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Research Article

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Arachidonic Acid and Prostaglandin Endoperoxide Metabolism in Isolated Rabbit and Coronary Microvessels and Isolated and Cultivated Coronary Microvessel Endothelial Cells

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ABSTRACT Isolated microvessels and isolated and cultured microvessel endothelial cells were prepared from rabbit cardiac muscle. Pathways of arachidonic acid metabolism were determined by measurement of exogenous substrate utilization ([1-^{14}C]arachidonic acid incorporation and release from intact tissue and cells; [1-^{14}C]prostaglandin H_2 (PGH_2) metabolism by broken cell preparations) and by quantification of endogenous products (immunoreactive 6-keto-prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$) and prostaglandin E (PGE) release) by selective radioimmunoassay. Rabbit coronary microvessels and derived microvascular endothelial cells (RCME cells) synthesized two major products of the cyclooxygenase pathway: 6-keto-$PGF_{1\alpha}$ (hydrolytic product of prostaglandin I_2) and PGE_2. A reduced glutathione requiring PGH-E isomerase was demonstrated in coronary microvessels and RCME cells, but not in rabbit circumflex coronary artery or aorta. In addition, a minor amount of a compound exhibiting similar characteristics to 6-keto-PGE_1 was found to be produced by microvessels and RCME cells. Measurement of endogenously released prostaglandins indicated that under basal and stimulated conditions, PGE release exceeded that of 6-keto-$PGF_{1\alpha}$. Microvessels and microvessel endothelial cells derived from cardiac muscle of rabbit exhibit pathways of arachidonate metabolism that are different from those of many large blood vessels and derived endothelial cells.

INTRODUCTION

Increased attention has been focused on the role of vascular endothelium in the generation of arachidonic acid (AA)[1] metabolites. The endothelium is a major source of prostaglandin I_2 (PGI_2) synthesis and release (1, 2) and the PGI_2 generated appears to play significant roles in the regulation of hemostasis and vascular smooth muscle contractility. The principal prostaglandin formed by the majority of large blood vessels (i.e., aorta, vena cava, etc.) is PGI_2 (3–6). A few reports have suggested other enzymatic pathways of prostaglandin generation in the macrovasculature including a reduced glutathione (GSH) requiring prostaglandin H (PGH)-E isomerase in the pigeon aorta (7) and a GSH requiring PGH-D isomerase in rat blood vessels (8, 9). The development of the methodology for the isolation of microvessels (i.e., <300 μm in diameter, a mixture of arterioles, venules, and capillaries) from various tissues (10, 11) gave investigators the ability to study the nature of AA metabolism in the microvasculature. A number of studies have described the synthesis of PGI_2 by cerebral microvessels (12–16). However, isolated microvessels also exhibit additional pathways of prostaglandin synthesis, dependent upon the vascular bed and species examined. Isolated microvessels from the cerebral cortex of the cow (13) and cat (Gerritsen, M. E.; unpublished observations) exhibit a PGH-prostaglandin E (PGE) isomerase, while microvessels from the rat cerebral cortex exhibit a

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[1] *Abbreviations used in this paper:* AA, arachidonic acid; DMEM, Dulbecco's modified Eagle medium; FITC, fluorescein isothiocyanate; 15-HPETE, 15-hydroperoxyeicosatrienoic acid; HPLC, high-pressure liquid chromatography; 9-OH PGDH, 9-hydroxy-prostaglandin dehydrogenase; PGA, PGD, PGE, PGF, PGH, and PGI, prostaglandins A, D, E, F, H, and I, respectively; *p*-HMB, *p*-hydroxymercurobenzoate; PRP, platelet-rich plasma; RCME, rabbit coronary microvessel endothelial; SEM, scanning electron microscopy; TxB_2, thromboxane B_2.

GSH-independent prostaglandin D (PGD) synthase (14). Microvessels from bovine cardiac muscle (11), adrenal cortex, and retina also demonstrate GSH-dependent conversion of PGH_2 to PGE_2 (Gerritsen, M. E.; unpublished observations).

In the past 3 to 4 yr, rapid progress has been made in the isolation and culture of microvascular endothelial cells (17–25). These studies have demonstrated that microvascular endothelial cells exhibit several fundamental differences potentially useful for the understanding and treatment of diseases with a microvascular component. Much of this information has been summarized in a recent review (26). These studies have indicated that several features may be common to large and small blood vessel endothelium, including the presence of angiotensin-converting enzyme, Factor VIII antigen, a monolayer appearance, Weibel-Palade bodies, a nonthrombogenic surface, and production of PGI_2. A number of the capillary endothelial cell lines appear to require an additional matrix component (collagen, gelatin, or fibronectin) for survival and growth (17–19, 21, 27), a requirement not shared by endothelium derived from larger blood vessels such as the aorta and umbilical vessels. Heparin-induced migration of capillary endothelium and formation of capillary endothelial tubes have been described in adrenal capillary endothelial cell cultures (28) and may be another unique feature of microvascular endothelium.

In this study, we prepared microvessels from the rabbit heart and we isolated and cultivated microvascular endothelium from the rabbit heart. Using these preparations, we demonstrated both PGI_2 synthase and PGH-PGE isomerase activities in the coronary microvascular endothelium, and we identified an additional product, most likely a metabolite of PGI_2, formed by the action of 9-hydroxy-prostaglandin dehydrogenase (9-OH PGDH) on PGI_2 or 6-keto-prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$). In isolated coronary microvessels and derived endothelial cells, PGE_2 is synthesized and released in much greater proportions than 6-keto-$PGF_{1\alpha}$, suggesting that PGE_2 may be the major product of AA metabolism in the rabbit coronary microvasculature.

METHODS

Isolation of rabbit coronary microvessels. Procedures used for the isolation of rabbit coronary microvessels were modified from those originally described for the isolation of bovine coronary microvessels (11). Hearts were removed from pentobarbital anesthetized male white New Zealand rabbits, and perfused with 60 ml phosphate-buffered saline (PBS) (pH 7.4). The atria, large coronary vessels, obvious fibrous and connective tissues, and the epi- and endocardial surfaces of the heart were removed. The remaining myocardial tissue was minced with scissors and homogenized (Sorval Omnimixer, DuPont Instruments-Sorvall Biomedical Div., Wilmington, DE) in 20 vol of PBS, pH 7.4, for four 10-s bursts at half-maximal speed. The homogenate was filtered over a 210-μm nylon mesh sieve (Tetko Inc., Elmsford, NY) and the retentate resuspended in 20 vol of PBS and homogenized further (Sorvall Omnimixer, then four to five passes in a Dounce homogenizer, "A" pestle, Kontes Glass Co., Vineland, NJ). The resulting homogenate was filtered over a 120-μm mesh sieve and washed extensively with PBS. The retentate was removed and examined for purity by phase-contrast microscopy. The 120-μm mesh retentate was routinely used without further purification. This preparation was virtually free of nonvascular contamination other than the presence of connective tissue that was closely associated with the microvessel networks (Fig. 1). Microvessels were either used immediately (e.g., [1-^{14}C]AA incorporation and release of radiolabeled products, immunoreactive PGE and 6-keto-$PGF_{1\alpha}$ release) or frozen on dry ice and stored at $-70°C$ for later use. Homogenates were prepared from frozen preparations by powdering using a liquid N_2-cooled stainless steel press, followed by homogenization of the powder in 50 mM Tris-HCl, 1 mM EDTA buffer (pH 8.1) with an all glass homogenizer (Potter-Elvehjem, Kontes Glass Co.), followed by sonication in a Branson sonifier equipped with a microtip (Branson Sonic Power Co., Dansbury, CT). The resulting suspension was subjected to centrifugation (1 min at 15,000 *g*) and the supernatant filtered through glass wool. The resulting filtrate was termed the homogenate. Protein concentration was determined by a modification of the Lowry method as described by Markwell and co-workers (29), with bovine serum albumin as standard.

