Prostacyclin Is the Major Prostaglandin Synthesized by Bovine Retinal Capillary Pericytes in Culture

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Prostaglandin synthesis by bovine retinal pericytes was investigated using high pressure liquid chromatography to separate and identify 3H-labeled prostaglandins released from 3H-arachidonic acid labeled pericyte monolayers. A dominant peak activity corresponding to 6-keto-PGF₁α was observed. This peak was eliminated when monolayers were pretreated with cyclooxygenase inhibitors and was augmented when monolayers were stimulated by the calcium ionophore A23187. Suspensions of pericytes and the cell-free media of monolayers incubated with arachidonic acid inhibited adenosine diphosphate-, collagen-, and archidonic acid-stimulated platelet aggregation in a bioassay for prostacyclin-like activity. This inhibitory activity was unstable at room temperature. Cultures of 7.5 to 10 X 10⁵ pericytes (7th passage near-confluence) released nanogram quantities of 6-keto-PGF₁α as measured by radioimmunoassay. These results are evidence that prostacyclin is the main prostaglandin synthesized by bovine retinal capillary pericytes in culture. Pericytes may influence the microcirculation via their production and release of this potent vasoactive arachidonic acid metabolite. Invest Ophthalmol Vis Sci 29:1511-1516, 1988

Although capillary pericytes are thought to play an important role in the microcirculation, and show striking pathologic changes in early diabetic microangiopathy, their function and metabolism are incompletely understood. Arachidonic acid metabolites of pericytes could be important, since these substances can affect microvascular blood flow and permeability and are known to be potent participants in the inflammatory and immune responses.¹² The cyclooxygenase pathway which transforms arachidonic acid to the prostaglandins and prostanoids (thromboxane A₂ and prostacyclin), and the lipoxygenase pathway which yields the leukotrienes and several hydroxylated products have been characterized in many tissues and cell types.³ The current investigation was undertaken to characterize the retinal capillary pericyte cyclooxygenase pathway. We present evidence that prostacyclin is the principal cyclooxygenase product of bovine retinal capillary pericytes in culture.

Materials and Methods

Preparation of Cell Culture

Primary cultures of bovine retinal capillary pericytes (BRCP) and subcultures of BRCP were prepared as previously described.⁴ The cells were replated in 35 mm or 100 mm dishes (Falcon, Beckton-Dickinson and Co., Lincoln Park, NJ) at a density of 2.5 × 10⁴ cells/cm² in growth media (DMEM containing 20% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin). The purity of subcultured pericytes was assessed by phase contrast microscopy, by the absence of indirect immunofluorescent staining for Factor VIII antigen, and by the lack of angiotensin-converting enzyme activity.⁴

Incubations With ³H-arachidonic Acid

Fifth passage cells at or near confluence in 100 mm culture dishes containing 1-2 × 10⁴ pericytes were used in labeling studies. After removal of maintenance media, monolayers were washed twice with phosphate-buffered saline (PBS), pH 7.4. Four milliliters of HB101 media containing ³H-arachidonic acid (1 μCi/ml, 83.8 Ci/mmol) were added to each dish, and the monolayers were incubated for 22 hr at

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37°C, under 5% CO₂. The incubation media were removed and monolayers were washed once with 2 ml of PBS. The incubation media and washings were combined for each monolayer and frozen at -20°C. Identical incubations were performed with monolayers pretreated for 30 min with 0.01 M acetylsericine acid or 100 μM indomethacin. The media containing these inhibitors were removed and monolayers were washed twice prior to the addition of labeled media.

In experiments with the calcium ionophore A23187, following the 22 hr labeling period and removal of labeled media, monolayers were washed again with 4 ml of PBS, and 4 ml of fresh HB101 medium was added to each culture. The calcium ionophore A23187 in ethanol, or ethanol alone was added to monolayers. The final concentration of ethanol was 1% and of ionophore, 5 μM. After an additional 1 hr incubation (37°C, 5% CO₂), media were removed, monolayers were washed with 2 ml PBS, and the combined media-wash solution from each monolayer was frozen at -20°C prior to processing for HPLC.

All monolayer incubations were performed in triplicate.

