Passage State Affects Arachidonic Acid Content and Eicosanoid Release in Porcine Aortic Endothelial Cells

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Porcine aortic endothelial cells were cultured through four passages from primary cultures. The arachidonic acid content of individual phospholipid classes and the release of 6-keto-prostaglandin F\textsubscript{1\alpha} and 15-hydroxyeicosatetraenoic acid in response to 1 \textmu M ionophore A23187 were assayed at each passage. The content of arachidonic acid in phosphatidylinositol and diacyl phosphatidylethanolamine remained constant at passage 1 but declined at passage 2 by approximately 29% and at passage 4 by approximately 59%. The release of 6-keto-prostaglandin F\textsubscript{1\alpha} was also unchanged at passage 1 but decreased by 60% at passage 2 and by 82% from its original value at passage 4. In contrast, the arachidonic acid content of diacyl phosphatidylycholine and of alkenyl phosphatidylethanolamine decreased with each passage, by 34% at passage 1, 59% at passage 2, 71% at passage 3, and 76% of the original value at passage 4. Stimulation with arachidonic acid reversed the passage effect. The release of 15-hydroxyeicosatetraenoic acid decreased by 82% at passage 1 and diminished to a 97% decrement from the original value by passage 4. When stimulated with arachidonic acid, 15-hydroxyeicosatetraenoic acid steadily decreased by approximately 70% at passages 3 and 4. The data indicate that passage state strikingly and nonuniformly affects phospholipid class arachidonic acid content and eicosanoid release in response to agonist stimulation. (Arteriosclerosis and Thrombosis 1991;11:167-173)

Endothelial cells possess the ability to release metabolites of arachidonic acid, including prostacyclin (PGI\textsubscript{2}) and 15-hydroxyeicosatetraenoic acid (15-HETE). PGI\textsubscript{2}, a product of the cyclooxygenase pathway, is a powerful vasodilator and a potent inhibitor of platelet aggregation. Its release provides at least one mechanism for maintaining a nonthrombogenic vascular surface. 15-HETE is the monohydroxylated metabolite of arachidonic acid produced by 15-lipoxygenase. The role of 15-HETE as a vascular autacoid has not been fully established, but it does modulate cellular lipid metabolism, influencing phospholipase A\textsubscript{2} activity,\textsuperscript{1} leukotriene synthesis,\textsuperscript{2} and PGI\textsubscript{2} synthesis.\textsuperscript{3-5}

Cultured endothelial cells provide investigators with a model that is both pure in cell type and easily manipulated. Previous studies have used cells over a wide variety of passage states ranging from primary cultures to 20th-passage cells. These studies have placed little emphasis on the influence of passage state on the capacity of endothelial cell culture to synthesize and release eicosanoids. In this study, we have examined the influence of cell passage on the release of PGI\textsubscript{2}, measured as 6-keto-prostaglandin F\textsubscript{1\alpha}(PGF\textsubscript{1\alpha}) and 15-HETE from cultured porcine aortic endothelial cells (PAECs) stimulated by the ionophore A23187. The arachidonic acid content of specific phospholipid classes was also measured. The present report demonstrates that cell passage differentially alters 6-keto-PGF\textsubscript{1\alpha} and 15-HETE production and causes nonuniform changes in the arachidonic acid content of specific phospholipid classes.