Isolation and cultivation of microvessel endothelial cells. The isolation procedure used was based on a modification of the method originally described by Simionescu and Simionescu (24). Male white New Zealand rabbits (~1 kg) were anesthetized with pentobarbital (50 mg/kg). The heart was removed and a sterile cannula introduced into the aorta. The heart was perfused with sterile 37°C PBS until blanching was observed. The atria were removed and the epicardial, subepicardial, and endocardial layers of the ventricles were cut away, as were all obvious large vessels, and connective and fibrous tissues. The remaining midportion of the myocardium was minced finely in a McIlwain tissue chopper equipped with three razor blades spaced with 0.1-mm washers. The stage was rotated 90° and the mincing repeated. The mince was incubated in Ca^{++}-free PBS containing 1 mg/ml collagenase (dissociation medium) and incubated at 37°C for 40 min with vigorous stirring. The suspension was diluted with 30 ml of Ca^{++}-free PBS and centrifuged for 10 min at 100 *g*. The supernatant was discarded and the pellet resuspended in 30 ml of dissociation medium and incubated at 37°C for an additional 30 min with gentle stirring. The resulting suspension was filtered over an 88-μm nylon mesh sieve and the filtrate homogenized in 15 ml of 37°C Ca^{++}-free PBS in a Dounce homogenizer with the "B" pestle. This homogenate was centrifuged at 100 *g* for 6 min, the supernatant discarded, and the homogenization-centrifugation step repeated two additional times. The final pellet was resuspended in 12 ml of Ca^{++}-free PBS and 6-ml fractions were layered over 4.5 ml of 6% bovine serum albumin (Sigma Chemical Co., St. Louis, MO; fraction V) in Ca^{++}-free PBS in 15-ml sterile centrifuge tubes, (Corning Glass Works, Corning, NY). After a 100 *g* centrifugation (5 min), the resulting pellet was resuspended in Dulbecco's modified Eagles medium (DMEM) containing 20% fetal calf serum, antibiotics, antifungal agents and supplemental L-glutamine. The suspension was pipetted into Corning 35-mm plastic culture dishes, and the dishes incubated at 37°C in 5% CO_2 in air.

After 30 min, the medium was removed and fresh DMEM added. The cells were fed every 3–4 d.

Transmission electron microscopy. Cells for transmission electron microscopy were grown on Corning 35-mm culture dishes. Monolayers were fixed for 30 min in 2.5% glutaraldehyde in 100 mM sodium cacodylate buffer (containing 0.16 M sucrose), pH 7.4, at 23°C for 30 min, and then postfixed in a 2% aqueous uranyl acetate 30 min at 4°C in the dark. The cells were rinsed with distilled water and dehydrated in a graded ethanol series (35, 50, 70, and 90% ethanol, 5 min each) then embedded in araldite as described by Brinkley and Chang (30). Thin sections were prepared with a Porter-Blum MT-2 microtome (Du Pont de Nemours, E. I. & Co., Inc./Sorvall Instruments Div., Newton, CT) and stained with lead citrate and uranyl acetate followed by examination and photography with an Hitachi America, Ltd. (New York) HS-9 electron microscope (Department of Anatomy, New York Medical College).

Scanning electron microscopy (SEM). Cells for SEM were grown on 35-mm optical plates (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA). Monolayers were rinsed three times with warm (37°C) 100 mM sodium cacodylate buffer containing 0.16 M sucrose, pH 7.4, followed by fixation in 2% aqueous osmium tetroxide. The cells were rinsed with distilled water, dehydrated in graded acetone, and prepared for SEM as described by Anderson (31). Specimens were viewed in a scanning electron microscope (Cambridge Instrument Co., Inc., Ossining, NY) with the assistance of Mr. Don Cooke, Union Carbide Corp., Elmsford, NY.

Assay for angiotensin-converting enzyme. Angiotensin-converting enzyme activity, assayed by the method of Ryan et al. (32), was kindly performed by Mr. Frank Palmieri and Dr. Patrick E. Ward of the Department of Pharmacology, New York Medical College. Confluent cells were washed five times with 1 ml PBS then removed with a rubber policeman and homogenates prepared by sonication (Branson sonifier, Branson Sonic Power Co.) in 10 mM Tris-HCl, 0.25 M sucrose, pH 7.5. Homogenates were incubated with [^{3}H]benzoyl Phe-Ala-Pro for periods of 5–60 min. Buffer blanks were used as controls.

Platelet adhesion. Platelet-rich plasma (PRP) was prepared from aliquots of citrated rabbit blood. Aliquots of PRP were diluted in Ca^{++}-free PBS and placed on the surface of the rabbit coronary microvessel endothelial cell (RCME cells) monolayers. After a 5-min incubation the PRP-buffer mixture was removed and the cells washed with 1 ml of buffer. The monolayers were examined under the inverted phase microscope, or prepared as above for SEM.

Factor VIII antigen. Confluent RCME cells were washed twice with 37°C PBS and fixed in methanol/acetone (1:1, vol/vol) at 23°C for 15 min. The culture dishes were washed three times with 1 ml PBS and then incubated with rabbit antiserum to bovine Factor VIII (diluted in PBS) for 30 min at 37°C. The cells were washed three times with PBS and incubated for an additional 30 min at 37°C with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG. The cells were washed three times with 1 ml PBS, a coverslip applied (with a drop of 50% glycerol in PBS) and examined for fluorescence under a microscope equipped with an epifluorescence light source (Carl Zeiss, Inc., Thornwood, NY). Controls for autofluorescence and nonspecific fluorescence used fixed cells with no antibody and cells treated with nonimmune rabbit serum followed by FITC-conjugated goat anti-rabbit IgG.

Metabolism of PGH_2 and arachidonic acid. [1-^{14}C]PGH_2 was prepared from [1-^{14}C]AA and purified as previously described (6). Immediately before incubation, 0.2 nmol of [1-^{14}C]PGH_2 was evaporated under a stream of N_2 (on ice) in a 1.5-ml centrifuge tube (Brinkmann Instruments, Inc., Westbury, NY). Aliquots of tissue or cell homogenates were diluted to 100 μl in Tris-HCl buffer (50 mM, with 1 mM EDTA, pH 8.1) with or without the addition of cofactors (e.g., reduced GSH) in the centrifuge tubes and the contents mixed vigorously, followed by incubation at 23°C for 5 min. The reaction was terminated and products extracted by the addition of 400 μl of ethyl acetate/methanol/0.2 M citric acid (15:2:1, vol/vol/vol), vigorous mixing, and rapid centrifugation (1 min at 15,000 g, Eppendorf microfuge). The organic phase was applied to thin-layer plates and the chromatograms developed in the organic phase of ethyl acetate/hexane/acetic acid/water, (56:24:12:60, vol/vol/vol/vol) and visualized by radiochromatogram scanning (Packard Radiochromatogram Scanner, model 7201, Packard Instrument Co., Downers Grove, IL). Prostaglandin standards were visualized by exposure to I_2 vapor. Quantitation was performed by scraping off the silica gel on appropriate areas of the chromatograms, addition of 10 ml scintillation fluid (PCS, Amersham Corp., Arlington Heights, IL), followed by liquid scintillation counting. Control incubations were also performed with 50 mM Tris-HCl, 1 mM EDTA buffer (pH 8.1), boiled homogenates (boiled control), and rabbit serum albumin (protein control). Experiments with [1-^{14}C]AA were performed as follows. Aliquots of [1-^{14}C]AA were evaporated to dryness under a stream of N_2, followed by reconstitution in 50 μl of 100 mM Na_2CO_3 and diluted to 0.2 Ci/ml (~6 μM) with DMEM. Cells or intact microvessels were incubated with 1 ml aliquots of [1-^{14}C]AA-DMEM for 4 h at 37°C. The medium was removed and the cells washed twice with DMEM without AA then incubated in 1 ml DMEM for an additional 4 h. The media from both incubations were acidified to pH 3.5 with formic acid and extracted twice with 2 vol chloroform. The organic phase was evaporated in vacuo and reconstituted in 300 μl ethyl acetate and applied to silica gel GHL thin-layer chromatography plates (Analtech, Inc., Newark, DE). Chromatograms were developed, visualized, and analyzed as described above.

Incorporation of [1-^{14}C]AA into membrane phospholipids was determined as follows. Cells or microvessels were homogenized in 20 vol chloroform/methanol (2:1)/g tissue with a Branson sonifier. The tissue homogenate was filtered over glass wool and the eluate washed twice with 2 vol of 0.13 M KCl. The organic layer was evaporated to dryness in vacuo and the extract reconstituted in 500 μl chloroform/methanol (1:1). Authentic phospholipid standards (Sigma Chemical Co.) were spotted along with a 200-μl aliquot of the tissue extract on silica gel GHL thin-layer plates. The chromatograms were developed in chloroform/methanol/ammonium hydroxide (70:35:5). Standards were visualized by exposure to I_2 vapor. Radioactivity was determined by scraping appropriate layers of silica gel followed by liquid scintillation counting.

