a flow rate of 1 ml/min: 34% B for 10 min, 34–40% B within 4 min, 40–50% B within 1 min, 50% B for 5 min, 50–75% B within 10 min, 75–100% B within 10 min, and 100% B for 10 min. The eicosanoids were identified by their retention times with the authentic radiolabeled standards.

RESULTS
Colocalization of PGIS with PGHS-1 in Resting BAECs—Constitutively expressed PGIS was localized in cytoplasm with a reticular pattern consistent with ER localization. It had a dominant localization at the perinuclear region and NE as detected by confocal microscopy (Fig. 1A, A). Weak staining signals were detected inside the nucleus. Constitutively expressed PGHS-1 had an identical location as PGIS (Fig. 1A, B), and image overlay was consistent with colocalization of these two enzymes in NE and ER (Fig. 1A, C). BAECs transfected with pPGIS-GFP also showed primarily NE and ER locations (Fig. 1A, D).

Subcellular Localization of PGIS and PGHS-1 in BAECs Infected with Ad-PGIS and Ad-PGHS-1—Since augmentation of PGI2 synthesis by adenovirus-mediated transfer of PGHS-1 and/or PGIS has a potential for therapy of vascular diseases (8), we determined whether the transgene products have a similar subcellular localization as the native gene products. Our unpublished data revealed that coinfection of endothelial cells with an equivalent m.o.i. of Ad-PGIS and Ad-PGHS-1 (50:50) resulted in a large increase of PGI2 and a concurrent suppression of PGE2 and other prostanoids.2 We therefore determined the subcellular localization of these two enzymes by coinfection of BAECs with 50 m.o.i. of Ad-PGIS and 50 m.o.i. of Ad-PGHS-1. Double staining and image overlay revealed colocalization of the overexpressed PGIS and PGHS-1 to ER and NE as the native enzymes (Fig. 1, compare b and a). These results indicate that adenovirus-mediated overexpressions of

2 S-K. Shyue, M-J. Tsai, J-Y. Liou, and K. K. Wu, unpublished data.
PGIS and PGHS-1 retain their native subcellular locations.

**Different Subcellular Locations of PGHS-2 in PMA-treated Versus Serum-treated or Ad-PGHS-2-infected BAEC**—PGHS-2 was visualized in our BAECs cultured in 10% FBS primarily in the cytoplasm as a diffuse reticular pattern consistent with ER localization, which was colocalized with PGIS (Fig. 2). By contrast, PGHS-2 in PMA-treated BAEC was localized to cytosolic dot-like structures with some localization in the nucleus (Fig. 2b). PGIS in PMA-treated BAEC remained localized in the cytoplasm, as shown by immunofluorescence microscopy (Fig. 2a). The localization of PGHS-2 was consistent with the notion that PMA-induced PGHS-2 has a different intracellular trafficking route that leads to a cytosolic location distinct from its native ER location. We suspected that this distinct location may influence prostacyclin biosynthesis. To test this possibility, we incubated PMA-treated, serum-treated, or Ad-PGHS-2-infected BAEC with [1-14C]AA for 10 min, extracted eicosanoids from the medium by a C18 cartridge, and analyzed the eicosanoids by HPLC. A predominant 6-keto-PGF1α peak was detected in serum-treated cells (Fig. 3). The addition of PMA did not significantly increase the 6-keto-PGF1α or alter the metabolic profile (Fig. 3). The 6-keto-PGF1α peak was reduced by ~95% when cells were pretreated with NS398, a selective PGHS-2 inhibitor (Fig. 3), consistent with PGHS-2 as the major source of 6-keto-PGF1α synthesis. Ad-PGHS-2-infected cells exhibited augmented 6-keto-PGF1α production (Fig. 4).

