

25-Hydroxycholesterol Increases Eicosanoids and Alters Morphology in Cultured Pulmonary Artery Smooth Muscle and Endothelial Cells

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Abstract—25-Hydroxycholesterol (25-OHC) is an oxidized derivative of cholesterol that has been implicated in the early development of arteriosclerosis. Changes in arterial smooth muscle cell (SMC) migration and proliferation have also been linked to the pathophysiology of arteriosclerosis. SMCs undergo “activation” in response to vascular injury by changing phenotypically and by increasing prostaglandin G/H synthase-2 (PGHS-2) protein levels and eicosanoid release. Activation is thought to be important in atheroma formation and arteriosclerosis progression. 25-OHC induces SMCs to change morphologically, increase PGHS-2, and increase eicosanoid release. Confluent monolayers were treated with 25-OHC (10 μg/mL) or the PGHS-2 inducer interleukin-1β (1 ng/mL) for 18 hours at 37°C. The 18-hour treatment resulted in morphological changes. After uptake of [14C]arachidonic acid, released radiolabeled arachidonic acid products were extracted and chromatographed by both normal and reverse-phase high-performance liquid chromatography systems. 25-OHC–treated cells increased their prostaglandin production, with the major component comigrating with a prostaglandin-E2 standard. HETEs and epoxyeicosatrienoic acids were not affected. Immunoprecipitation analysis of treated and control cell lysates using anti–PGHS-1 and -2 and anti–α-actin primary antibodies indicated PGHS-2 induction over control and no change in contractile proteins. These changes are consistent with SMC activation, which occurs in vascular injury models. The notion that oxysterols can activate vascular SMCs may be important in ultimately understanding the pathophysiology of atheroma formation. (Arterioscler Thromb Vasc Biol. 1999;19:2901-2908.)

Key Words: prostaglandins ■ prostaglandin G/H synthase ■ hydroxyeicosatetraenoic acids ■ α-actin

Vascular endothelial cell (EC) injury and atheroma formation are considered early events in the pathophysiology of arteriosclerosis.1,2 Likewise, changes in arterial smooth muscle cell (SMC) migration, proliferation, and phenotype are considered important parts of the disease process.3–8 The “activation” of SMCs to secretory, growing phenotypes occurs in response to injury early in the development of arteriosclerosis.7,9

Eicosanoids and oxysterols, the oxidized derivatives of cholesterol, are also thought to play a role in the initial stages of arteriosclerosis.1,2,10,11 25-Hydroxycholesterol (25-OHC) is a much-studied oxysterol that is both formed endogenously and found in the diet.12,13 25-OHC is also present in atheromatous plaques.1,14 We recently reported that 25-OHC enhances eicosanoid production in coronary artery ECs by increasing the amount of prostaglandin (PG) G/H synthase-2 (PGHS-2) protein.15 We postulated that 25-OHC–induced prostanoids could modulate vascular tone either indirectly through endothelial prostanoids or directly by an effect on SMCs.

SMC PGHS-2 mRNA and protein are increased after activation in an experimental model of vessel injury, resulting in marked increases in PG production.9 Here, we show that cultures of SMCs treated with the oxysterol 25-OHC exhibit morphological changes, increases in PGHS-2 protein, and increased eicosanoid release that are consistent with the phenomena of SMC activation described in vascular injury models. Furthermore, the effects of 25-OHC on the pulmonary endothelium mirror those previously described for bovine coronary artery endothelium.15 This is the first time that an oxysterol has been shown to activate SMCs in culture.