9-OH PGDH assay. The activity of 9-OH PGDH in homogenates of RCME cells was measured by a modification of the procedure detailed by Tai and Yuan (33) as described by Wong et al. (34). The assay is based on the transfer of [9-^{3}H] of PGI_2 to lactate by coupling the 9-OH PGDH and lactate dehydrogenase reactions. For these assays RCME and microvessel homogenates were prepared as described above, with 50 mM Tris-HCl buffer containing 1 mM EDTA and 0.1 mM dithiothreitol. The incubation mixture contained sodium pyruvate (5 nmol), NAD^+ (2 nmol), lactate dehydrogenase (50 μg), and 10,000 cpm of [9-^{3}H]PGI_2 in a total volume of 1 ml. The reaction was initiated by the addition

of substrate and terminated by the addition of 300 μl of a dextran-coated charcoal suspension (10% charcoal in 1% dextran in water), followed by centrifugation at 1,000 g for 5 min. The supernatant was decanted into scintillation vials and 10 ml PCS scintillation fluid was added, followed by scintillation counting. The observed counts per minute were converted to disintegrations per minute with a quench correction curve and external standards. Activity is expressed as picomoles of 6-keto-PGE_1 formed per milligram of protein per hour.

Radioimmunoassay of PGE and 6-keto-$PGF_{1\alpha}$. The concentration of immunoreactive 6-keto-$PGF_{1\alpha}$ (the stable hydrolytic product of PGI_2) and immunoreactive PGE were determined in nonextracted samples of PBS that had been incubated with RCME cells or isolated coronary microvessels. Antisera were kindly supplied by Dr. Lawrence Levine (Brandeis University, Waltham, MA). The radioimmunoassay procedure was performed as detailed by Granström and Kindahl (35). The detection limit of both the 6-keto-$PGF_{1\alpha}$ and PGE antisera was 2 pg/tube and intra- and interassay variation was routinely <10%. Values are expressed as the amount of PG released per 10^5 cells (or milligrams of microvessels, wet weight) per 15 min. The anti-6-keto-$PGF_{1\alpha}$ antisera demonstrated some cross-reactivity with 6-keto-PGE_1 (3.1%), 6,15-diketo-$PGF_{1\alpha}$ (1.0%), and $PGF_{2\alpha}$ (0.3%). The remaining prostanoids tested (13,14-dihydro-6,15-diketo-$PGF_{1\alpha}$, PGE_2, thromboxane B_2 [TxB_2], PGA_2, and AA) exhibited cross-reactivity of <0.03%. The antisera directed against PGE_2 were not as specific, exhibiting complete cross-reactivity with PGA_2 (150%), and detectable cross-reactivity with PGE_1 (25.6%), PGD_2 (0.4%), $PGF_{2\alpha}$ (0.3%), 6-keto-PGE_1 (10.1%), and 15-keto-PGE_2 (2.6%). The cross-reactivity with other metabolites of the AA cascade (i.e., TxB_2, 6-keto-$PGF_{1\alpha}$, 13,14-dihydro-6,15-diketo-$PGF_{1\alpha}$) and AA was <0.01%. Measurements of 6-keto-$PGF_{1\alpha}$ and PGE release from RCME cells was determined by the following protocol. Media containing fetal calf serum (FCS) were removed from confluent RCME cells. The cells were washed twice with 2 ml of warm (23°C) PBS, then incubated for 15 min at 23°C in room air in 1 ml PBS (per 17.5-mm culture well). At the end of the first incubation, the buffer was removed and 1 ml of warm PBS with or without drugs was added. The cells were incubated an additional 15 min under the above conditions, the buffer removed by aspiration, and samples from both incubations frozen on dry ice and stored at −20°C until assay.

High-pressure liquid chromatography (HPLC). Separation of PG and related compounds by HPLC was performed by a modification of the procedure originally described by van Rollins et al. (36). The method used isocratic separation with 70% phosphoric acid (pH 2.95) and 30% acetonitrile, a Waters C_{18} microbondapak HPLC column (Waters Associates, Millipore Corp., Milford, MA), and a flow rate of 1.0 ml/min. Elution of authentic standards was monitored at 192 nM and elution of radiolabeled products was monitored by liquid scintillation counting of 0.5–1.0-ml fractions eluted from the column. The HPLC system used included a Waters Associates variable wavelength spectrophotometer (model 450). Fractions eluting from the column were collected with an Eldex universal fraction collector (Eldex Laboratories, Inc., Menlo Park, CA). Fractions for injection were prepared in acetonitrile and filtered through a 0.4-μm Teflon filter (Gelman Sciences, Inc., Ann Arbor, MI, TF-200).

Synthesis of 15-hydroperoxyeicosatrienoic acid (15-HPETE). 15-HPETE was synthesized from AA by incubation with soybean lipoxidase as previously described (37). 2.68 mg AA and trace [1-^{14}C]AA (600,000 dpm) were incubated with soybean lipoxidase (1 mg) in 11 ml of 47 mM sodium borate buffer, pH 9.0, for 5 min at 22°C. The 15-HPETE produced was extracted into diethyl ether after acidification with 0.2 M citric acid and purified by silicic acid chromatography. Purity of the preparation was determined by thin-layer chromatography in ether/hexane (8:2, vol/vol); the presence of the peroxy group was indicated by a positive reaction with freshly prepared ferrous thiocyanate. The compound was reduced to a more polar compound (i.e., 15-hydroxyeicosatetraenoic acid) by treatment with $NaBH_4$ in methanol. The concentration of the purified 15-HPETE was determined by liquid scintillation counting based on the initial specific activity of the AA. 15-HPETE was stored at −20°C in absolute ethanol.

Materials. Collagenase (CLS type I, 145–150 U/mg) was obtained from Worthington Biochemical Corp., Freehold, NJ. DMEM, FCS, antibiotics (Pen-Strep), fungizone, and L-glutamine were obtained from Flow Laboratories, Inc. (McLean, VA). Electron microscope preparative reagents (glutaraldehyde, sodium cacodylate, uranyl acetate, araldite, lead citrate) were from Polysciences, Inc. (Warrington, PA). The substrate for the angiotensin converting enzyme assay, [^{3}H]benzoyl Phe-Ala-Pro was purchased from Ventrex Laboratories, Portland, ME. Antiserum to bovine Factor VIII was kindly provided by J. Brown, University of California, San Diego. [1-^{14}C]AA (56 mCi/nmol) and [5,8,9,11,12,14,15-^{3}H]6-keto-$PGF_{1\alpha}$ (150 Ci/mmol), [9-^{3}H]PGI_2 (12 Ci/nmol), and [5,6,8,11,12,13,15-^{3}H]PGE_2 (150 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Citric acid, Trsi-HCl, sodium pyruvate, NAD^+, GSH (reduced and oxidized), nordihydroguaretic acid, dithiothreitol, indomethacin, *p*-hydroxymercuribenzoate (*p*-HMB), *N*-ethylmaleimide, dextran, EDTA, soybean lipoxidase, rabbit serum albumin, silicic acid (SIL-A-200), and phospholipid standards were from Sigma Chemical Co. 2-Mercaptoethanol was purchased from Eastman Kodak Co. (Rochester, NY). All solvents were obtained from J. T. Baker Chemical Co., (Phillipsburg, NJ) and were either of reagent (thin-layer and column chromatography) or HPLC grade. Bovine serum albumin (Pentex) was from Miles Laboratories Inc. (Elkhart, IN). All prostaglandins were kindly provided by Dr. John Pike, Upjohn Co., Kalamazoo, MI. FITC-conjugated goat anti-rabbit IgG was from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA. Unlabeled AA was obtained from Nu-Chek Prep Inc. (Elysian, MN). Thin-layer chromatography plates (silica gel GHL) were from Analtech, Inc. (Newark, DE). Bacto lipopolysaccharide (*Salmonella enteritidis*) was from Difco Laboratories Inc., Detroit, MI.

RESULTS

Isolation and identification of RCME cells

Isolated rabbit coronary microvessels were prepared virtually free of nonvascular contamination, (Fig. 1). Viable endothelial cells, as assessed by trypan blue exclusion (80–90% of the purified RCME cells excluded the dye) were harvested from the rabbit coronary microcirculation as described in Methods. Cells attached to the culture dishes within a few minutes of the initial inoculation and began to flatten out on the bottom of the dish. Removal of the medium and unattached cells 30 min after inoculation helped to

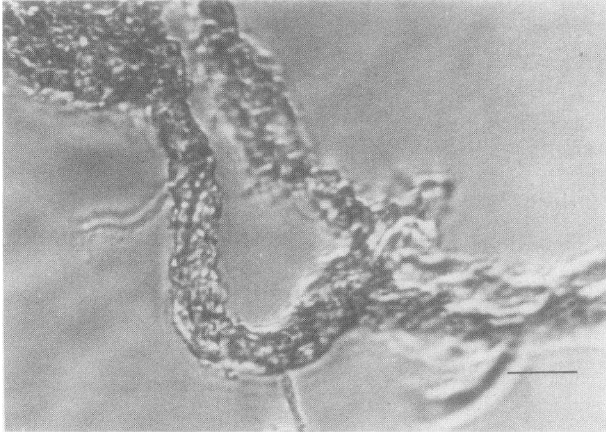

FIGURE 1 Isolated microvessels from the rabbit heart (phase contrast; bar, 15 μm). The microvessels consist of a mixture of arterioles, venules, capillaries, and associated connective tissue. When isolated, the microvessels aggregate and appear as strands of "tubes."