Methods

Isolation and Culture of Porcine Aortic Endothelial Cells

PAECs were isolated and cultured as previously reported\textsuperscript{6,7} based on modifications of established techniques.\textsuperscript{8,9} Briefly, freshly isolated porcine thoracic aortas were obtained from a local abattoir and
transported in \(N\)-2-hydroxyethylpiperazine-\(N'\)-2-ethansulfonic acid (HEPES)-buffered Hanks' balanced salt solution (pH 7.4; Sigma Chemical Co., St. Louis, Mo.) containing 100 \(\mu\)g/ml penicillin G and 100 \(\mu\)g/ml streptomycin (Sigma). Aortas were rinsed in the above solution and cut longitudinally. A single scrape of a sterile razor blade removed the endothelial lining, which was dispersed by incubating the scrapings for 20 minutes in 3 ml of a 0.1% solution of collagenase (type 1, Sigma) in phosphate-buffered saline (pH 7.4) containing 1 g/l glucose and 1% minimum essential amino acids (GIBCO, Grand Island, N.Y.). The resultant cell suspension was centrifuged at 3000g for 10 minutes, the supernatant fluid was removed, and the pellet was resuspended in 5 ml Dulbecco's modified Eagle's medium (DMEM, Sigma) containing the above antibiotics and 10% plasma-derived porcine serum (HyClone Laboratories, Logan, Utah). This suspension was plated onto 60×15-mm gelatin-coated dishes. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO\(_2/\)95% air. After 24 hours, the medium was changed to DMEM, antibiotics as above, and 10% fetal bovine serum (complete medium; J.R. Scientific, Irvine, Calif.). The medium from then on was changed every 2 days. All experiments were conducted 24 hours after a medium change. At confluence, the monolayers displayed typical cobblestone morphology and stained positively for the factor VIII-related antigen and the acetylated low density lipoprotein (LDL) receptor. Only those cells achieving confluence within 5–6 days of isolation were used for further study. These were defined as passage 0.

Subsequent passages were achieved by exposing confluent cultures to 2 ml 0.5% trypsin (type V, Sigma) and 0.2% EDTA in \(\text{Ca}^{++}\),\(\text{Mg}^{++}\)-free Hanks' balanced salt solution (pH 7.4) after a rinse in Hanks' balanced salt solution. Trypsin was inhibited by the addition of a fivefold excess (10 ml) of complete medium. The split ratio was a constant 1:4. Subsequent confluencies were achieved within 3–4 days of subculture. Media were changed every 48 hours. Only unstimulated cultures were used for propagation.

**Stimulation of Porcine Aortic Endothelial Cells**

A23187 (1 \(\mu\)M) was prepared from a concentrated stock solution in dimethyl sulfoxide and dissolved in DMEM containing 0.05 mg/ml bovine serum albumin (BSA, fraction V, Sigma). Arachidonic acid (10 \(\mu\)M) (NuChek Prep, Inc., Elysian, Minn.) was prepared from concentrated stock solution in ethanol and dissolved by vortex mixing in the DMEM/albumin mixture as above. Five milliliters of the solution was applied to 60-mm dishes of confluent monolayers and incubated at 37°C. At times from 1 to 30 minutes, 400-\(\mu\)l samples of medium were removed and frozen for subsequent analysis of eicosanoids. Corrections were made for volumes removed. The kinetics of release of 6-keto-PGF\(_{1\alpha}\) and 15-HETE were not affected by passage (data not shown). Only the absolute amounts of metabolites released were affected by passage. The release of all products was complete by 30 minutes; therefore, this time point was chosen for comparisons of the effect of passage state on eicosanoid release.

**Radioimmunoassay**

6-keto-PGF\(_{1\alpha}\) and 15-HETE were measured by radioimmunoassay in the medium samples removed in the above time course. Antiserum to 6-keto-PGF\(_{1\alpha}\) (from Lawrence Levine, Brandeis University, Waltham, Mass.) displayed less than 5% cross-reactivity to any major prostanoid tested. Standard curves were performed with DMEM plus 0.05 mg/ml BSA. Other details of the assay have been reported elsewhere.\(^7,10\) Antiserum to 15-HETE (Advanced Magnetics, Cambridge, Mass.) displayed less than 5% cross-reactivity to related eicosanoids. A standard curve was performed in DMEM plus 0.05 mg/ml BSA. Other aspects of the assays were completed according to the manufacturer's instructions.

**Phospholipid Analyses**

Lipids were extracted from confluent cultures of PAECs, and major phospholipid classes were separated as previously described.\(^6,11\) Individual phospholipid fractions were assayed for phosphorus\(^12\) after hexane/water extractions (three times, 1:1, vol/vol) to remove solvent phosphorus.