Methods

Materials

Cell culture media and materials were purchased from GIBCO. 25-OHC, 22(S)-OHC, 20-OHC, indomethacin, A23187, SDS-PAGE reagents, and buffer salts were from Sigma. Secondary antibodies were from Bio-Rad. PG standards, 6-keto-PGF1α, PGE2, thromboxane B2, 11-hydroxyeicosatetraenoic acid (11-HETE), 15-HETE, and 12-hydroxyheptadecatrienoic acid (12-HHT) standards were purchased from Cayman Chemical. Arachidonic acid was obtained from Nu Check. [14C(U)]arachidonic acid (866 mCi/mmol) was from DuPont NEN. Interleukin-1β (IL-1β) was purchased from Boehringer Mannheim. All organic solvents were high-performance liquid chromatography (HPLC) grade and were purchased from...
Burck and Jackson or Sigma. Octadecasyl silica (Bond Elut) extraction columns were obtained from Varian. Anti–PGHS-1 antibody (PG-20) and purified PGHS-1 and -2 proteins were from BioDiagnostics Research. Anti–PGHS-2 antibody used in these experiments was a generous gift from Prof. Jacques Maclouf (Paris, France).

Cell Cultures
Confluent monolayers of rabbit pulmonary artery ECs and rabbit pulmonary artery SMCs were cultured as previously described.\(^{15,17}\) Cells were maintained in fed medium consisting of RPMI-1640 supplemented with 15% FCS, 1 mmol/L L-glutamine, 25 mmol/L HEPES, and 1% (vol/vol) antibiotic-antimycotic and antibiotics (penicillin G, tylosin, nystatin, and gentamicin). Cells were isolated and cultured by a modification of methods previously described.\(^{16,17}\)

Pulmonary arteries were dissected to their most distal ends within the lung, and all branch vessels were ligated at their origin from the main pulmonary artery. The arteries were then removed and placed in RPMI medium containing 25 mmol/L HEPES, 1 mmol/L L-glutamine, and antibiotics for 15 minutes. The rinsed arteries were placed in a Petri dish and covered with collagenase in RPMI supplemented with 1% collagenase type IV solution and trypsin. Generally, the cultures were maintained in feed medium consisting of RPMI-1640 supplemented with 15% FCS, 1 mmol/L L-glutamine, and antibiotics for 15 minutes in an atmosphere of 95% air and 5% CO\(_2\). EC feed medium was replaced daily for the first 3 days and twice weekly thereafter. Cells were then plated onto 25-cm\(^2\) culture flasks coated with 1% gelatin.

Measurement of Arachidonic Acid Metabolites
Eicosanoid production by treated and control cultures was determined by incubating cells with \(^{14}\)C-arachidonic acid and resolving the \(^{14}\)C-metabolites by HPLC. The SMC and EC cultures (75-cm\(^2\) flasks) were incubated at 37°C for 5 minutes, at which time \(10\mu\)mol/L unlabeled arachidonate was added to the flasks. The cells were then incubated at 37°C for 5 minutes, at which time 10 \(\mu\)mol/L A23187 was added. After 10 minutes at 37°C, the cells were mechanically disrupted with a rubber spatula, and the mixture containing the cells and buffer was transferred to a conical centrifuge tube. The \(^{14}\)C-arachidonic acid metabolites were then extracted with octadecasyl silica extraction columns (Varian) as previously described.\(^{15}\)

**High-Performance Liquid Chromatography**
Extracted \(^{14}\)C-arachidonic acid metabolites were identified and quantified by both reverse-phase (RP) and normal-phase (NP) HPLC.\(^{15,17}\) RP-HPLC was used to isolate the PGs, and NP-HPLC was used to isolate the HETEs.

To separate the PGs from the other major eicosanoids and unmetabolized arachidonic acid, an RP-HPLC system with a Nucleosil-C18 column was used. For method 1, solvent A was water and solvent B was 0.01% glacial acetic acid in acetonitrile. The products were eluted by a linear gradient of 50% solvent A in solvent B to 100% solvent B over 40 minutes. Flow rate was 1 mL/min, and absorbance was monitored at 235 nm. Column effluent was collected in fractions, and radioactivity in the fractions was determined by liquid scintillation spectrometry. The retention time of the radioactive peaks was compared in all cases with known eicosanoid standards separated under identical chromatographic conditions.

