Sterol Efflux to Apolipoprotein A-I Originates from Caveolin-Rich Microdomains and Potentiates PDGF-Dependent Protein Kinase Activity†

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ABSTRACT: The kinetics of sterol efflux from human aortic smooth muscle cells equilibrated with a [^{3}H]-benzophenone-modified photoactivable free cholesterol analogue (^{3}H-FCBP) did not differ significantly from those labeled with free cholesterol (^{3}H-FC). Trypsin digestion of caveolin cross-linked by photoactivation of FCBP led to association of radiolabel in a single low molecular weight fraction, indicating relative structural homogeneity of caveolin-bound sterol. These findings were used to investigate the organization of sterols in caveolae and the ability of these domains to transfer sterols to apolipoprotein A-I (apo A-I), the major protein of human plasma high-density lipoproteins (HDL). During long-term (4−5 h) incubation with apo A-I, caveolin-associated ^{3}H-FC and ^{3}H-FCBP decreased, in parallel with an increase in apo A-I-associated sterol. Assay of caveolin-associated labeled sterols indicated that caveolae were a major source of sterol lost from the cells during HDL formation. Short-term changes of sterol distribution in caveolae were assayed using platelet-derived growth factor (PDGF). PDGF was without effect on FC efflux in the absence of apo A-I, but when apo A-I was present, PDGF increased FC efflux ~3-fold beyond the efflux rate catalyzed by apo A-I alone. At the same time, caveolin-associated FC decreased, and PDGF-dependent protein kinase activity was stimulated. Parallel results were obtained with ^{3}H-FCBP-equilibrated cells, in which apo A-I potentiated a PDGF-mediated reduction of radiolabel cross-linked to caveolin following photoactivation. These results suggest that sterols within caveolae are mobile and selectively transferred to apo A-I. They also suggest a novel role for sterol efflux in amplifying PDGF-mediated signal transduction.

Caveolae are cell surface microdomains rich in sphingolipids, free cholesterol (FC),[1] the structural protein caveolin, and extracellular receptor kinases including those reactive with platelet-derived growth factor (PDGF), adrenalin, or insulin (*1*). Caveolae also copurify with intermediates of the MAP kinase, protein kinase A, and protein kinase C signaling pathways. Two different roles for caveolae in signal transduction have been proposed. Caveolae may facilitate the assembly of plasma membrane-associated complexes (*2*−*3*). Alternatively, they may represent reservoirs of functionally inactive signaling proteins (*4*). Caveolin-1, the major structural protein of caveolae, is itself a kinase substrate and potential intermediate in one or more signaling pathways (*5*).

Caveolae have also been implicated in the regulation of cellular FC homeostasis (*6*). FC binds to caveolin directly (*3*, *7*−*8*). FC within caveolae, unlike most plasma membrane FC, is accessible both to extracellular cholesterol oxidase and to acceptors such as lipid-poor (pre-β-migrating) HDL and lipid-free apo A-I (*9*−*11*). Within the cell, both newly synthesized and preformed recycling FC are preferentially directed to caveolae (*10*, *12*−*13*). Caveolin was recently identified as a component of the recycling endosomes which return internalized lipoprotein FC to the cell surface (*14*). Finally, the expression of caveolin is sensitively regulated by FC. Loading with FC from low-density lipoprotein upregulated caveolin mRNA and caveolar expression (*15*−*16*) while FC depletion or oxidation inhibited over time the expression of caveolae at the cell surface (*15*, *17*).

Changes in cell FC modify the association of signaling proteins, including PDGF and insulin receptor proteins as well as signaling intermediates with caveolae (*2*, *3*, *18*), but the molecular mechanisms of these changes have not been identified. Caveolae are dynamic membrane microdomains, not discrete organelles, and as a result, definition of the physiological distribution of FC and caveolar proteins in the living cell has been difficult.

Photoactivable lipids can provide unique information on the organization of lipids and proteins in transient or unstable complexes by forming permanent covalent links that can serve to identify the binding sites in such complexes (*19*−*21*). In the present study, a novel FC analogue containing a photoreactive benzophenone group (FCBP) has been syn-

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[1] Abbreviations: apo A-I, apolipoprotein A-1; FC, free cholesterol; FCBP, 22-(*p*-benzoylphenoxy)-23,24-bisnorcholan-5-en-3β-ol; PDGF, platelet-derived growth factor; PL, phospholipid; SMC, smooth muscle cells.

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thesized and used to study the effects of lipid efflux on the distribution of FC between caveolae and noncaveolar membrane microdomains. In parallel studies, advantage was taken of the observation that transport of FC to cell surface caveolae from intracellular pools is strongly inhibited below 18 °C (*13*, *22*). This suggested that decreased temperature, like cross-linking, could be used to limit changes in caveolar sterol distribution during analysis. Human aortic smooth muscle cells (SMC) were used because their kinetics of FC efflux, and the formation of HDL, have been studied in detail (*11*). PDGF-mediated signaling from these cells is also well defined (*23*, *24*).