**Fatty Acid Analysis**

Individual phospholipid classes were isolated by high-performance liquid chromatography as previously described.\(^6\) Fatty acid methyl esters were prepared by heating the individual phospholipids (with an internal standard, 17:0; added for quantification of mass) at 90°C for 1 hour in 6% acetyl chloride in methanol. After addition of a second internal standard to monitor recovery (15:0 methyl ester), the preparation was dried under \(\text{N}_2\) and redissolved in hexane, and an aliquot was injected into a Perkin-Elmer Model 3500 gas chromatograph (Perkin-Elmer Corp., Norwalk, Conn.). The chromatograph was fitted with a 6-ft glass column packed with 10% SP2330 on 100/120 Chromosorb W AW (Supelco, Inc., Bellefonte, Pa.), using helium as a carrier gas. Flame ionization detection was used. The areas under the curves were integrated with a Shimadzu integrator, Shimadzu Corp., Kyoto, Japan. The oven temperature was programmed from 160°C to 220°C at a heating rate of 5°C/min with a hold at 220°C for 10 minutes.

Diacyl- and 1-O-alkenyl-2-acylphosphatidylethanolamine (diacyl PE, alkyl PE) as well as diacyl- and 1-O-alkyl-2-acylphosphatidylcholine (diacyl PC, alkyl PC) fractions were separated by the formation of diradylglycerol ether acetates as previously described\(^11\) with subsequent thin-layer chromatography on silica gel G plates. The plates were developed in hexane/diethyl ether (60:40) for 7 cm and dried and redeveloped in
FIGURE 1. Line plot showing effect of passage (0–4) on the arachidonic acid content (% AA) of cultured porcine aortic endothelial cells (PAECs). PAECs were cultured through four passages as described in “Methods.” Total lipid extracts were transmethylated and analyzed for AA content by gas chromatography using 17:0 and 15:0 methyl ester as internal standards. Data are expressed as percent AA compared with passage 0 cultures. Mean±SEM, n=5.

The arachidonic acid content of PI and diacyl PE is shown in Figure 2A. The initial arachidonic acid level in PI (passage 0) was 206.8±13.3 pmol/10⁵ cells (29.6±1.9 mole %). The levels of arachidonic acid declined with passage continually and linearly, such that the level at passage 4 was reduced by 56.1% compared with the original. Diacyl PE levels of

the same direction with toluene. Spots were visualized with I₂ vapor (diacyl \( R_f = 0.38 \); 1-O-alkyl \( R_f = 0.43 \); 1-O-alkenyl \( R_f = 0.48 \)). The spots were scraped and the silica gel extracted in hexane/water (3:1).

Total Protein Assay

Protein assays were performed as previously described.¹

Cell Counting

Cell counts were routinely performed manually after trypsinization of the sample by the hemacytometer method. All data have been standardized to values derived from 10⁵ cells.

Results

Throughout all stages of this study, confluent cultures of PAECs exhibited no changes in outward morphology or growth characteristics. Consecutive cultures maintained identical levels of total cell protein (17.55±0.13 µg/10⁵ cells; passage 0 value; mean±SEM, n=5), the same level of total lipid phosphorus (1.210±0.030 µg lipid phosphorus/10⁵ cells; passage 0 value; mean±SEM, n=5), and identical distributions of phosphorus among the cellular phospholipids (percent of total; passage 0 value; mean±SEM, n=5): phosphatidylcholine, 53.4±4.8; phosphatidylethanolamine, 25.5±3.2; phosphatidylinositol (PI), 5.5±0.7; phosphatidylserine, 4.0±0.5; and others including sphingomyelin, cardiolipin, and lysocompounds, 11.6±1.3.