Extraction of Prostaglandins

Media were extracted following incubation of pericytes with 3H-arachidonic acid, by a modification of the method of Powell. The combined media-wash solution from each monolayer was acidified with phosphoric acid to pH 3.0 ± 0.1, and applied to 10 × 10 mm octadecylsilyl silica cartridges (SEP-PAK, Waters Associates, Milford, MA). Cartridges were eluted sequentially with 20 ml each of H₂O, 15% ethanol, petroleum ether and ethyl acetate. The ethyl acetate fractions were evaporated to dryness under N₂ and redissolved in 0.5 ml of acetonitrile/1.7 mM H₃PO₄ (33:67), pH 3.5. In studies using authentic standards, prostaglandin (PG) recoveries were determined to be: PGD₂ 65 ± 1%, PGE₂ 84 ± 4.5%, PGF₂α 49 ± 1%, thromboxane B₂ 46 ± 1%, and 6-keto-PGF₁α 37 ± 1%. The major loss of PGs occurred in the 15% ethanol column wash.

High Pressure Liquid Chromatography

A reverse-phase high pressure liquid chromatography (rp-HPLC) method, with slight modification, was used for analysis of 3H-labeled arachidonic acid products extracted from the culture media. A C₁₈, 4.5 mm × 25 cm column (Supelco Co., Bellefonte, PA) was equilibrated with acetonitrile/1.7 mM H₃PO₄ (33:67), pH 3.5. Aliquots (0.25 ml) of each extraction were injected and stepwise isocratic elutions with acetonitrile/1.7 mM H₃PO₄ (33:67), pH 3.5, 20 min, acetonitrile/1.7 mM H₃PO₄ (50:50), pH 3.5, 20 min, and 100% acetonitrile, 20 min, were performed at a flow rate of 1.7 ml/min. Fractions of 0.6 ml were collected, combined with 5 ml of Budget-solve (Research Products International Corp., Mount Prospect, IL) and counted in a Beckman LS-100C liquid scintillation counter. Retention times of peak activities were compared to those of unlabeled authentic PG standards detected by UV absorbance (192 nm) in the same HPLC system.

Bioassay

Suspensions of pericytes and the cell-free media from pericyte monolayers were tested for their ability to inhibit platelet aggregation induced by collagen (95 μg/ml), adenosine diphosphate (ADP, 2 μM), or arachidonic acid (0.4 mM) in a modification of a bioassay described by Weksler et al. Citrated venous blood obtained from healthy donors was centrifuged for 15 min at 150 g, and then for 15 min at 2000 g, to obtain platelet-rich plasma (PRP) and platelet-poor plasma (PPP), respectively. The platelet count of PRP was adjusted to 200,000/ml with PPP.

Pericytes were removed from culture dishes with a rubber policeman after a 5 min incubation with 0.1% collagenase at 37°C. Cells were transferred to plastic tubes and diluted 1:10 with a HEPES-salt solution (HS, 10 mM HEPES, pH 7.5, 1 mM MgCl₂, 1.8 mM CaCl₂, 1 mM MgCl₂, 150 mM NaCl, 5 mM glucose), centrifuged at 50 g for 3 min and, after removal of the supernatant, resuspended in 1 ml of HS. One-tenth milliliter of each cell suspension was removed for manual cell counts. Suspensions yielding 2-5 × 10⁶ cells/ml were used for bioassay within 2 hr of preparation. Aliquots (50 μl) of pericyte suspensions were added to cuvettes containing 400 μl of PRP. Pericytes and PRP were stirred at 1200 rpm, 37°C, in a Biodata model PAP-4 aggregometer for 5 min. Aggregating agent (collagen, ADP, or arachidonic acid) then was added, and platelet aggregation monitored (% light transmission through cuvette) over time. The final volume in each cuvette was 0.5 ml, consisting of PRP, pericyte suspension, aggregating agent solution and HS (when the volume of aggregating agent was less than 50 μl). Control experiments were performed substituting equal numbers of granulocytes for pericytes, or substituting HS for pericytes.

The cell-free media from confluent monolayers of 1-2 × 10⁶ pericytes were similarly tested for anti-aggregating activity. Monolayers were washed twice with 2 ml of HS after removal of maintenance media. Two ml of HS then were added to each monolayer, and aliquots (50 μl) were removed for bioassay after 5
min incubations at room temperature. In some experiments, 20 μM arachidonic acid was added to the HS over monolayers for 5 min prior to removing aliquots for bioassay. For each experiment, duplicate aliquots were removed and set aside at room temperature for 30 min before bioassay.