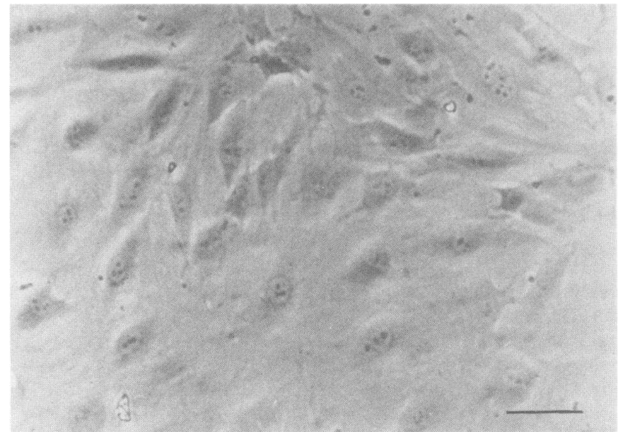

FIGURE 2 Light micrograph of a primary culture of RCME cells, near confluence. The cells exhibit a polygonal appearance with indistinct cell borders. Bar, 100 μm.

select for endothelial cells. Although previous studies with capillary endothelium have reported the requirement for gelatin, collagen, or other substratum materials (19, 27), in our hands the RCME cells attached as well to untreated culture plates. The primary cultures required high serum concentrations (15–20%) to support growth. Subsequent passages could be maintained at somewhat lower serum concentrations (10–15%). Cell division was apparent 4–8 h after addition of the isolated RCME cells to the culture dishes, and confluent monolayers were obtained in 10–20 d, dependent upon the initial plating density. Initially, cells appeared to grow out from small clusters and individual cells were elongated with a broad peripheral cytoplasm and indistinct cell borders. Near confluence, the cells exhibited a monolayer appearance and a rectangular-like shape (Fig. 2). Primary cultures obtained by the procedures outlined in Methods appeared to be homogeneous populations with the rare appearance of contaminating cells with a smooth muscle-like morphology, as evidenced by overgrowth of cells on top of one another, forming hills and valleys. Selective cloning of cells with endothelial morphology was accomplished with cloning rings and treatment with trypsin-EDTA; subsequent cultures were derived from clusters of cells with endothelial morphology. Cells prepared in this manner have continued to grow in culture and maintain their endothelial characteristics for at least six subsequent passages.

Additional evidence for the endothelial origin of the RCME cells was provided by the presence of Factor VIII antigen and of angiotensin converting enzyme (Fig. 3). The RCME cells offered a nonthrombogenic surface to rabbit platelets; platelets were observed attaching only to regions where the underlying substrate had been exposed (Figs. 4 and 5).

Examination of the cells by transmission electron microscopy revealed large numbers of vesicles, smooth and rough endoplasmic reticulum, fine filaments, free ribosomes, and many large structures that were apparently endoplasmic reticulum with dilated cisternae (Fig. 6 *A*). When viewed in the scanning electron microscope, the cultured RCME cells appeared homogeneous. They were large cells (~20 × 60 μm) that exhibited numerous blebs and pits apparently on the surface of the cells; these structures seemed most prominent in the perinuclear region of the cells (Fig. 6 *B*).

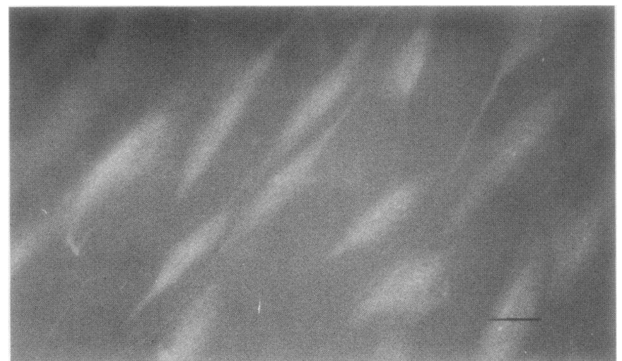

FIGURE 3 Positive Factor VIII indirect immunofluorescent staining of cultured RCME cells. Control incubations with nonimmune serum were completely black. Bar, 50 μm.

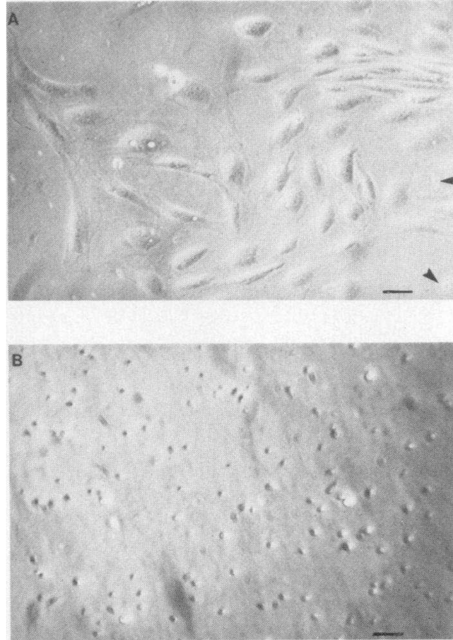

FIGURE 4 (*A*) RCME cells were incubated with PRP prepared from citrated rabbit blood. After a 5-min incubation the PRP was removed and the cells washed with 1 ml PBS. A few adherent platelets can be seen in the lower right-hand corner (arrows) in regions devoid of cells. Bar, 50 μm. (*B*) Region of culture dish without RCME cells (same culture dish as in *A*). Platelets can be seen adhering to the surface of the dish. Bar, 50 μm.

Prostaglandin synthesis and release by RCME cells and isolated rabbit coronary microvessels

Incorporation and metabolism of [1-^{14}C]AA by intact cultured RCME cells and intact isolated microvessels. The RCME cells rapidly incorporated [1-^{14}C]AA into the cellular phospholipids. After a 1-h incubation 50–60% of the added [1-^{14}C]AA had been incorporated, predominantly into the phosphatidyl ethanolamine fraction of the phospholipids, but radioactivity was also observed co-migrating with phosphatidyl choline, phosphatidylinositol, and phosphatidylserine standards. After the 4- and 8-h incubations with [1-^{14}C]AA, the incubation media was removed, extracted, and chromatograms developed as described in the Methods. Three major products were observed, co-migrating with PGE_2, 6-keto-$PGF_{1\alpha}$, and $PGF_{2\alpha}$ (Fig. 7 *A*). The major product, co-migrating with authentic PGE_2 was identified as PGE_2 by co-migration with authentic standard in a second solvent system (ethyl acetate/acetic acid [99:1, vol/vol]) and by treatment with 0.1 N HCl to form a less polar compound co-migrating with prostaglandin A_2 (PGA_2). The compound co-migrating with 6-keto-$PGF_{1\alpha}$ also comigrated with authentic 6-keto-$PGF_{1\alpha}$ in the second solvent system, and was resistant to acid treatment. The compound co-migrating with $PGF_{2\alpha}$ did not comigrate with authentic $PGF_{2\alpha}$ in ethyl acetate/acetic acid (99:1, vol/vol), and acid treatment resulted in the formation of a less polar compound migrating in the region of PGA_2. Authentic $PGF_{2\alpha}$ was not affected by acid treatment. These observations suggested that the $PGF_{2\alpha}$-like compound was not $PGF_{2\alpha}$, but some other metabolite of AA. Formation of all three products was inhibited by preincubating the cells with 5 μM indomethacin for 2 h before the addition of [1-^{14}C]AA and maintaining concentration of indomethacin in the incubation media with [1-^{14}C]AA (Fig. 7 *B*).

Additional nonpolar products of AA metabolism migrated in the regions of PGA_2 (Fig. 7 *C*). The synthesis of these compounds was not prevented by pretreatment of the RCME cells with indomethacin (Fig. 7 *B*), but was reduced by nordihydroguaretic acid (100 μM) pretreatment (Fig. 7 *C*), suggesting that these compounds may be hydroxyacids formed by the lipoxygenase pathway.