EXPERIMENTAL PROCEDURES

Synthesis of 22-(p-Benzoylphenoxy)-23,24-bisnorcholan-5-en-3β-ol (FCBP). 3α,5-Cyclo-22-iodo-5α,23,24-bisnorcholan-6β-ol 6-methyl ether, prepared by modifications of the sequence of Partridge et al. (*25*) as previously described (*26*), was subjected to reaction with 4-hydroxybenzophenone under phase transfer conditions, followed by acidic hydrolysis of the *i*-steroid moiety to generate FCBP, mp 146−147 °C. Radiolabeled ^{3}H-FCBP was prepared by analogous reaction of the C_{22} iodide with 3,5-dibromo-4-hydroxybenzophenone (*27*). Catalytic debromination with $^3H_2$ and acidic hydrolysis of the *i*-steroid (NEN Life Sciences, Boston, MA) yielded ^{3}H-FCBP (specific activity 44.5 Ci/mmol). Details of these procedures and complete characterization of all new compounds have been described (*28*). 1,2-^{3}H-FC, also from NEN, had a specific activity of 48.3 Ci/mmol.

Cell Culture. SMC were cultured in smooth muscle cell growth medium (basal medium, BM) (Clonetics, San Diego, CA) supplemented with 2% v/v fetal bovine serum (FBS) (*11*). Rapidly dividing SMC, following serum starvation, respond to PDGF by acute upregulation of tyrosine-dependent protein kinase activities. Confluent cells are much less responsive to PDGF (*29*). To distinguish long-term effects of FC depletion from transient effects mediated by PDGF, two protocols were developed. For confluent cultures, 2×10^5 cells were plated per 3.5 cm dish; the cells were used in individual experiments after 10 days. To obtain cultures responsive to PDGF, 3×10^5 cells were plated and grown for 48 h in BM plus 2% FBS. All dishes were transferred to basal medium without FBS, containing 1 mg/mL high molecular weight dextran (Dextran T-500, Pharmacia, Parsippany, NY) as oncotic agent, for 48 h, prior to use in individual experiments.

To prepare cells labeled isotopically, ^{3}H-FC or ^{3}H-FCBP was added per dish at 37 °C in ethanol to complete medium (*11*). Final ethanol concentration was <0.5% v/v. The cells were incubated with ^{3}H medium for 24−48 h, in the case of FCBP-labeled cells under dark room conditions. Under these conditions, equilibration of the isotope between cellular and medium sterol was complete (*11*).

To exchange cellular FC mass for FCBP in SMC, the total sterol content of FBS was increased 2-fold by slowly adding FCBP (1 mg/mL) in ethanol solution to undiluted serum. FBS enriched with FC was prepared in the same way. Following incubation (30 min, 37 °C) each preparation was diluted to 2−25% v/v FBS in BM and incubated with SMC. In some experiments, ^{3}H-FCBP or ^{3}H-FC was mixed with FCBP or FC, prior to its incorporation into cell culture medium.

Determination of Sterol Efflux and Influx. Human apo A-I, purified from centrifugally isolated, delipidated HDL, was >98% pure by SDS gel electrophoresis (*30*). Cell cultures were washed (twice) with BM and then incubated for up to 5 h at 37 °C in BM with 1 mg/mL dextran. For individual experiments, incubations were carried out in the presence or absence of a saturating concentration of apo A-I (10 μg/mL) to stimulate apo A-I complex formation from cell surface phospholipids (PL) via ATP binding cassette A1 (ABCA1) transporter activity (*11*). After incubation, the medium was collected and centrifuged (1000*g*, 10 min). The supernatant was assayed for its content of ^{3}H-FC or ^{3}H-FCBP. After removal of the incubation medium, the cells were washed, dissolved overnight in 0.1 N NaOH, and extracted with chloroform and methanol. Their sterol and radioactive content was assayed in portions of the chloroform phase. Cell FC mass was measured using cholesterol oxidase (*31*). Under the conditions described the cells contained negligible esterified sterol (<5% total sterol). Sterol efflux from cells equilibrated with either ^{3}H-FC or ^{3}H-FCBP was linear over 5 h. Efflux rates are expressed as percent cell radiolabel transferred to the medium over the same period. Cells labeled to equilibrium with ^{3}H-lipid were incubated at temperatures between 15 and 37 °C. Efflux rates were computed in terms of the Arrhenius plot (ln v vs $1/T$), where T is the absolute temperature (K).

Analysis of Cross-Linked FCBP. To study the effects of sterol efflux to apo A-1 on the caveolin-associated pool, SMC equilibrated with ^{3}H-FCBP were incubated at 37 °C in the presence or absence of apo A-1 under dark room conditions. Following incubation, the medium was collected and transferred to empty cell culture dishes. The cells were washed with PBS. Both medium and cells were cooled on ice while being illuminated for 0.25−1 h under UV light (365 nm) from a distance of 5 cm. There was no change in the level or distribution of ^{3}H-label if photoactivation was extended to 5 h under the same conditions.