Fractions corresponding to the peaks containing \(^{14}\)CPGs (retention time of 3 to 10 minutes, method 1) and \(^{14}\)CHETEs (22 to 25 minutes) were collected separately. After evaporation of the acetonitrile under a nitrogen stream and acidification of the pooled fractions, the radioactive metabolites were extracted into cyclohexane/ethyl acetate (50:50). The organic layers containing the separated metabolites were dried under a nitrogen stream, and the residues were stored at \(-40^\circ\)C for further HPLC analysis.

The PG fraction (3 to 10 minutes, method 1) was resolved into its component PGs with the same Nucleosil-C18 column with RP-HPLC, method 2. Method 2 used solvent C (0.025% phosphoric acid in water) and solvent D (acetonitrile). Elution was carried out isocratically over 40 minutes with 31% solvent D in solvent C followed by a 20-minute linear gradient to 100% solvent D and a 10-minute isocratic elution at 100% solvent D. The flow rate was 1 mL/min, and absorbance was monitored at 207 nm. The column effluent was collected in 0.5-mL fractions and analyzed for radioactivity by liquid scintillation spectrometry.

Method 3 is used to separate the HETE fraction (22 to 25 minutes, method 1). A Nucleosil silica (5-mm, 4.6×250-mm) NP-HPLC column was used. For method 3, solvent E was hexane containing 0.1% acetic acid, and solvent F was hexane containing 2% isopropanol and 0.1% acetic acid. A linear gradient of 25% solvent F in solvent E to 100% solvent F over 45 minutes was used. The flow rate was 3 mL/min, and 0.6-mL fractions of the column effluent were collected. Absorbance was monitored for method 3 at 235 nm, and fractions were analyzed for radioactivity.

**Electrophoresis and Immunoprecipitation**
To measure changes in PGHS-1, PGHS-2, and \(\alpha\)-actin protein in M199 medium, SDS-PAGE was performed by the method of Laemmli\(^{18}\) with 12% resolving gels and 4% stacking gels. Samples were prepared for electrophoresis by removing medium from 75-cm\(^2\) tissue culture flasks and washing cell monolayers 3 times with 10 mL HEPES buffer, pH 7.4, at 37°C. Cells were then scraped and pelleted. Cold lysis buffer (1 mL, buffer A) consisting of 10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% (vol/vol) Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecylsulfate, and 0.25 mmol/L PMSF was added to the pelleted SMCs or ECs, and the mixture was incubated at 4°C for 5 minutes. The crude cell lysate was briefly sonicated, then centrifuged at 15 000 g for 15 minutes at 4°C. The supernatant was stored at \(-20^\circ\)C until further use. Protein was determined on all samples by the method of Bradford,\(^{19}\) and the amount of sample in each lane was normalized to protein content of crude cell lysate. Cell lysates were then preclariﬁed with protein A Sepharose (Pierce) for 45 minutes at 4°C, incubated with a 1:100 dilution of primary antibody for 2 hours at 4°C, and then precipitated with protein A Sepharose. The pelleted beads were washed extensively with buffer A, resuspended in 25 \(\mu\)L of sample buffer, boiled 3 minutes, and subjected to SDS-PAGE as above. Immunoprecipitated proteins were visualized with Coomassie blue R-250, Kaleidoscope prestained standards (7000 to 208 000...
Figure 1. Effects of 25-OHC and IL-1β treatment on SMC morphology. Phase-contrast photomicrographs (×10) of monolayers of rabbit pulmonary artery SMCs. A, Vehicle control; B, after treatment with 10 ng/mL IL-1β for 18 hours; and C, after treatment with 10 μg/mL 25-OHC for 18 hours.
Figure 2. Effects of 25-OHC and IL-1β treatment on EC morphology. Phase-contrast photomicrographs (×10) of monolayers of rabbit pulmonary artery ECs. A, Vehicle control; B, after treatment with 10 ng/mL IL-1β for 18 hours; and C, after treatment with 10 μg/mL 25-OHC for 18 hours.
kDa; Bio Rad) were used for molecular-weight determinations. Coomassie-stained gels were quantified, where indicated, by densitometry with an AMBIS gel documentation system.