Isolated coronary microvessels rapidly incorporated [1-^{14}C]AA into the phospholipid fraction, with 80–90% of the label taken up within 1 h. However, subsequent incubation of the microvessels for periods of 4–14 h resulted in the release of relatively (compared with the RCME cells) small amounts of labeled material co-migrating with 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, and PGE_2. The majority of the radiolabeled material released appeared to consist of free AA and several other nonpolar compounds, probably hydroxy fatty acids (Fig. 8).

FIGURE 5 Cells with a smooth-muscle-like morphology were clonally derived from primary cultures of RCME cell preparations. These cells were incubated in a manner identical to those in Fig. 4. Numerous platelets can be seen adhering to the surface of these smooth-muscle-like cells. At later stages of growth, these cells exhibited a hill-and-valley appearance and the cells overgrew one another. Bar, 50 μm.

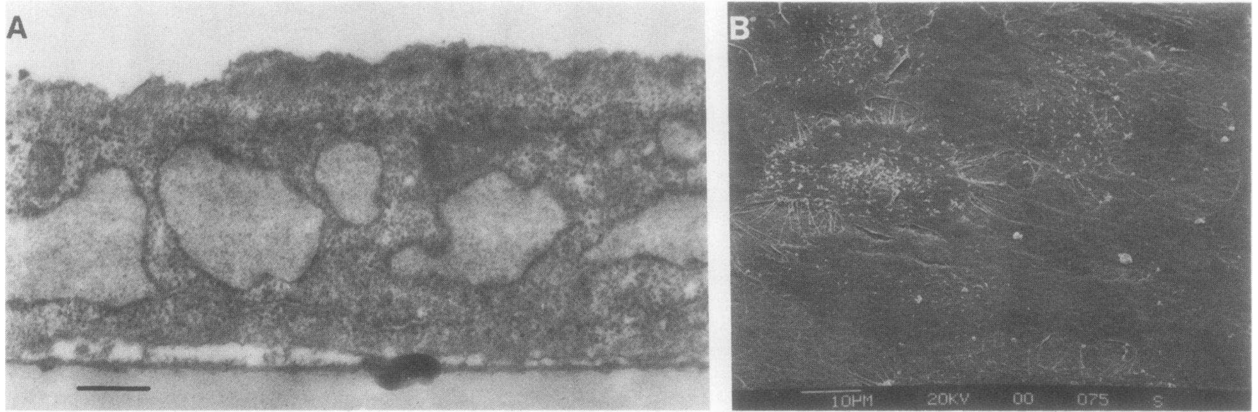

FIGURE 6 (*A*) Electron micrograph of RCME cells (obtained from the primary cultures). Marker = 400 nm. Cells contain number large endoplasmic reticulum structures with dilated cisternae. (*B*) Scanning electron micrograph of RCME cells (obtained from primary cultures). Marker = 10 μm. All exhibit numerous blebs in the perinuclear region.

Metabolism of [1-^{14}C] PGH_2 by broken cell preparations of RCME cells and rabbit coronary microvessels. The metabolism of [1-^{14}C]PGH_2 by RCME homogenates was performed under rigorous conditions enabling the distinction of enzymatic transformation vs. nonenzymatic decomposition of PGH_2 to prostaglandins and 12-hydroxyheptadecatrienoic acid. The profile of prostaglandin products obtained with 50 μg of RCME cell homogenate protein (equivalent to 0.5 mg protein/ml, final concentration) in the absence and presence of 2 mM GSH indicated at least two enzymatic products of PGH_2 metabolism: 6-keto-$PGF_{1\alpha}$ and PGE_2 (Fig. 9 *A* and *B*). Enzymatic formation of 6-keto-$PGF_{1\alpha}$, the hydrolysis product of PGI_2, was easily identified, as this product is not formed nonenzymatically by PGH_2 decomposition. Enzymatic formation of PGE_2 is more difficult to establish. To define a GSH-requiring PGH-PGE isomerase, we established the following criteria:

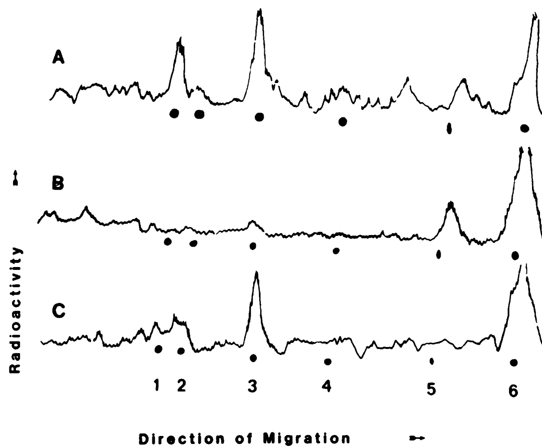

FIGURE 7 RCME cells (obtained from primary cultures) were incubated with [1-^{14}C]AA (0.2 Ci, 51.2 mCi/mmol) for 4 h at 37°C in 5% CO_2 in room air. Radiolabeled products were extracted by acidification to pH 3.5 with 88% formic acid followed by extraction into 2 × 2 vol of chloroform. The organic phase was evaporated in vacuo, and the residue resuspended in ethyl acetate to an adjacent region of the thin-layer chromatography plate before development of the chromatogram. The direction of migration of the solvent is from left to right, and increasing radioactivity is shown in the left-hand margin. (*A*) RCME cells incubated as above without drugs. (*B*) RCME cells pretreated with 5 μm indomethacin (2 h), followed by incubation for 4 h with [1-^{14}C]AA and 5 μm indomethacin. (*C*) RCME cells pretreated with 100 μM nordihydroguaretic acid (2 h) before addition of fresh media containing [1-^{14}C]AA. Standards: (*1*) 6-keto-$PGF_{1\alpha}$, (*2*) $PGF_{2\alpha}$, (*3*) PGE_2, (*4*) PGD_2, (*5*) PGA_2, (*6*) AA.

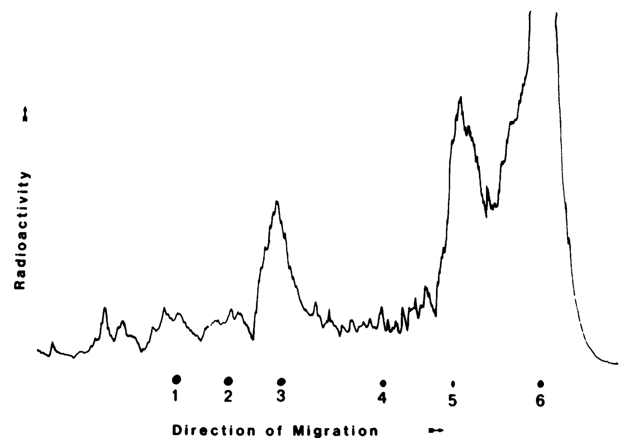

FIGURE 8 Representative radiochromatogram scans obtained from isolated rabbit coronary microvessels (60 mg wet wt) incubated with [1-^{14}C]AA (0.2 μCi) for 4 h. Numbers indicate migration of authentic standards: (*1*) 6-keto $PGF_{1\alpha}$, (*2*) $PGF_{2\alpha}$, (*3*) PGE_2, (*4*) PGD_2, (*5*) PGA_2, (*6*) AA.

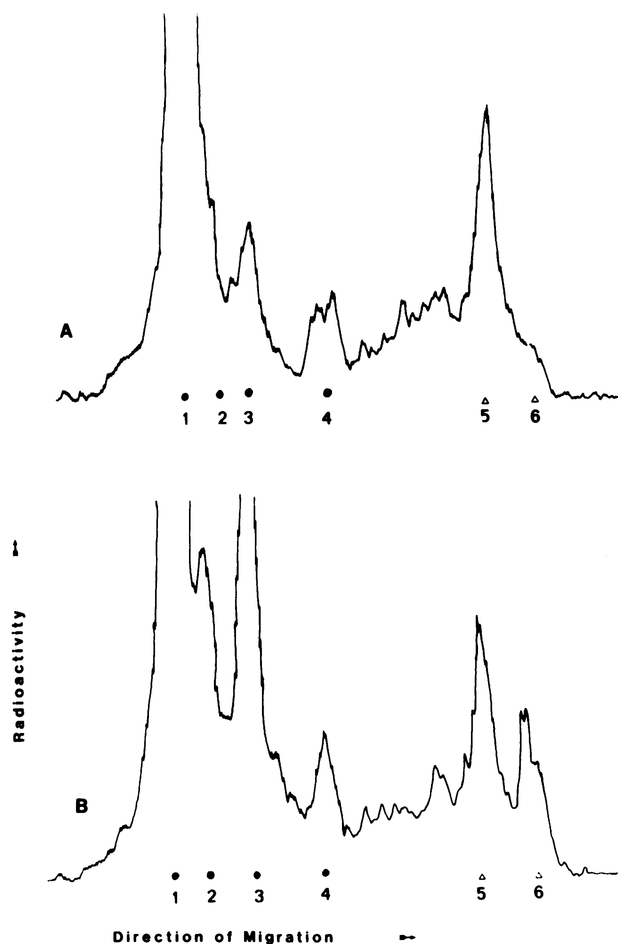

FIGURE 9 Representative radiochromatogram scans obtained from incubations of RCME homogenates (equivalent to 100 μg protein) with [1-^{14}C]PGH_2 as described in Methods in the absence (*A*) and presence (*B*) of 2 mM reduced glutathione. Migration of authentic standards is indicated as (*1*) 6-keto-$PGF_{1\alpha}$, (*2*) $PGF_{2\alpha}$, (*3*) PGE_2, (*4*) PGD_2. Δ indicates where unreacted PGH_2 (*5*) and 12-hydroxyheptadecatrienoic acid (*6*) migrate. In the presence of GSH, there is a marked enhancement of PGE_2 formation.