To analyze the incorporation of ^{3}H-FCBP into total cell protein, cell suspensions were collected in PBS at 0 °C and then extracted for 24 h at −20 °C with 50 volumes of 2/1 v/v ethanol−diethyl ether and then with diethyl ether (twice) under the same conditions. Additional ethanol−ether or ether extraction released no further label from the precipitate, which was dried on ice under N_2 and dissolved in 20 μL of electrophoresis buffer (pH 7.4) containing 2% SDS and 0.1 M 2-mercaptoethanol. After electrophoresis in the presence of low or high molecular weight protein markers (Rainbow, low and full range, Amersham/ Pharmacia) and electrotransfer to 0.2 μm pore nitrocellulose membrane, caveolin (22 kDa) was identified with polyclonal rabbit anti-caveolin antibody. The intensity of the plus bands, visualized with Supersignal chemiluminescent substrate (Pierce, Rockford, IL), was measured using a Molecular Dynamics scanning densitometer. To determine the distribution of cross-linked ^{3}H-FCBP in total cell extracts, 2 mm strips of each lane were collected and assayed by scintillation counting.

Proteolysis of ^{3}H-FCBP Cross-Linked Caveolin. Digestion was carried out using sequencing grade modified trypsin (Promega, Madison, WI). Immunoprecipitates with caveolin antibody (see below) were washed with 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM $CaCl_2$ and then incubated in the same buffer for 12 h at 37 °C with trypsin (10 μg per

25 μL of sample). After digestion, the product was analyzed by SDS electrophoresis in 16.5% w/v Tris−Tricine polyacrylamide gels (Readigels; Bio-Rad, Richmond, CA) together with low molecular weight protein standards. Extended trypsin digestion (up to 24 h) had no further effect.

Immunofractionation of Sterol−Caveolin Complexes. SMC equilibrated with ^{3}H-FC were incubated with lysis buffer for 40 min at 4 °C (*18*) and passed 15 times through a 27G needle. The lysate was centrifuged (12000 rpm, 10 min) and 2.5 μg of polyclonal caveolin antibody (BD-Transduction Laboratories) added to the pooled supernatant from two 3.5 cm dishes. After incubation (2 h, 4 °C) 25 μL of protein A−agarose (Santa Cruz Biotechnology) was added for 2 h under the same conditions. To measure caveolin-associated ^{3}H-FC, samples were layered over 0.8 mL of 10% sucrose in 10 mM Tris-HCl (pH 7.5) plus 5 mM EDTA. After centrifugation (12000*g*, 6 min) to sediment the beads through the sucrose layer, the upper phase was removed. The infranate was washed with the same volume of Tris−EDTA buffer and removed. Finally, the sucrose layer was removed and ^{3}H-FC associated with the packed beads assayed by liquid scintillation spectrometry. No caveolin was detectable in the supernatant fractions, indicating that antibody binding was complete under the conditions described.

In some experiments cross-linked ^{3}H-FCBP was assayed in immunoprecipitates of caveolin from the postnuclear supernatant of cell homogenates. After photoactivation, the cells were incubated at 4 °C with lysis buffer. Immunoprecipitation with caveolin antibody and determination of cross-linked ^{3}H-FCBP following SDS gel electrophoresis were carried out as described above.

Effects of PDGF. Recombinant PDGF-BB was purchased from CalBiochem, San Diego, CA. ^{3}H-Equilibrated or unlabeled SMC were preincubated with BM-dextran (48 h). PDGF (100 ng mL^{-1}) was then added. To some dishes was added apo A-I (10 μg mL^{-1}) 2 min prior to the addition of PDGF to initiate HDL formation (*11*). Medium was collected at zero time and at intervals up to 30 min following the addition of PDGF. Radioactivity associated with caveolin was assayed following its precipitation from cell lysates with caveolin antibody as described above. Caveolin-associated ^{3}H-FC or ^{3}H-FCBP was determined as described above. In other experiments, after removal of medium from the cells and washing with cold PBS, 100 μL of boiling SDS sample buffer plus 0.1 M mercaptoethanol was added and the cell lysate collected. Cell proteins were fractionated by SDS electrophoresis on 12% w/v gels. Following electrotransfer, tyrosine phosphorylation among cell proteins was quantitated with p-tyrosine monoclonal antibody (PY99, Santa Cruz Biotechnology) using techniques described above.

RESULTS

Synthesis and Properties of FCBP and ^{3}H-FCBP. The structure of the photoactivable FC analogue FCBP is shown in Figure 1A. Figure 1B shows schematically the predicted structure of covalently modified protein following photoactivation, during which the carbonyl carbon atom of the benzophenone had been cross-linked to the α-position of a proximate amino acid (*19*). FCBP was dissolved in ethanol prior to incorporation into cell culture media.

Confluent SMC were equilibrated with ^{3}H-FC or ^{3}H-FCBP. Preliminary experiments established that ^{3}H-FCBP, like ^{3}H-FC, in cells and medium reached complete equilibration within 48 h (*11*). Assays involving FCBP were carried out under dark room conditions. Efflux of both sterols was linear over 5 h and at rates that did not differ significantly (Table 1). The extent of activation by apo A-I over baseline rates and inhibition by sodium vanadate, a marker for caveola-dependent FC efflux (*11*), were also similar.