6-keto-PGF₁α Radioimmunoassay

6-keto-PGF₁α antiserum was kindly provided by Dr. J. B. Smith, Temple University (Philadelphia, PA). Early confluent monolayers were washed twice with 2 ml HS after removal of media. Two milliliters of HS, HS with 20 μM arachidonic acid or HS with 5 μM calcium ionophore A23187, were added to monolayers. After 30 min incubations (37°C, 5% CO₂), supernatants were removed and immediately placed on ice. Radioimmunoassay (RIA) was performed within 2 hr on 100 μl aliquots of the unextracted supernatants as described by Czervionke et al.⁸ The detection limit of this RIA was 3 pmole/ml. Cross-reactivities of antiserum with thromboxane B₂, PGD₂, PGE₂ and PGF₂α were less than 1%. Results were expressed as means ± standard error of the mean (SEM) ng 6-keto-PGF₁α/ml/30 min for groups of three identically treated monolayers. The significance of the differences between group means was determined with the student t-test (two-tailed) for unpaired data, with the Bonferroni method to account for multiple comparisons.

Materials

Acetylsalicylic acid (ASA), calcium ionophore A23187, bovine gamma globulin (Cohn fraction V), adenosine diphosphate, PGD₂, PGE₂, PGF₂α, thromboxane A₂, and 6-keto-PGE₂ were obtained from Sigma Chemical Co. (St. Louis, MO). HPLC-grade ethyl acetate, water, acetonitrile, and phosphoric acid were from Allied Fisher Scientific Co. (Fairlawn, NJ). Arachidonic acid and collagen were from Biodata Corp. (Haboro, PA). ³H-Arachidonic acid (83.8 Ci/mmol) was from New England Nuclear (Boston, MA). HB101 Cell culture media and 0.1% collagenase (Washington Class III) were from Hana Biological (Berkeley, CA).

Results

rp-HPLC

rp-HPLC of the media removed from 5th passage pericyte monolayers incubated with ³H-arachidonic acid for 22 hr and extracted for prostaglandins, revealed a single large peak of radioactivity with a retention time corresponding to 6-keto-PGF₁α. Two very small peaks eluting with retention times identical to those of PGF₂α and PGE₂ also were detected. When ASA (0.01 M) or indomethacin (100 μM) were added to the culture media prior to ³H-arachidonic
Bioassay

Inhibition of platelet aggregation by ADP, arachidonic acid and collagen was observed after 5 min incubations of platelet-rich plasma with 1–2.5 × 10⁵ pericytes (Fig. 2). Only slight or no inhibition of aggregation occurred when human granulocytes replaced pericytes. Aliquots of supernatants from confluent, unstimulated pericyte monolayers did not inhibit platelet aggregation. In contrast, 50 µl of supernatants from monolayers stimulated with 20 µM arachidonic acid produced nearly complete inhibition of platelet aggregation (Fig. 3). Supernatants removed from stimulated monolayers and left at room temperature for 30 min prior to bioassay did not inhibit aggregation, demonstrating the lability of the inhibiting factor.

Radioimmunoassay

Unstimulated cultures consisting of approximately 10⁶ pericytes produced nanogram quantities of 6-keto-PGF₁α after 30 min incubations (Table 1). Cultures stimulated with 5 µM of the calcium ionophore A23187 or 20 µM arachidonic acid produced 6-fold or 12-fold increases, respectively, in 6-keto-PGF₁α production (Table 1). These differences were significant at the P < 0.05 level.

Discussion

We provide evidence that prostacyclin (PGI₂) is the principal cyclooxygenase product of 5th–7th passage cultures of bovine retinal capillary pericytes (BRCP).
First, a predominant labeled arachidonic acid metabolite co-eluting with 6-keto-PGF_{1α} (a stable metabolite of PG-I_{2}) was identified by rp-HPLC. This was eliminated by pretreatment of the cultures with cyclooxygenase inhibitors, and was augmented by the calcium ionophore A23187. Second, bioassay for PG-I_{2}-like activity demonstrated that BRCP produced a substance with a short half-life at room temperature and physiologic pH, that inhibited platelet aggregation by several stimuli. Third, a specific radioimmunoassay was used to quantitate and confirm the identity of 6-keto-PGF_{1α} produced by BRCP monolayers.

Unstimulated confluent cultures of 0.75-1.0 × 10^6 pericytes produced nanogram amounts of 6-keto-PGF_{1α}. Incubation with arachidonic acid or the calcium ionophore A23187 induced significant increases in 6-keto-PGF_{1α} production, presumably by providing more substrate for pericyte cyclooxygenase. In contrast to 6-keto-PGF_{1α}, rp-HPLC analysis of extracted culture media showed only small peaks corresponding to PGF_{2α} and PGE_{2}, and the absence of PGD_{2} and thromboxane B_{2} peaks, in stimulated and unstimulated monolayers. Since 6-keto-PGF_{1α} loss during extraction exceeded the losses of the other prostaglandins, this difference may be underestimated in Figure 1.