The PGE-forming activity should (*a*) exhibit a specific requirement for GSH as cofactor, (*b*) be inhibited by boiling or by pretreatment with sulfhydryl-directed reagents such as *N*-ethylmaleimide or *p*-HMB, and (*c*) exhibit saturation with increasing substrate (PGH_2) concentrations. These criteria are based on earlier studies of PGH-PGE isomerase activity in bovine seminal vesicles (37) and bovine cerebral and coronary microvessels (11, 13). The GSH-enhanced PGE-forming activity of the RCME cell homogenates was destroyed either by heating at 100°C for 5 min, followed by quenching on ice, or by preincubation with 5 mM *p*-HMB (Tables I and II) and exhibited specificity for GSH as cofactor (Table I). In addition, the GSH-enhanced PGE-forming activity could be saturated at substrate concentrations exceeding 50 μM (data not shown).

A third major product comigrating with $PGF_{2\alpha}$ was also observed (Fig. 9). The position of the radiolabeled $PGF_{2\alpha}$-like compound appeared to be related to the amount of 6-keto-$PGF_{1\alpha}$ formed. This was indicated at higher protein concentrations where little PGE_2 was formed (in the absence of GSH), and the majority of the PGH_2 was converted to 6-keto-$PGF_{1\alpha}$ and the $PGF_{2\alpha}$-like compound. Preincubation of the RCME cell homogenates with 15-HPETE, an inhibitor of PGI_2 synthase, inhibited the formation of both 6-keto-$PGF_{1\alpha}$ and the $PGF_{2\alpha}$-like compound, suggesting that this unknown compound may arise from the PGI_2 synthase pathway. The evidence of the lack of acid and base stability and the co-migration of the $PGF_{2\alpha}$-like compound with authentic 6-keto-PGE_1 in the two solvent systems described above suggested that the unknown compound may be 6-keto-PGE_1, the metabolite of PGI_2 via 9-OH PGDH (34). Additional evidence for the formation of 6-keto-PGE_1 was provided by HPLC (Fig. 10), where radiolabeled product co-eluted with authentic 6-keto-PGE_1. In another series of experiments, we measured the activity of 9-OH PGDH, with [9-^{3}H]PGI_2 as substrate. Estimates of this activity ranged from 9 to 30 pmol/mg protein per h for RCME cell homogenates. In addition, RCME cells were incubated with [1-^{14}C]5,8,11-eicosatrienoic acid, under the identical conditions as described above for [1-^{14}C]AA incorporation and metabolism (Fig. 11). Cells incubated with [1-^{14}C]5,8,11-eicosatrienoic acid did

TABLE I
Effects of Various Thiols and Sulfhydryl-directed Reagents on the Formation of PGE_2 from PGH_2 by Homogenates of RCME Cells

Group	Reagents	PGE_2 formed
		pmol
1	Control, no drugs	44±2
2	2 mM reduced GSH	96±3*
3	5 mM oxidized GSH	46±3
4	5 mM dithiothreitol	50±3
5	1 mM *p*-HMB + 2 mM reduced GSH	44±4
6	Boiled homogenate	50±1
7	Boiled homogenate + 2 mM reduced GSH	48±2

Homogenates of RCME cells, equivalent to 100 μg protein were incubated with [1-^{14}C]PGH_2 (0.2 nmol) at 23°C for 5 min. Products were extracted and quantified as described in Methods. Values are the mean of five determinations (±SEM).

* $P < 0.05$, significantly different from group 1; the *t* test for nonpaired values.

TABLE II

[1-^{14}C]PGH_2 Metabolism by Homogenates Prepared from Rabbit Coronary Microvessels, RCME Cells (Freshly Isolated and Cultured), Aorta, Circumflex Coronary Artery, Rabbit Serum Albumin, and Boiled RCME Cell Homogenates

Preparation	2 mM GSH	6-keto-$PGF_{1\alpha}$	$PGF_{2\alpha}$	PGE_2	PGD_2	PGH_2 + HHT
Coronary microvessels	−	2±1	28±3	20±1	35±2	80±3
	+	4±1	22±3	37±1*	34±1	68±2*
RCME cells (fresh)	−	10±1	18±3	46±1	34±1	70±6
	+	22±3*	34±2*	61±1*	35±2	32±2*
RCME cells (cultured)	−	46±6	18±3	40±3	36±2	43±3
	+	23±4*	10±2	96±1*	20±3	36±3
Circumflex coronary artery	−	7±2	26±2	55±3	41±1	62±1
	+	10±2	38±1*	60±6	46±1	30±1
Aorta	−	27±1	32±1	40±6	26±3	41±2
	+	56±6*	17±1*	40±5	37±1*	35±1
Rabbit serum albumin	−	-0-	36±2	46±3	41±1	42±1
	+	-0-	50±1	46±1	42±2	29±3
RCME cells (boiled)	−	-0-	35±2	50±1	42±1	40±3
	+	-0-	40±1	48±2	40±1	38±2

Homogenates equivalent to 100 μg of protein and boiled controls and rabbit serum albumin (100 μg protein) were incubated with 0.2 nmol [1-^{14}C]PGH_2 at 23°C for 5 min in the absence and presence of reduced GSH. Values are expressed as pmol product formed (mean±SEM).

* Significant ($P < 0.05$) change from the corresponding incubation in the absence of GSH. The t test, nonpaired values; $n = 5$.

not demonstrate formation of radiolabeled material co-migrating with 6-keto-$PGF_{1\alpha}$ or $PGF_{2\alpha}$.

Homogenates prepared from rabbit coronary microvessels also demonstrated PGI_2 synthase (formation of 6-keto-$PGF_{1\alpha}$) and PGH-PGE isomerase (GSH-enhanced PGE formation) (Table II). A variable amount of radiolabeled material also co-migrated with $PGF_{2\alpha}$, some of which could be 6-keto-PGE_1. Assays of 9-OH PGDH activity indicated activities of 10–13 pmol/mg protein per h, further supporting this possibility. We compared PGH_2 metabolism by homogenates of rabbit coronary microvessels, freshly isolated and cultured RCME cells, circumflex coronary artery, and aorta (Table II). GSH-enhanced PGE formation could be demonstrated in the homogenates of coronary microvessel and of isolated and cultured RCME cells, but not in homogenates of rabbit circumflex coronary artery or aorta. PGI_2 synthase could be demonstrated in all of the vascular preparations. No evidence for 6-keto-$PGF_{1\alpha}$ or GSH-enhanced PGE formation by control incubations with rabbit serum albumin or boiled RCME homogenates could be demonstrated (Table II).

Release of immunoreactive 6-keto-$PGF_{1\alpha}$ and PGE by isolated coronary microvessels and RCME cells. Isolated coronary microvessels and cultured (primaries through sixth passage) RCME cells released detectable amounts of 6-keto-$PGF_{1\alpha}$ and PGE into the incubation buffer (PBS). Under basal conditions (i.e., in the absence of drugs), release of PGE exceeded that of 6-keto-$PGF_{1\alpha}$. Freshly isolated microvessels released 0.99±0.13 pg 6-keto-$PGF_{1\alpha}$/mg wet wt per 15 min and 10±2.31 pg PGE/mg wet wt per 15 min ($n = 12$). RCME cells also released more PGE than 6-keto-$PGF_{1\alpha}$ (Table III). Release of both PG from the RCME cells could be increased by treatment with AA, or the calcium ionophore A 23187, melittin, and lipopolysaccharide (Table III). We tested a second antiserum directed against PGE_2 purchased from Seragen Inc. (Boston, MA). This antiserum exhibits minimal cross-reactivity with PGA_2. Estimates of PGE release under basal and AA-stimulated conditions with this antiserum were not significantly different from those obtained using Dr. Levine's anti-PGE antiserum, indicating that cross-reacting materials such as PGA are probably not contributing to the high PGE levels observed. Preincubation of the cells for 20 min with 5 μM indomethacin inhibited the release of 6-keto-$PGF_{1\alpha}$ and PGE_2 by 80–85%. We also measured the release of 6-keto-$PGF_{1\alpha}$ and PGE_2 from rings of rabbit circumflex coronary artery, and observed 0.24 ng 6-keto-$PGF_{1\alpha}$/mg wet wt per 15 min and 0.08 ng PGE/mg wet wt per 15 min ($n = 3$) released.