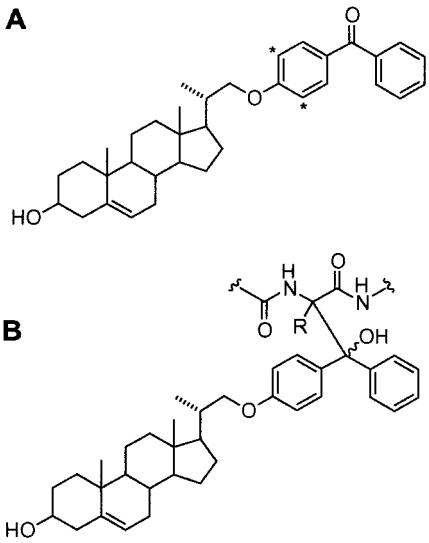

FIGURE 1: (A) Structure of FCBP. Asterisks (*) indicate sites substituted by tritium in ^{3}H-FCBP. (B) Covalent linkage of FCBP to an α-carbon of a peptide.

Table 1: Comparison of ^{3}H-FC and ^{3}H-FCBP as Tracers of Cell Sterol[a]

sterol	efflux (%)[b]	fold activation[c]	inhibition[d]
^{3}H-FC	2.6 ± 0.2	3.1 ± 0.1	75 ± 3
^{3}H-FCBP	2.4 ± 0.5	3.3 ± 0.2	69 ± 1

[a] SMC were labeled to equilibrium (48 h; *11*) with either ^{3}H-FC or ^{3}H-FCBP. Total cell label was (4.0−4.5) × 10^5 dpm in different experiments. The cells were maintained at 37 °C during equilibration and subsequently. After incubation (18 h) with basal medium plus dextran, the cells were changed to fresh medium plus dextran and incubated for 5 h in the presence or absence of apo A-I (10 μg mL^{-1}) and 10 mM sodium orthovanadate solution to inhibit FC efflux (*11*). [b] Apo A-I-dependent efflux was measured as the difference in medium FC label in the presence and absence of apo A-I, expressed as percent of initial cell label. Efflux was linear over the incubation period for both labels. [c] Fold activation is the ratio of medium label in the presence and absence of apo A-I. [d] Inhibition of efflux is expressed as percent reduction of apo A-I stimulated activity in the presence and absence of vanadate. Data shown are means ± 1 SD, $n = 4$.

To determine the effects of replacing FC mass with FCBP, SMC were preincubated (48 h) in medium containing 25% v/v FBS in which free sterol had been increased (×2.0) with FC or with FCBP. In the course of this procedure there was no change in the total sterol content of cells containing FCBP relative to controls (5.0 ± 0.2 μg in each case). However, in the same cells, 1.8 ± 0.1 μg of total sterol was now represented by FCBP, representing a mass ratio (relative to FC) of (1.8)/(5.0 − 1.8) or 0.47. In these experiments, the initial ratio of total FCBP and FC (cells plus medium) was 0.46 ± 0.02. These data indicated that FCBP partially but substantially substituted for FC in SMC and that this was without effect on cellular sterol homeostasis.

Efflux to apo A-I of radioactive sterol from cells containing a FCBP/FC mass ratio of 0.45 fully equilibrated with ^{3}H-

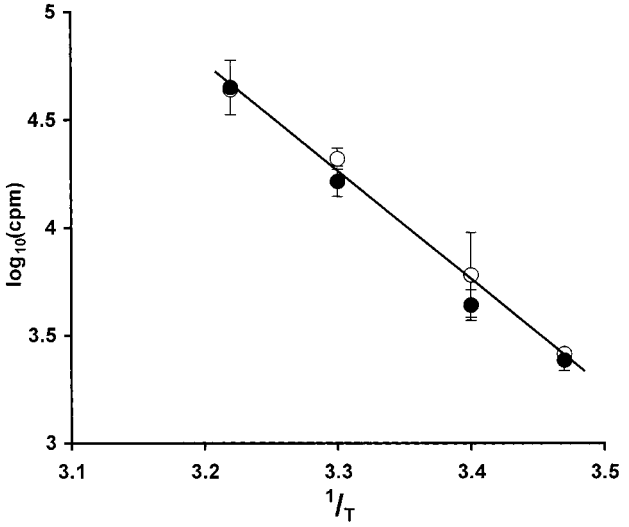

FIGURE 2: Temperature dependence of sterol efflux from cells labeled with ^{3}H-FC (closed circles) or ^{3}H-FCBP (open circles) over the temperature range 16−37 °C. Incubation was for 3 h in the presence of apo A-I (10 μg mL^{-1}). Efflux was linear with time at each point. Background efflux rates [(−) apo A-I] were subtracted, and apo A-1-dependent efflux was expressed as a function of 1/*T*, where *T* is the absolute temperature (K).

FCBP was 2.0 ± 0.2% h^{-1} relative to total cell radioactivity. In parallel experiments, efflux from cells containing tracer levels of ^{3}H-FCBP was 2.5 ± 0.2% h^{-1}. Finally, the temperature dependence of FC and FCBP efflux to apo A-I was compared. The slopes obtained did not differ significantly (Figure 2). Efflux at 16 °C was reduced approximately 10-fold compared to that measured at 37 °C, consistent with previous measurements of the temperature dependence of FC efflux (*13*, *32*). These data also indicated that FCBP, either at tracer levels or replacing FC at a mass ratio of up to 0.47, was transferred out of SMC to apo A-I with kinetics closely resembling those of FC.