The level of arachidonic acid, the common precursor of 6-keto-PGF₁α and 15-HETE, was measured in various phospholipids and expressed as a function of passage. The change in the total amount of arachidonic acid is shown in Figure 1. Initial passage 0 cultures contained 2,550.1±252.2 pmol/10⁵ cells (17.9±1.8 mole %; mean±SEM, n=3). This level was reduced by 21.8% within one passage and was reduced further by 36.3%, 49.2%, and 66.0% in consecutive passages. The pattern of changes in the levels of arachidonic acid in various phospholipid classes as a function of passage is shown in Figure 2.
Arachidonic acid did not change from passage 0 (384.2 pmol/10⁵ cells; 26.7±2.7 mole %) to passage 1 but abruptly dropped 30.0% at passage 2 and had a total decrease of 60.3% at passage 4.

The arachidonic acid content of diacyl PC and alkanyl PE is shown in Figure 2B. The initial passage 0 level of arachidonic acid in diacyl PC was 826.0 pmol/10⁵ cells (14.0±1.4 mole %). This level was diminished by 36.4% of the passage 0 content at passage 1, by 59.3% at passage 2, by 72.1% at passage 3, and by 75.7% at passage 4. The arachidonic acid content of alkanyl PE was 609.5 pmol/10⁵ cells (38.5±2.6 mole %) at passage 0 but declined sharply by 30.1%, 58.7%, 66.8%, and 74.3% in consecutive passages.

In data not shown, the level of arachidonic acid in phosphatidylserine remained relatively low throughout the passages: 39.6 pmol/10⁵ cells (7.8±2.3 mole %) at passage 0 to 4.6±2.1 mole % at passage 4, a decrement of 41.0%. The arachidonic acid content of alkyl PC, by contrast, rose with continued passage. From an initial value of 179.2 pmol/10⁵ cells (28.0±2.9 mole %), the level increased with consecutive passage by 106.8%, 153.6%, 153.2%, and 159.6%.

Arachidonic acid levels in the culture medium with serum supplements (at 10% levels) were 11.6±0.5 M and 15.2±0.5 µM for fetal bovine and plasma-derived porcine serum, respectively. This medium is sufficiently enriched in arachidonic acid to prevent the appearance of the deficiency triene 20:3n9,13 which was present only in trace amounts in cellular lipid analyses and never exceeded levels found in the serum itself. In general, an increase in saturated and monounsaturated fatty acids compensated for the loss of arachidonic acid. Detailed fatty acid analyses are available on request from the authors.

Primary cultures of PAECs released large amounts of 6-keto-PGF₁α in response to the ionophore A23187 as shown in Figure 3. This level of production was maintained after the first passage. At the second and third passages, 6-keto-PGF₁α production was reduced by approximately 60% and by the fourth passage had diminished by approximately 82% from its initial value.

Passage effects on 6-keto-PGF₁α release were abated when 10 µM arachidonic acid was used as an agonist. Expressed as the mean±SEM (n=3 individual aortic preparations), values from consecutive passages were 278.6±67.1, 257.6±21.6, 233.5±39.1, 243.1±18.0, and 268.9±19.2 pg/ml/10⁵ cells (30-minute incubations).

15-HETE release by primary cultures in response to A23187 did not differ from that of 6-keto-PGF₁α (Figure 3). The pattern of release in subsequent passages, however, was strikingly different. After a single passage, release of 15-HETE was diminished by 82% compared with that in passage 0. In subsequent cultures, net 15-HETE release had further diminished, such that a 97.3% reduction from the initial level was observed by passage 4 cultures.

Exogenous arachidonic acid as an agonist did not reverse the passage effects on 15-HETE release. 15-HETE levels in consecutive passages after arachidonic acid stimulation for 30 minutes were 660.2±57.3, 511.7±32.3, 439.5±75.5, 198.1±29.1, and 217.4±11.4 pg/ml/10⁵ cells (mean±SEM, n=3 individual aortic preparations).

**Discussion**

In the present study, we have demonstrated that the level of arachidonic acid in PAECs is lowered as the cultures are passed. These findings are in agreement with other authors who have studied the effects of cell culture conditions on fatty acid levels within the cells of interest. We observed an overall lowering of the arachidonic acid content in the major phospholipids, but the rates and patterns of the loss within the individual phospholipid class or subclass were distinctly different. Concomitantly, the passage state strongly and differentially influenced the metabolism and release of two major eicosanoids.