DISCUSSION

In this report, we describe a method for the primary and continuous subculture of microvascular endothe-

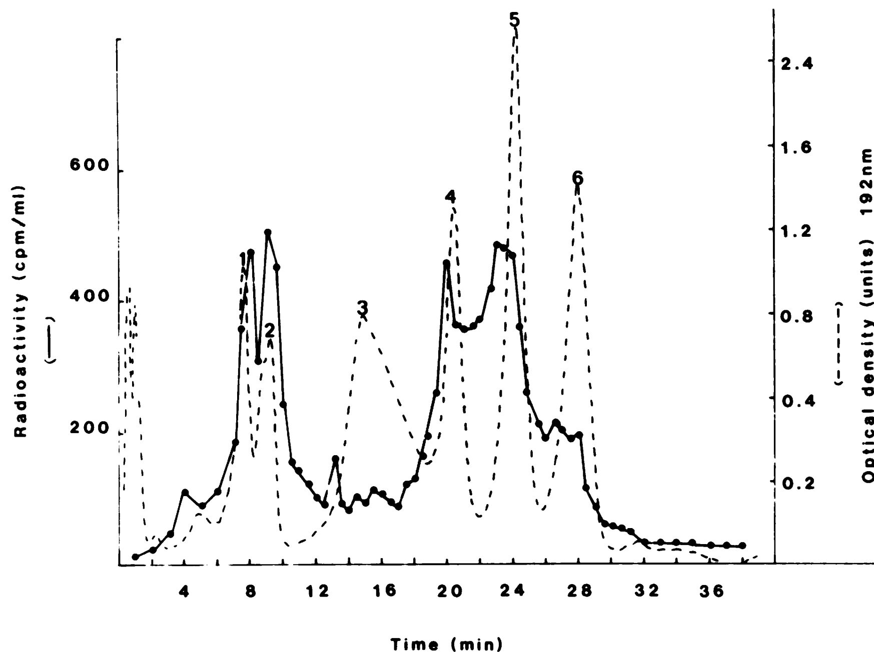

FIGURE 10 Homogenates prepared from RCME cells (250 μg protein) were incubated with 0.5 nmol of [1-^{14}C]PGH_2 in the absence of GSH. After a 10-min incubation at 23°C, products were extracted, buffered, and evaporated in vacuo. The residue was resuspended in acetonitrile and injected in the HPLC system (as described in Methods). Authentic standards were injected along with the sample, and absorption of standards was monitored at 192 nm. Materials eluting from the column were collected in 0.25–0.5-ml fractions, mixed with OCS (New England Nuclear) and radioactivity determined by liquid scintillation counting (- - -) absorption at 192 nm. (●——●) radioactivity, counts per minute per milliliter. Numbers refer to authentic standards: (*1*) 6-keto $PGF_{1\alpha}$, (*2*) 6-keto PGE, (*3*) TxB_2, (*4*) $PGF_{2\alpha}$, (*5*) PGE_2, (*6*) PGD_2.

lial cells derived from the rabbit myocardium. These cultures exhibit many characteristics common to endothelial cells in culture such as the production of Factor VIII antigen and other less specific endothelial markers, including angiotensin-converting enzyme, monolayer morphology, and platelet adhesion-resistant cell surface (31).

The precise origin of the RCME cells cannot be ascertained at this time. The selection for microvascular endothelium is based on the architecture of the coronary vasculature; removal of the epicardial, subepicardial, and endocardial surfaces and large superficial vessels removes potential contamination by mesothelial cells of the epicardium and the endothelium of lymphatics, large vessels, or endocardium (24). Although the bulk of the remaining vasculature would consist of capillaries, endothelium derived from arterioles and venules would contribute to the isolated RCME cells obtained after the isolation procedures outlined in Methods.

Prostaglandin biosynthesis by RCME cells was monitored by several procedures: endogenous release (as determined by radioimmunoassay), incorporation and metabolism of exogenous substrate ([1-^{14}C]AA), and enzymatic transformations of [1-^{14}C]PGH_2 by broken cell preparations. The results demonstrate the ability of these cells to synthesize and release products derived from the PGI_2 synthase (6-keto-$PGF_{1\alpha}$ and possibly 6-keto-PGE_1) and PGH-PGE isomerase (PGE_2) pathways of PGH_2 metabolism. These pathways could be demonstrated in isolated coronary microvessels, freshly isolated RCME cells, and cultured RCME cells, indicating that adaptation to growth under culture conditions did not lead to the expression of new or alternate patterns of PG production.

The generation of the compound tentatively identified as 6-keto-PGE_1 from PGI_2 (or 6-keto-$PGF_{1\alpha}$) described in this report is the first indication of microvascular PGI_2 metabolism. The identity of 6-keto-PGE_1 was established by the identical thin-layer chromatographic behavior of the unknown compound with authentic 6-keto-PGE_1 in two solvent systems, by its conversion to a less polar compound upon acid treatment, and by co-elution of the radiolabeled metabolite and 6-keto-PGE_1 by HPLC. In addition, the enzyme that converts PGI_2 or 6-keto-$PGF_{1\alpha}$ to 6-keto-PGE_1,

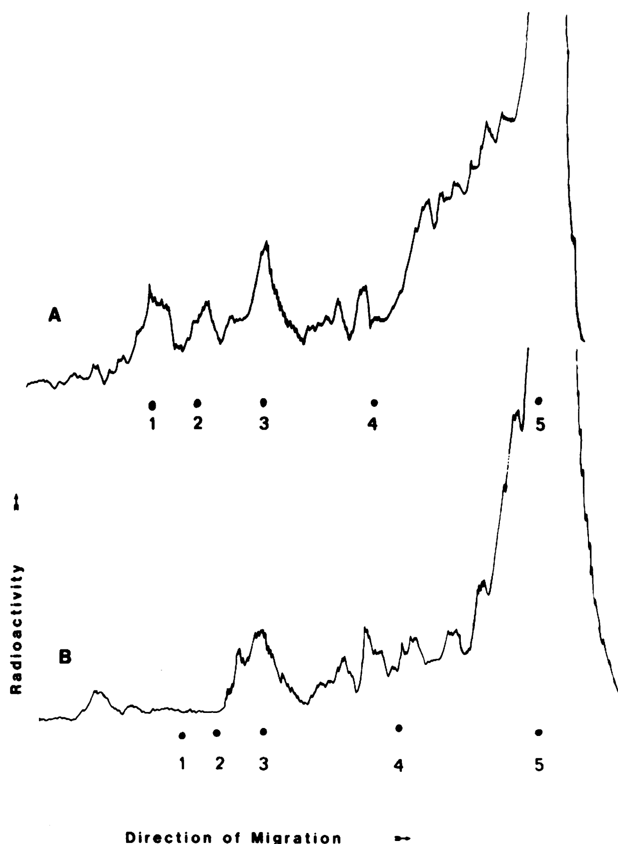

FIGURE 11 Representative radiochromatogram scan obtained from incubation of RCME cells with (*A*) [1-^{14}C]arachidonic acid and (*B*)[1-^{14}C]eicosatrienoic acid (0.2 μCi, 54.5 mCi/mmol) under identical conditions as described in Fig. 7. Migration of authentic standards indicated as (*1*) 6-keto $PGF_{1\alpha}$, (*2*) $PGF_{2\alpha}$, (*3*) PGE_2, (*4*) PGD_2, (*5*) AA. The 1-series PG (PGE_1, $PGF_{1\alpha}$, etc.) exhibits identical migration characteristics as the 2-series counterparts in this solvent system.

9-OH PGDH, was identified in broken cell preparations of coronary microvessels and RCME cells, and the activity of this enzyme was sufficient to account for the amount of 6-keto-PGE_1 formed. The 6-keto-PGE_1 was not formed from 5,8,11-eicosatrienoic acid. This fatty acid is not a substrate for PGI_2 synthase, and thus provides further evidence for the formation of 6-keto-PGE_1. However, absolute identification of 6-keto-PGE_1 as authentic 6-keto-PGE_1 will have to await gas chromatography-mass spectrometry analysis.