Caveolin Cross-Linking to FCBP. SMC equilibrated with ^{3}H-FCBP were exposed to UV light as described under Experimental Procedures. The cells were collected, delipidated, and then fractionated by SDS−polyacrylamide gel electrophoresis. Covalently linked ^{3}H-label was recovered in fractions with MW_{app} of 14−150 kDa. A ∼22 kDa fraction comigrated with caveolin identified by Western blotting (Figure 3A). This peak represented 25.2 ± 3.4% of total label recovered (n = 4). While the presence of additional sterol binding proteins in SMC is not unexpected, further research will be needed to identify these.

To determine the effect of sterol efflux to apo A-I on the pattern of cross-linked proteins, SMC equilibrated with ^{3}H-FCBP were first incubated with apo A-I (10 μg/mL). After 5 h, 3 ± 1% of total cell ^{3}H-FCBP-1 had been transferred to the medium. There was a much larger proportional decrease (−47%, −50%, two experiments) in the radioactivity recovered in the 22 kDa band after SDS gel electrophoresis than in the loss of total cell ^{3}H-FCBP. There was no significant change in label in the other peaks. These data suggested that a 22 kDa protein among those cross-linked to ^{3} H-FCBP contributed disproportionately to sterol efflux in this system.

Postnuclear supernatants from SMC equilibrated with ^{3}H-FCBP were precipitated with caveolin antibody. The level of ^{3}H-FCBP cross-linked to caveolin was determined. The only significant peak of radioactivity recovered in these experiments comigrated during electrophoresis with caveolin (Figure 3B).

Table 2: Comparison of Caveolin-Associated FC and FC Efflux

parameter	18 °C	37 °C
FC efflux (mass)[a]	23.2 ± 2.5	237 ± 4.5
FC efflux (%)[b]	0.31 ± 0.05	3.2 ± 0.1
caveolin-assoc FC (mass)[c]	62.5 ± 8.3	77.2 ± 15.0
caveolin-assoc FC (%)[d]	0.83 ± 0.11	1.03 ± 0.20
decrease in caveolin-assoc FC mass after incubation[e]	18.5 ± 3.2	36.9 ± 5.0
decrease in caveolin-assoc FC (% of efflux)[f]	79 ± 15	16 ± 4

[a] Efflux in the presence of apo A-I is expressed as ng (5 h)$^{-1}$ and was calculated from medium ^{3}H-FC and cell FC specific activity for each experiment. [b] Efflux (%) is expressed relative to total cell FC. [c] Caveolin-associated FC mass (ng) is FC recovered bound to caveolin antibody beads following purification under sucrose as described under Experimental Procedures. [d] Caveolin-associated FC mass as a percent of total cell FC. [e] Difference in caveolin-associated FC (ng) after 5 h incubation in the presence or absence of apo A-I (10 μg mL^{-1}). [f] Decrease in caveolin-associated FC relative to efflux is expressed as the ratio between lines a and e. Values shown are means ± 1 SD (n = 4).

The medium from ^{3}H-FCBP-equilibrated cells previously incubated with apo A-I was delipidated and fractionated by SDS gel electrophoresis. The major peak of label comigrated with authentic apo A-I protein (MW 29 kDa) (Figure 3C).

When the sterol−protein complex obtained by the immunoprecipitation of caveolin from photoactivated, ^{3}H-FCBP-equilibrated cells without apo A-I incubation was digested with trypsin and the product fractionated by SDS gel electrophoresis, >95% of label was recovered in a fraction with MW_{app} of 5 ± 1 kDa (Figure 4). The product was now unreactive by Western blotting with anti-caveolin antibody. Additional experiments using biophysical techniques such as mass spectrometry will be needed to determine more precisely the positions of covalent attachment. However, the present data suggest that cross-links between FCBP and caveolin are formed within a single domain representing 20−25% of the primary sequence.

Origin of FC Transferred to Apo A-I. The experiments above suggested that the ^{3}H-FCBP transferred to apo A-I was preferentially drawn from a caveolin-associated pool. To further investigate this possibility, dishes of SMC equilibrated with ^{3}H-FC were incubated for 5 h in BM plus dextran at 37 °C in the presence or absence of apo A-I (10 μg mL^{-1}). The rate of FC efflux was determined (Table 2). Caveolin-associated FC was assayed following precipitation of cell lysate with caveolin antibody as described under Experimental Procedures. A significant decrease in caveolin-associated FC was measured when apo A-I was included in the incubation medium. As a percentage, this was 5-fold greater than that of FC efflux (16% vs 3%), consistent with the conclusion of some selectivity of FC efflux for caveolar FC. However, the total mass of FC transferred from the cell was much greater than the loss of caveolin-associated FC (Table 2).