Results
Morphological changes were observed in both SMCs and ECs treated with 25-OHC. Photomicrographs of control and IL-1β– and 25-OHC–treated SMC cultures are shown in Figure 1. Disorganized orientation, cytoplasmic swelling, and increased vacuolization were noted in the 25-OHC–treated SMC cultures after 18 hours of incubation. No morphological changes were noted in control or IL-1β–treated SMC cultures. Morphological changes of ECs are shown in Figure 2. The 25-OHC–treated ECs show cytoplasmic swelling, increased vacuolization, and membrane changes. These effects were noted after 48 hours of treatment. As in SMC cultures, no morphological changes were noted in the control or IL-1β–treated EC monolayers. In both SMC and EC cultures, the chronic addition of indomethacin (10⁻⁵ mol/L) during 25-OHC treatment did not block the morphological changes noted (data not shown).

[¹⁴C]arachidonic acid metabolism was studied in EC cultures treated with vehicle and 25-OHC. Photomicrographs of control and IL-1β– and 25-OHC–treated SMC cultures are shown in Figure 1. Photographs of control and IL-1β– and 25-OHC–treated SMC cultures are shown in Figure 1. Disorganized orientation, cytoplasmic swelling, and increased vacuolization were noted in the 25-OHC–treated SMC cultures after 18 hours of incubation. No morphological changes were noted in control or IL-1β–treated SMC cultures. Morphological changes of ECs are shown in Figure 2. The 25-OHC–treated ECs show cytoplasmic swelling, increased vacuolization, and membrane changes. These effects were noted after 48 hours of treatment. As in SMC cultures, no morphological changes were noted in the control or IL-1β–treated EC monolayers. In both SMC and EC cultures, the chronic addition of indomethacin (10⁻⁵ mol/L) during 25-OHC treatment did not block the morphological changes noted (data not shown).

[¹⁴C]arachidonic acid metabolism was studied in EC cultures treated with vehicle and 25-OHC and in SMC cultures treated with vehicle, 25-OHC, or IL-1β. Figure 3 shows the separation of the major EC [¹⁴C]eicosanoids by RP-HPLC (method 1). Endothelial 25-OHC treatment results in a large increase in eicosanoid production compared with control cultures. PGs, HHT, and HETEs are all increased, as evidenced by the increase in radioactivity in the PG (3 to 10 minutes’ retention time), HHT (15 to 18 minutes), and HETE (22 to 25 minutes) fractions. No change in epoxyeicosatriene-
treated, and IL-1β–treated EC and SMC lysates were performed with anti–PGHS-1 and -2 specific antibodies. Cellular α-actin, a major SMC-specific protein, was determined by immunoblot analysis to determine whether 25-OHC or IL-1β treatment induced the contractile protein. Figure 7 shows representative immunoblots of the 3 proteins obtained from EC and SMC lysates after treatment with vehicle, 25-OHC, or IL-1β.

PGHS-1 protein content did not change after 25-OHC or IL-1β treatment in either SMCs or ECs. PGHS-2 protein was increased by 25-OHC in both SMC and EC lysates compared with control cultures. IL-1β effects on SMCs and ECs were different. Although IL-1β increased PGHS-2 in ECs, it had no significant effect on PGHS-2 in SMC lysates. α-Actin was determined in SMC cultures only. It increased 39% after IL-1β treatment and 10% after 25-OHC treatment.

Discussion
The migration and proliferation of vascular SMCs is thought to play an important role in the pathogenesis of arteriosclerosis. SMCs are the predominant cell type in atherosclerotic plaques, and their proliferation leads to vascular occlusion. The response of SMCs to vascular injury is considered important in the initial pathophysiological sequence of events leading to atheroma formation. Vascular SMCs respond to injury by changing from a contractile, nongrowing phenotype to a secretory, growing phenotype by a process called activation. PGHS-2 induction and increased eicosanoid release are characteristic of activation. In this article, we report that the oxysterol 25-OHC enhances differential eicosanoid release in rabbit pulmonary artery SMCs and ECs. Furthermore, gross morphological changes are noted, eicosanoid release is enhanced, and PGHS-2 protein contents are increased after oxysterol treatment. All of these effects of 25-OHC treatment on SMCs are consistent with SMC activation. This is the first time that oxysterol-mediated SMC activation has been described.