Basal release of immunoreactive PGE exceeded that of 6-keto-$PGF_{1\alpha}$ from both RCME cell and isolated coronary microvessels. In contrast, basal release of 6-keto-$PGF_{1\alpha}$ from rings of circumflex coronary artery exceeded that of PGE. Incubation with AA or A 23187 resulted in a significant increase in the release of both prostaglandins from the RCME cells, with the production of PGE significantly greater than that of 6-keto-$PGF_{1\alpha}$. Measurement of 6-keto-$PGF_{1\alpha}$ as an index of PGI_2 synthase activity probably underestimates the products of this pathway, owing to the formation of 6-keto-PGE_1, which would not be determined with the 6-keto-$PGF_{1\alpha}$ antisera. Independent measurements of 6-keto-PGE_1 formation ([1-^{14}C]AA and PGH_2 metabolism, 9-OH PGDH activity) indicate that under basal conditions measurements of 6-keto-PGE_1 formation underestimates total products of PGI_2 synthase by 10–50%.

Recent studies by a number of investigators (38–41) have indicated that vascular smooth muscle and endothelium from several sources have the ability to synthesize both PGE_2 and PGI_2, and that the ratio of these products may alter when the cellular environment changes. For example, adaptation and growth of pig aorta smooth muscle and endothelial cells in culture results in an apparent loss of PGI_2 synthase activity and an augmentation of PGE_2 formation (39–41). Similar alterations may occur with aging. Chang et al. (40) found that the predominant prostaglandin formed by aortic smooth muscle cells from young rats was PGI_2, whereas that of older rats was PGE_2. Augmentation of one pathway vs. another may be a feature of environmental adaptation in large vessel and microvascular endothelium; indeed, the synthesis and release of PGE_2 may be directly related to the redox state of the endothelial cell. Furthermore, PGE synthesis may be augmented after insult, injury, or disease (42).

Interestingly, several other investigators have reported that PGI_2 is the major prostaglandin produced by the heart. As the majority of endothelium perfused in intact hearts is of the microvascular compartment, and the principal source of prostaglandins released by perfused hearts is thought to be of vascular origin (11, 43), it is perhaps surprising that PGE_2 is the primary AA metabolite synthesized by the rabbit coronary microvascular endothelium. There are several explanations for these apparent discrepancies. A study by Nowak et al. (44) monitored the cardiac synthesis of infused [1-^{14}C]AA in man. These authors concluded that 6-keto-$PGF_{1\alpha}$ constituted the major [1-^{14}C]-prostaglandin formed. However, these authors observed substantial amounts of products co-migrating with 13,14-dihydro-15-keto-PGE_2, PGE_2, and $PGF_{2\alpha}$. The identity of the putative PGE_2 metabolite was not investigated further, although this unidentified product constituted 32% of the total ^{14}C-PG, while [^{14}C]6-keto-$PGF_{1\alpha}$ levels were 23% of the total. Another problem complicating interpretation of this study is the presence of 9-keto-prostaglandin reductase in blood (45) capable of reducing PGE_2 to $PGF_{2\alpha}$. Other studies (5, 46) have monitored release of prostaglandins from isolated perfused hearts. An elegant study by DeDeckere and co-workers (46)

TABLE III
Release of Immunoreactive PGE and 6-keto-PGE^{1α} from RCME Cells

Group	Incubation conditions	6-keto-$PGF_{1\alpha}$ released	PGE released
		pg/15 min/10⁵ cells	
1	PBS, no drugs added	36±1	1,065±95
2	A 23187, 2.5 μM	113±20*	3,933±946*
3	A 23187, 5.0 μM	152±7*	5,033±916*
4	AA, 3μM	708±35*	6,650±660*
5	AA, 30 μM	1,316±76*	29,400±2,637*
6	Melittin, 0.1 μg/ml.	89±5*	835±100
7	Melittin, 10 μg/ml.	1,190±43*	2,816±542*
8	Lipopolysaccharide, 100 μg/ml	124±15*	1,800±167*

* $P < 0.05$ from group 1, t test for nonpaired values, $n = 6$. Buffer blanks containing the drugs (e.g., A23187 and AA) used in the incubations were also analyzed by radioimmunoassay for immunoreactive 6-keto-$PGF_{1\alpha}$ and PGE activity. Blank levels fell below the detection limit of the radioimmunoassay (i.e., 2 pg/tube) for all drug concentrations used. Cells used for the above study were from the first passage after primary culture.

underlines the need for careful evaluation of such investigations. DeDeckere et al. monitored release of prostanoids by a combination of biological activity profiles and gas chromatography from two components of the outflow of perfusate from the rabbit heart—that ejecting from the right ventricle (Qrv) and a small proportion escaping the interstitium and dripping from the heart (Qi). Qrv (representing material released into the circulation by the coronary macro- and microvasculature) levels of PGI_2 synthase products (PGI_2 and 6-keto-$PGF_{1\alpha}$) were similar to those of PGE_2. In contrast, Qi (representative of contributions from lymphatics, pericardium, mesothelial cells) levels of PGI_2 synthase products were 16-fold that of PGE_2. These observations strongly support our findings, indicating that release of PGE_2 by the microvascular compartment contributes to the total venous effluent of the heart. In addition, these results indicate that studies monitoring perfusate allowed to drip over the surface of isolated heart after ejection from the right ventricle, and including that perfusate escaping the interstitium, do not accurately reflect coronary vascular PGI_2 and PGE_2 synthesis. A recent study by Coene et al. (47) indicated that substantial amounts of PGI_2 are elaborated from the pericardium, pleural membranes, and derived mesothelial cells; thus, small amounts of adherent membranes or mesothelial cells on the surface of perfused hearts may further complicate interpretation of these studies.

It cannot be ruled out, however, that injury of the microvascular endothelium during isolation and subsequent cultivation may result in some inactivation of PGI_2 synthase activity. However, the PGE_2 isomerase activity was demonstrated in isolated microvessels as well as microvascular endothelium, whereas no evidence of such activity was indicated in large coronary vessels or aortae from the rabbit. PGE release from isolated coronary microvessels and microvascular endothelium exceeded that of 6-keto-$PGF_{1\alpha}$. In contrast, 6-keto-$PGF_{1\alpha}$ release from the isolated circumflex coronary artery of the rabbit was greater than that of PGE_2. Addition of injury-related substances such as lipopolysaccharide and melittin elicited similar increases in both 6-keto-$PGF_{1\alpha}$ and PGE_2 from RCME cells, indicating that short-term responses to injury result in the elaboration of both prostaglandins.

It is intriguing to speculate about the function of PGI_2 and PGE_2 synthesis by the microvascular endothelium. Perhaps the most reasonable role is revealed by the specific biological activities of these two prostanoids. A major action of PGI_2 synthesis and release would be directed at the platelet; PGI_2 is a potent antiaggregatory agent, whereas PGE_2 exhibits weak or opposite actions to that of PGI_2 on platelet behavior (48). Indeed, several studies have suggested in vivo influences of PGI_2 on platelet aggregation in both the macro- and microcirculation (49–51). A second major function regulated or modulated by products of the AA cascade is that of vascular reactivity. PGI_2 and PGE_2 can directly increase arteriolar diameter, while inhibition of PG synthesis can lead to reduced skeletal muscle vasodilitation induced by exercise or ischemia (51–53). PGE_2, but not PGI_2, can reduce the vasoconstrictor response to norepinephrine in the cremaster microcirculation (54, 55). A third action of both prostaglandins, the inhibition of norepinephrine release from sympathetic nerves (56–58) may be important in the local control of blood flow. Prostaglandins also exert other influences on the microcirculation, such as the alteration of erythrocyte deformability (59, 60) and capillary permeability (61).

In conclusion, we have demonstrated that coronary microvascular endothelium from the rabbit can be isolated and grown in primary and subsequent subculture. These endothelial cells have the ability to synthesize PGI_2 and PGE_2. These observations strongly suggest that a major source of the PGI_2 synthase and PGH-PHE isomerase activities observed in isolated microvessel preparations is the microvascular endothelium. The microvascular endothelium also exhibits the capacity to convert a small proportion of PGI_2 or 6-keto-$PGF_{1\alpha}$ to a compound tentatively identified as 6-keto-PGE_1. The role of these AA metabolites in the physiology of the coronary microcirculation is poorly understood at this time. The isolated and cultivated RCME cells should provide a useful model for further studies concerned with the regulation of and roles of AA metabolism in the coronary microcirculation.

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