The rest of the cellular FC lost from the cell could have originated from noncaveolar membrane domains. Alternatively, it could reflect a partial replenishment, during incubation at 37 °C, of caveolar FC from internal FC pools. To distinguish these possibilities, the immunoprecipitation

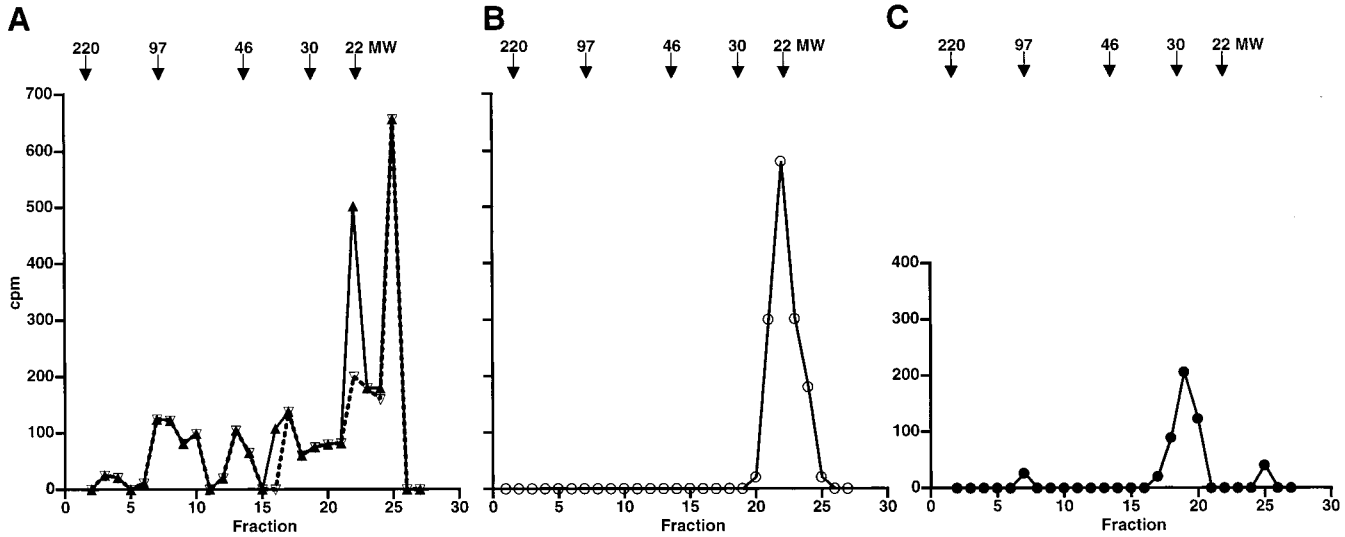

FIGURE 3: SDS gel electrophoresis of fractions from ^{3}H-FCBP-labeled SMC. (A) Whole cell lysate extracted with ethanol−ether after incubation of the cells for 5 h at 37 °C in the presence (open triangles) or absence (closed triangles) of apo A-I. (B) Distribution of cross-linked ^{3}H-FCBP−caveolin in complexes formed from the incubation of SMC lysate with caveolin antibody. The figure shows the presence of a single major species comigrating with caveolin identified by Western blotting. (C) Delipidated medium from the experiment with apo A-I shown in panel A. The migration rates of protein standards of known MW are shown.

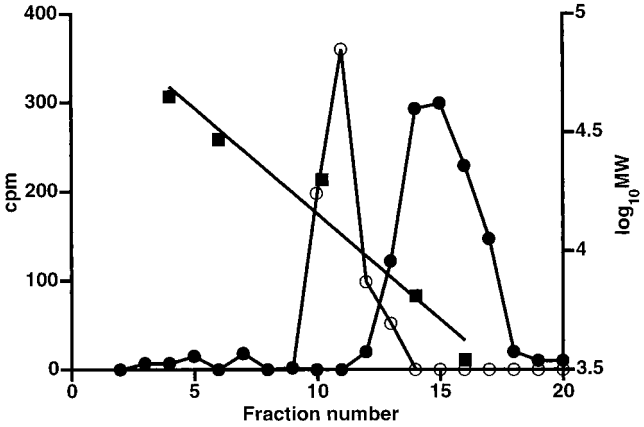

FIGURE 4: Effects of digestion with trypsin on caveolin-bound ^{3}H-FCBP. SDS gel electrophoresis of caveolin (open circles) and trypsin-digested caveolin (closed circles). Also shown (closed squares) are the migration rates of molecular mass standards (range 3.5−45 kDa) in the same experiment. The peak for digested caveolin has an apparent molecular mass of 5 ± 1 kDa (three experiments).

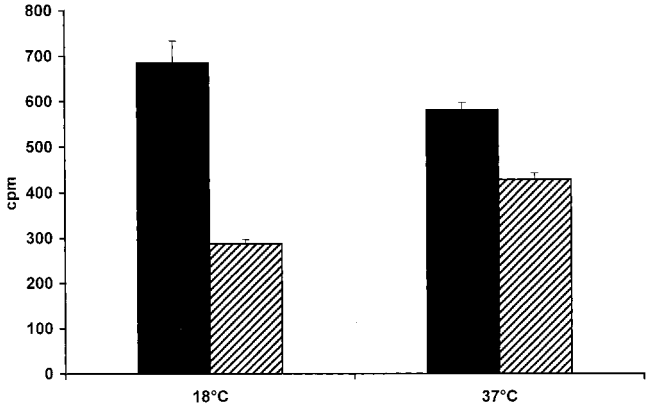

FIGURE 5: Cellular caveolin-bound ^{3}H-FCBP following incubation ± apo A-1 at 18 and 37 °C. Total cell extract was immunoprecipitated with caveolin antibody as described under Experimental Procedures. Key: solid bars, incubation in the absence of apo A-I; hatched bars, incubation in the presence of apo A-I. Rates are means ± 1 SD (n = 4).

and efflux studies were repeated at 18 °C, conditions where intracellular FC transport to caveolae is strongly inhibited (*13*, *22*). Efflux over 5 h was ~10-fold decreased compared to FC released from the cells at 37 °C. At 18 °C, the mass of FC that was caveolin-associated was similar to that measured at 37 °C (Table 2). The difference did not reach significance, suggesting that FC was not redistributed between caveolae and other cellular pools as temperature was reduced. Following incubation with apo A-I for 5 h at 18 °C, the decrease in FC associated with caveolin was equivalent to 79 ± 15% of total efflux (Table 2). These results indicated that, under conditions where replenishment of caveolar FC was inhibited, most FC efflux was associated with a comparable reduction in caveolin-associated FC.