Eicosanoid production in experimental arteriosclerosis has been investigated in a variety of vascular preparations. The majority of observations suggest that biosynthesis of eicosanoids is reduced in arteriosclerotic vessels. Initially,
newborn rat SMCs, with cellular rounding up and cytoplasmic swelling. Rabbit pulmonary artery ECs also exhibited gross morphological changes after 25-OHC treatment. These changes paralleled those previously described for bovine coronary ECs. EC cytoplasmic swelling and increased vacuolization were noted. 25-OHC effects on endothelial morphology seem to be consistent between species and tissue types. It should be noted that as previously reported, EC cultures were treated for 48 hours with 25-OHC and SMCs were treated for 18 hours. Confluent EC cultures, in our hands, required longer treatment than SMC cultures to elicit the morphological changes described.

25-OHC dramatically increased eicosanoid release in both ECs and SMCs. Eicosanoid production by SMCs increased 4-fold after 25-OHC treatment, primarily reflecting increases in the PG fraction. PGE$_2$ was the predominant PG produced by SMCs, followed by 6-keto-PGF$_{1alpha}$, HHT and HETE production were not noted to be increased above control levels in 25-OHC- or IL-1β-treated SMC cultures. IL-1β, a known inducer of PGHS-2, showed an identical eicosanoid pattern of release to 25-OHC, with a 2-fold increase in total eicosanoids. Both the morphological and eicosanoid-stimulating effects of 25-OHC treatment were found to be dose-dependent (data not shown). 25-OHC was used at 10 µg/mL because this concentration yielded maximal morphological, enzyme, and eicosanoid changes without causing cell death.

Interestingly, chronic addition of indomethacin during 25-OHC treatment had no effect on the phenotypic changes observed but completely blocked the eicosanoid increases noted. This suggests that morphological changes and eicosanoid enhancement mediated by 25-OHC may be independent and perhaps unrelated events.

Because increases in PGHS-2 mRNA and protein are characteristic of SMC activation after injury, we studied PGHS-2 protein in SMC and EC lysates before and after 25-OHC treatment. These results were compared with identical cultures treated with the known PGHS-2 inducer IL-1β. Treatment with 25-OHC results in an increase in PGHS-2 in SMCs and ECs. No change was noted in PGHS-1 protein, the constitutive form of the enzyme. Interestingly, IL-1β treatment increased PGHS-2 only in ECs and not in SMC cultures. This may suggest that the mechanism by which IL-1β increases PGHS-2 in ECs is not present in SMCs.

Because PGs, 11-HETE, 15-HETE, and HHT may be PGHS-derived, we believe that the eicosanoid increases with 25-OHC are due predominantly to PGHS-2 induction. Along these lines, the production of 11- and 15-HETE is greater with PGHS-2 than PGHS-1.55 Because activated SMCs are also characterized by a loss of contractile function and decreases in levels of contractile proteins,7 experiments comparing the content of α-actin in control, IL-1β-treated, and 25-OHC–treated SMCs were performed. IL-1β increased α-actin content in SMCs by 40% over control in our experiments. However, 25-OHC also increased α-actin 10% compared with control SMCs. Thus, in our hands, contractile proteins are not inhibited by 25-OHC but rather are slightly elevated.

In summary, the characteristics of SMC activation secondary to intravascular injury that have been described are observed with 25-OHC treatment: morphological changes,
enhanced eicosanoid release, and increases in PGHS-2. This study indicates that oxysterols may activate SMCs in vitro in a manner similar to that associated with early arteriosclerotic development demonstrated in vivo. We must remember, however, that arteriosclerosis is a chronic-progressive disease. Cell culture experiments, as described here, reflect predominantly acute responses of cell phenotypes that may differ from those occurring in vivo. These findings may be important in understanding the role of oxysterols and related oxidized cholesterol derivatives in the pathophysiology of arteriosclerosis.

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References
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