**Abnormal Subcellular Locations of PGIS and PGHS-1 in ECV304 Cells**—ECV304 was reported as an immortal cell line derived from human umbilical vein endothelial cells that exhibits features of these cells. This cell line has recently been noted to be contaminated with bladder cancer cells. Our cultured ECV304 cells did not produce detectable 6-keto-PGF1α or other eicosanoids on reverse-phase HPLC analysis when they were treated with [1-14C]arachidonate (data not shown). These cultured cells were positively stained for von Willebrand factor as shown by immunofluorescence microscopy (Fig. 5a). Western blot analysis did not detect PGHS-1 or PGIS protein in...
resting ECV304. However, when these cells were subject to double staining for PGIS and PGHS-1, both enzymes were visualized in bizarre filamentous structures widely distributed in the cytoplasm (Fig. 5b, A and B). Image overlay showed an almost complete colocalization to the filaments (Fig. 5b, C). To determine whether these filaments contained actin or vimentin, we stained the cells with actin or vimentin antibodies and did not detect colocalization of PGHS-1 or PGIS with actin or vimentin (data not shown). We then determined whether overexpressed PGIS and PGHS-1 by adenovirus-mediated gene transfer in this cell line also had the unusual localization. To our surprise, the overexpressed PGIS and PGHS-1 were colocalized to the perinuclear region and cytoplasm with a reticular appearance as the native enzymes in BAECs (Fig. 5c, A–C), and the overexpressed PGHS-1 and PGIS proteins were highly detectable by Western blotting (Fig. 6A). Furthermore, co-overexpression of these two enzymes resulted in a monophasic overproduction of PGI2 detected as 6-keto-PGF1α on HPLC (Fig. 6B).

**DISCUSSION**

Prostacyclin synthase, a member of the cytochrome P450 superfamily, is constitutively expressed in endothelial cells (10–12). We have previously shown that PGIS anchors to the ER membrane by a single transmembrane domain located at its N-terminal region, with the enzyme mass located at the cytosolic side of the membrane (6). In the present study, we provide new information by confocal analysis that PGIS is localized not only on the ER but also at the perinuclear region including NE. Given the recent observation that cytochrome P450 is mobile in ER (13), the NE location of PGIS may be an extension of that from ER. It is expected that the membrane anchoring topology of PGIS in NE will be similar to that in ER.

The present study has also shown colocalization of PGIS with PGHS-1. Since PGHS-1 is located within the ER and probably also within the lumen of NE through hydrophobic interaction (14), colocalization of these two enzymes implies a close physical relationship on the membrane of ER and NE. This would facilitate the transfer of PGE2 generated by PGHS-1 associated with the inner membrane of the lumen to enter PGIS facing the opposite side of the membrane.

We previously reported that retrovirus-mediated and adenovirus-mediated transfer of PGHS-1 cDNA into human endothelial cells increased PGHS-1 levels to be in large excess of PGIS (8, 15). This resulted in augmentation of not only prostacyclin but also PGE2 and other prostanoid synthesis (8, 15). We have recently evaluated the influence of the ratio of PGHS-1 to PGIS overexpression as conferred by co-transfer of different m.o.i. values of Ad-PGHS-1 and Ad-PGIS on the PGI2 synthesis. The results show that when the m.o.i. ratio of co-transfected Ad-PGHS-1 to Ad-PGIS is approximately 1:2:1, the 6-keto-PGF1α level was singularly augmented while the level of other prostanoids became undetectable.2 These metabolic data together with our confocal results further support coupling of PGHS-1 with PGIS.

Our report of PGIS and PGHS-1 colocalization to NE and ER and the previous separate reports of similar subcellular locations of cytosolic phospholipase A2 (3) and PGHS-1 (4) lead to a plausible conclusion that all three PGI2 synthetic enzymes are colocalized to NE and ER. It is generally believed that PGI2 and other prostanoids produced in ER are secreted into the extracellular milieu to act as an autacoid. The fate and the role of PGI2 and other prostanoids generated in NE are less clear. There is an emerging theory that the nuclear membrane may transmit distinct signals to nucleus (16, 17). PGI2 and prostan-
oids produced in NE may thus serve as signaling molecules for nuclear function. This notion is supported by the demonstration of functional PGE$_2$ receptors on nuclear membrane (18, 19). It is important to investigate whether the PGL$_2$ receptor is expressed on nuclear membrane and whether it transmits signals to nucleus for distinct functions.