^{3}H-FCBP-equilibrated cells were incubated at 18 or 37 °C for 5 h in the presence or absence of apo A-I, as described above for ^{3}H-FC-labeled SMC, except that after incubation, the cells were photoactivated. Caveolin was immunoprecipitated from the cell extracts as described above (Figure 5). The reductions in caveolin-bound ^{3}H-FCBP after incubation of cells with apo A-I that were measured were similar to those found for caveolin-associated ^{3}H-FC in Table 2. These data are consistent with the hypothesis that apo A-I depleted the caveolin-associated sterol pool and that caveolin-associated FC is replenished at 37 °C.

Effect of PDGF on Caveolin-Associated Sterol and Efflux. The experiments described above assayed chronic effects (5 h) of apo A-I on caveolin-associated FC. To determine if acute changes in the distribution of cell surface sterol involved the same pool, the levels of caveolin-associated FC were assayed 2.5−30 min following the addition of PDGF. Short-term effects of PDGF are considered to be directly related to signal transduction via the kinase activity of PDGF receptor protein (*33*, *34*). Longer term effects reflect the induction of mitosis. The cell surface PDGF receptor is localized mainly to caveolae (*35*). Rapid changes, complete within 10 min, in the association of this receptor with

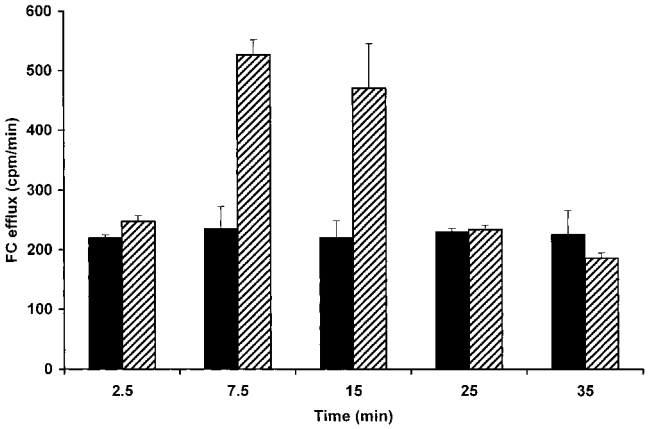

FIGURE 6: Effects of PDGF on efflux from ^{3}H-FC-equilibrated SMC. Rates of efflux were assayed over the time course shown following the addition of apo A-I (10 μg mL^{-1}) in the absence of PDGF (closed bars) or the presence of PDGF (100 ng mL^{-1}) (hatched bars). Efflux was assayed over 5 min periods; the midpoint of each assay period is shown on the horizontal axis. Baseline efflux rates (in the absence of apo A-I) have been subtracted. PDGF was without effect on FC efflux in the absence of apo A-I. Rates are means ± 1 SD (n = 3).

membrane FC have been reported following the addition of PDGF (*18*), and the effects of PDGF on cell protein kinase activities were complete within a similar time frame (*24*).

FC efflux from ^{3}H-FC-equilibrated SMC in the absence of apo A-I was 4−5 ng h^{-1} μg^{-1} of cell FC. This basal rate was unchanged by addition of PDGF (100 ng mL^{-1}). In the presence of apo A-I alone, the rate of FC efflux was increased ~3-fold above baseline (Table 1). When apo A-I was present together with PDGF, FC efflux increased a further 2.9 ± 0.3-fold within 5 min (n = 4) and 2.3 ± 0.3-fold after 10 min. Twenty minutes following addition of PDGF, FC efflux had declined to the rate measured in the presence of apo A-I alone (Figure 6). These data indicate that, in the presence of apo A-I, PDGF acutely stimulated FC efflux.

Effect of PDGF and Apo A-I on Protein Kinase Activity. Protein phosphotyrosine levels in SMC were assayed as a function of time in the presence or absence of apo A-I (10 μg mL^{-1}) and PDGF (100 ng mL^{-1}) by scanning the signal from Western blots of cell extracts incubated with phosphotyrosine antibody (see Experimental Procedures). In these experiments, apo A-I was without significant effect on phosphoprotein levels. Within 10 min, the addition of PDGF was associated with a transient 7−8-fold increase in phosphorylation, but in the presence of both PDGF and apo A-I, protein phosphorylation was increased a further 2.5 ± 0.5-fold (Figure 7).