Few previous reports have demonstrated such a striking capacity for endothelial cells to release 15-
HETE. Setty and Stuart,5 using human umbilical artery endothelial cell membrane fractions, observed approximately 545 pg/10 min/10^6 cells on an equivalent cellular protein basis. Equivalent measures of 6-keto-PGF_1α release from that study were 5,413 pg/10 min/10^6 cells, yielding a 15-HETE/6-keto-PGF_1α ratio of 0.10. Mayer et al4 found approximately 10,090 pg 15-HETE/10 min/10^6 cells and 13,260 pg 6-keto-PGF_1α/10 min/10^6 cells (0.76 ratio) for bovine aortic endothelial cell homogenates based on similar protein adjustments using 10 μM A23187 as an agonist. Our data, adjusted for total volume, show a release of 6,905.3 pg 15-HETE/10 min/10^6 cells and 8,639.5 pg 15-HETE/30 min/10^6 cells from primary cultures of PAECs. Similarly, we found a release of 6,477.0 pg 6-keto-PGF_1α/10 min/10^6 cells and 9,327.5 pg 6-keto-PGF_1α/30 min/10^6 cells (0.80 and 0.93 ratios for 10 minutes and 30 minutes, respectively). Our values cited previously are derived from incubations using 1 μM A23187 as the agonist, which in our experience provided the greatest general eicosanoid release of any compound tested. Our data are in good agreement with these two studies. Other investigators17,18 have reported metabolite ratios of 0.10–0.48 that appear to be dependent on the species, type of endothelial cell, preparation, and stimulation.

The pattern of decrease in 6-keto-PGF_1α release closely resembles the pattern of loss of arachidonic content in the two diacyl phospholipids, PI and diacyl PE. In contrast, the pattern of decrease in 15-HETE release closely parallels the pattern of arachidonic acid loss from diacyl PE and 1-O-alkeny1 PE. We have previously shown that in fourth-passage PAECs prelabeled with 1-[14C]arachidonic acid, stimulation with A23187 led to a rapid loss of radiolabel from PI and diacyl PE.6 This time course coincided with the appearance of radioactivity in the 6-keto-PGF_1α and several other cyclooxygenase-derived products. Later in the time course, significant losses of [14C]arachidonic acid from alkanyl PE and diacyl PC corresponded to the appearance of 15-HETE and other minor lipoxigenase products. We have suggested that certain phospholipid pools of arachidonic acid may be linked to one or the other oxygenating enzymes, that is, PI and diacyl PE to cyclooxygenase and alkanyl PE and diacyl PC to lipoxigenase. Our current findings support this view.

Our lipid phosphorus data are in good agreement with previous investigations15,16,19 that have examined other sources of endothelial cells in primary culture. Our finding, that no change in the distribution and absolute amount of lipid phosphorus occurs as a function of passage, indicates that changes in arachidonic acid content of phospholipid classes represents remodeling of the classes and not net change in phospholipid mass.

That arachidonic acid exists in pools specialized for eicosanoid release and eventual metabolism has been studied previously in endothelial cells. Although the authors have not always agreed on the phospholipid source of arachidonic acid, they agree that specialized pools are probable. Using PAECs prelabeled with [3H]arachidonic acid and [14C]stearic acid, Hong and Deykin25 concluded that ionophore A23187 and bradykinin stimulation elicited arachidonic acid release from PI and that this release resulted in PG1₂ production. Thomas and colleagues,21-23 performing similar experiments with cultured human umbilical vein endothelial cells, proposed that A23187 induced release of arachidonic acid selectively from phosphatidylcholine and suggested that this release was the important phospholipase A₂ activity leading to eventual PG₁₂ production. They observed little change in PI arachidonic acid levels, however. We have hypothesized that arachidonic acid released from diacyl PE and from PI was used by cyclooxygenase and that arachidonic acid originating from diacyl PC and alkanyl PE gave rise to 15-HETE.6 In contrast, Colard et al24 observed that agonist-induced release of arachidonic acid in platelets did not proceed directly from diacyl PC but was preceded by a transfer of arachidonic acid from diacyl PC to primarily diacyl PE before release.