Prostacyclin (PG-I_{2}) is a potent vasodilator and inhibitor of platelet aggregation and platelet adherence to endothelium.10-14 A gradient of decreasing PG-I_{2} release from the inner (intimal) to outer (adventitial) layers of arteries has been demonstrated15 and correlates with the observed thromboreistance of vascular endothelium relative to the media and adventitia. In cell cultures derived from the layers of large arteries and veins (eg, aorta, umbilical vein), the greatest amounts of PG-I_{2} are produced by endothelial cells, followed by medial smooth muscle cells.16 In contrast, PG-I_{2} was not the principal PG released from small vessels or small vessel endothelial cells from rabbit myocardium,9 bovine cerebral capillaries,17,18 or newborn human foreskin.20 Arachidonic acid metabolism by intact retinal capillaries or cultured retinal capillary endothelial cells has not been characterized and it is not known whether PG-I_{2} is the major PG. Pericytes may release a significant proportion of the PG-I_{2} produced by the microvascular membrane under normal or pathologic conditions.

The role of PG-I_{2} and other PGs in the microcirculation has recently been reviewed.20 PGs may influence microvascular blood flow by a direct relaxant effect on vascular smooth muscle, they can modulate the effects of other vasoactive mediators such as noradrenaline, angiotension II and vasopressin, and they can alter microvascular responsiveness to sympathetic nerve stimulation.20 PG-I_{2} and PGE_{2}, the most potent vasodilator PGs, are known to regulate several aspects of immunologic responses and inflammation. They are necessary for the increases in vascular permeability and edema formation induced by C5a (the chemotactic peptide fragment of the fifth component of complement), leukotriene B_{4}, and the synthetic chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine.21 Polymorphonuclear leukocytes (PMNs) also are required for this response.21 PMN adherence in vitro is inhibited by PG-I_{2},22 and may be augmented by thromboxane A_{2}.23 PG-I_{2} may inhibit lymphocyte transformation24 and the differentiation of murine 3T3-L1 fibroblasts into adipocytes in response to insulin and indomethacin.25 The intimate association of retinal pericytes and endothelial cells, and the proximity of pericytes to the microvascular lumen26 (and ultimately to blood cells) might facilitate interactions between these cells via PG-I_{2} and other arachidonic acid metabolites.27 Retinas from diabetic patients exhibit loss of capillary pericytes with microaneurysm formation in those vessels lacking pericytes.28 Retinal vasconstriction in response to 100% oxygen breathing is impaired in diabetes mellitus.29 The finding of actin filaments in BRCP and of BRCP actin activation of skeletal muscle myosin Mg\(^{2+}\)-ATPase is evidence that BRCP possesses the machinery to generate contraction.30 These observations support a role for retinal capillary pericytes in the regulation of microvessel caliber and stability.

PG-I_{2} released by pericytes may feed back to regulate pericyte contractility and thus modulate microvascular blood flow and permeability.31 Pericyte PG-I_{2} could potentiate histamine and bradykinin-induced increases in endothelial permeability,32 since pericytes are also found along postcapillary venules.26 Histamine and bradykinin stimulate PG-I_{2} release from endothelial cells, and might also influence pericyte PG-I_{2} release. The loss of pericyte PG-I_{2} or the altered release of PG-I_{2} by pericytes in the metabolic environment of diabetes mellitus might contribute to the abnormal flow and permeability characteristic of early diabetic microangiopathy. Finally, pericyte

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<th>Stimulus</th>
<th>ng 6-keto-PGF_{1α}/dish/30 min†</th>
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<tr>
<td>None</td>
<td>3.87 ± 0.02</td>
</tr>
<tr>
<td>Calcium ionophore A23187 (5 μM)</td>
<td>17.3 ± 1.9</td>
</tr>
<tr>
<td>Arachidonic acid (20 μM)</td>
<td>45.9 ± 4.8</td>
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* 0.75-1.0 × 10^6 cells per dish from the 7th passage.
† Results are presented as the mean ± SEM for three culture dishes.
PGI₂ is a potent inhibitor of platelet aggregation and may normally counterbalance the action of platelet-activating agents, eg, thromboxane A₂. The early loss of microvascular pericytes and pericyte PGI₂ may promote the formation of microvascular platelet thrombi in diabetic microangiopathy.

Key words: prostacyclin, prostaglandins, retinal pericyte, arachidonic acid metabolism

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References