Our findings of ER and nuclear locations of PGHS-2 in serum-treated BAEC are in agreement with a previous report (4). Our results further reveal colocalization of PGHS-2 with PGIS in BAEC cultured in the presence of 10% FBS. Thus, under this culture condition, PGHS-2 has a similar relationship with PGIS as PGHS-1. This relationship is retained when PGHS-2 is overexpressed by transgenes but is drastically altered when cells are stimulated by PMA. PMA causes transport of PGHS-2 out of ER into vesicle-like structures (20). The nature of these vesicles remains to be determined. Recent studies suggest that caveolins are key proteins in cytosolic vesicle formation (21, 22). It will be important to determine whether PMA-induced dot-like structures contain caveolins and whether PGHS-2 interacts directly with caveolins in the vesicles. Work is now in progress in our laboratory to address these important questions. Work is also in progress to determine whether interleukin-1 stimulation also induces PGHS-2 localization in cytosolic vesicles in endothelial cells. Given the recent discovery of diverse pathophysiological roles of PGHS-2, it is conceivable that PGHS-2 compartmentalization may contribute significantly to its diverse activities. Characterization of its subcellular localization and its interacting enzymes under various pathophysiological stimuli will shed further light on the mechanisms by which it triggers these pathophysiological processes.

The unusual location of PGHS-2 and a lack of colocalization with PGIS in PMA-treated cells versus the “native” colocalization of PGHS-2 and PGIS in serum-treated or Ad-cyclooxygenase-2-infected cells significantly influence prostacyclin synthesis. The high passage BAECs used in our experiments express PGHS-2 and produce a considerable amount of prostacyclin when cultured in the presence of 10% FBS. Prostacyclin synthesis was not augmented by PMA. By contrast, overexpressed PGHS-2 by transgenes augments PGI$_2$ synthesis. These results further support the importance of colocalization and coupling of prostanoid synthetic enzymes. HPLC analysis failed to detect appreciable amounts of other eicosanoids in PMA-treated cells. It is possible that PGHS-2 is catalytically inactive when localized to the vesicles. An alternative explanation is that uncoupling of PGHS-2 from other synthetic enzymes disrupts the transfer of substrates for PGI$_2$ synthesis. To our knowledge, localization of PGHS-2 to cytosolic vesicles has not been previously reported. It should be interesting to determine the catalytic activity of these enzymes and their pathophysiological implications.

ECV304 cells used in our experiments retained a key feature of endothelial cells, i.e. expression of von Willebrand factor. However, they did not express detectable PGHS-1 or PGIS proteins nor did they synthesize PGL$_2$. This is attributable to abnormal subcellular locations of PGHS-1 and PGIS. Both enzymes are colocalized to a filamentous structure not containing actin or vimentin and are tightly associated with the filaments rendering them not solubilized by lysis buffer for protein blotting. It is possible that both proteins undergo erroneous post-translational modifications which lead to their transport to an aberrant destination in the cell. This assumption is probably unlikely since overexpressed PGHS-1 and PGIS by adenovirus-mediated transfer have normal subcellular locations as well as normal functional capacity. If abnormal post-translational modification were to be the culprit, overexpressed proteins would be expected to have a similar fate. The abnormal locations of these two enzymes in native cells could be due to a common genetic defect which causes an abnormal trafficking of these proteins. The transgenes, on the other hand, would not have such defect and, therefore the encoded proteins would have normal subcellular locations. ECV304 cells will be valuable for unraveling the intracellular trafficking of these two enzymes.

In conclusion, constitutively expressed and transgenically overexpressed PGIS is colocalized with PGHS-1 at nuclear envelope and endoplasmic reticulum of cultured endothelial cells and this innate colocalization property is critical for synthesis of physiologically important prostacyclin and other prostanoids. PGIS is also colocalized with PGHS-2 in serum-treated and transgenic overexpressed PGHS. By contrast, PGIS is not colocalized with PGHS-2 induced by PMA, resulting in an altered prostanoid synthetic profile, which may contribute to the diverse pathophysiological roles of PGHS-2. These findings underscore the importance of appropriate colocalization and possible coupling of synthetic enzymes in regulating prostanoid biosynthesis.

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