We have previously described a coenzyme A–independent transfer of arachidonic acid from diacyl PC to alkanyl PE and diacyl PE in platelets.25 This transfer, a direct transacylation, occurs after the apparent action of phospholipase A₁ that forms a 2-lyso PE compound as the acceptor. In the platelet, this occurs only with agonist stimulation, but in the cultured endothelial cell, the action appears to be continuous,11 perhaps because of stimulating substances in the fetal calf serum of the growth medium. In this study, we observed an apparently similar coupling of diacyl PC and alkanyl PE. If the content of arachidonic acid found in both phospholipids at each passage is converted to the percent of arachidonic acid at passage 0, then the decrease in each phospholipid agrees point for point, suggesting a tightly coupled mechanism, that may serve as the precursor reservoir for HETE formation. It is of interest that in the platelet, we have found that exogenous arachidonic acid cannot serve as a source for transacylation.

A decrease in substrate (arachidonic acid) may not be the only mechanism involved in decreased release of eicosanoids. The potential exists for a decrease in the synthesis, expression, or activity of endothelial cyclooxygenase, PG₁₂ synthetase, and 15-lipoxygenase that would limit metabolite elaboration. Many changes in cellular characteristics have been reported to be influenced by the passage state of endothelial cells. These include the expression of heparin and heparin-like molecules26 and angiotensin converting enzyme activity.27,28 Moreover, cation levels and fluxes have been shown to be altered by passage.29 Fry et al30 reported decreases in prostaglandin production from cultured adult human aortic endothelial cells and vena cava endothelial cells with passage. Also noted were increases in thrombin-induced platelet adherence, whereas cyclo-
oxygenase antigen levels diminished. MacIntyre et al\(^{31}\) reported that PAECs in culture produced diminishing amounts of PGI\(_2\), but that incubation in cell-free plasma or with high levels of arachidonic acid restored production. Indeed, research from our laboratory has previously shown that even the time in culture may deplete arachidonic acid levels and prostaglandin synthesis in methylcholanthrene-transformed mouse fibroblasts.\(^{14}\) Since the present study uses a contact-inhibited monolayer culture, a more complex mechanism than arachidonic acid starvation is required to explain our findings. In the present study, we were able to reverse the effect of passage on 6-keto-PGF\(_{1\alpha}\) production with additional arachidonic acid, although at a reduced absolute level when compared with that produced by A23187 stimulation. This may represent the introduction of arachidonic acid into a miscible pool, available for cyclooxygenation, but distinct from a more active pool coupled to a specific phospholipase. The additional substrate/agonist, however, did not reverse the passage effect on 15-HETE levels. We are currently exploring the possibility that specific changes in cellular enzyme levels may further explain our data.

Recent evidence indicates that changes occur in endothelial cell function in vivo, using a model that is suggestive of changes occurring with passage. Shimokawa et al\(^{32,33}\) observed that coronary arteries of pigs that have undergone balloon angioplasty and that had not been previously denuded of endothelium. These investigators observed that vessels with regenerated endothelium displayed an impaired inhibition of ergonovine-induced contractions and also a reduced ability to release endothelium-derived relaxing factor in response to serotonin and UK 14304. Pertussis toxin-sensitive G proteins were found to be dysfunctional in regenerated endothelium and may be involved in the above phenomena. These observations indicate the need for further careful examination of the functional capacity of regenerated endothelium and of regenerated (subcultured) cells in general.

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References


KEY WORDS • arachidonic acid • passage state • endothelial cells • eicosanoids • 15-hydroxyeicosatetraenoic acid • 6-keto-prostaglandin F$_1$$_

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