Effect of PDGF on Caveolin-Associated FC. The effect of PDGF in the presence or absence of apo A-I on caveolin-associated FC was assayed under the conditions described for the estimation of phosphotyrosine levels. Ten minutes after addition of the growth factor to ^{3}H-FC-equilibrated cells at 37 °C, caveolin was precipitated from the postnuclear supernatant fraction. The complex of caveolin with ^{3}H-FC was purified using caveolin antibody, as described under Experimental Procedures.

When apo A-I alone was added to BM, there was little change in FC radioactivity precipitated with caveolin relative to control (Figure 8, panel A). When PDGF was added in the absence of apo A-I, FC associated with caveolin was

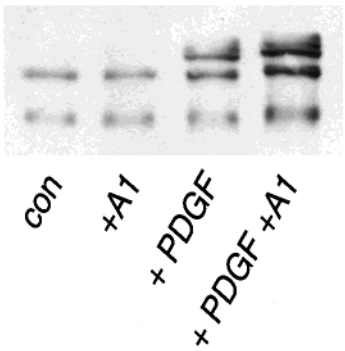

FIGURE 7: Effects of PDGF ± apo A-I on cell protein kinase activity. Subconfluent cells were incubated (10 min, 37 °C) in the presence or absence of apo A-I (10 μg mL^{-1}) and PDGF (100 ng mL^{-1}). The protein phosphotyrosine content of cell extracts was determined following Western blotting with anti-phosphotyrosine antibody as described under Experimental Procedures. Left to right: control cells (no additions); +apo A-I only; +PDGF only; +apo A-I + PDGF.

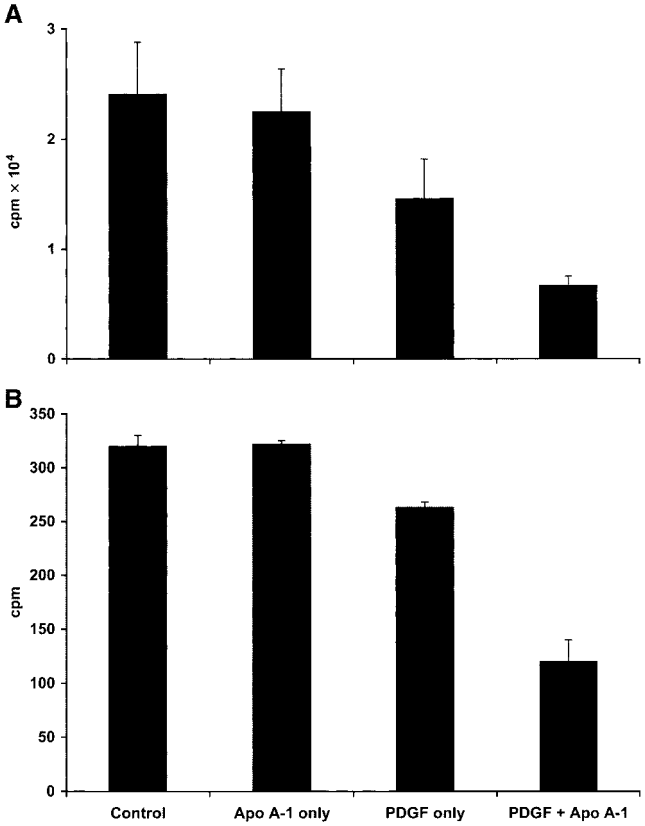

FIGURE 8: (A) Effects of PDGF ± apo A-I on caveolin-associated ^{3}H-FC. Data shown are ^{3}H-FC precipitated with anti-caveolin antibody from cell homogenates prepared as described under Experimental Procedures. Values shown are means ± 1 SD (n = 3). Over the period of incubation (10 min, 37 °C) the effect of apo A-I on caveolin-associated FC was undetectable. (B) Effects of PGFG (100 ng mL^{-1}) ± apo A-I (10 μg mL^{-1}) on caveolin-associated ^{3}H-FCBP. Cells were labeled as described under Experimental Procedures. Following a 10 min incubation with PDGF and apo A-I, cross-linking was carried out for 15 min on ice, and caveolin immunoprecipitated from cell homogenates. The washed product was fractionated by SDS gel electrophoresis. The data shown represent the ^{3}H-label associated with the caveolin band.

significantly lower, though as described above, this was not associated with any increase in ^{3}H-FC efflux into the medium. When PDGF was added together with apo A-I, an

additional 28 ± 3% of caveolin-associated FC was lost. These findings indicate that, following short-term stimulation with PDGF, additional FC efflux was associated with reduced caveolin-associated FC.

SMC equilibrated with ^{3}H-FCBP were incubated with PDFG in the presence or absence of apo A-I and then photoactivated. When the radioactivity associated with caveolin was assayed, a decrease (−16%) in cross-linked ^{3}H-FCBP occurred over 10 min in the presence of PDGF alone. A much greater decrease (−60%) was found when apo A-I and PDGF were present together (Figure 8, panel B). There was no significant change in levels of radioactivity over the same period when apo A-I alone was included.

DISCUSSION

In the present study, the relationships among caveolar sterol, sterol efflux, and PDGF-mediated protein kinase activity were investigated, using both FC itself and FCBP, a photoactivable FC analogue, whose binding to protein can be made permanent by cross-linking. Enzyme−substrate complexes, and the organization of proteins within lipid bilayers, have previously been studied using a variety of photoactivable lipids (*36*, *37*). Recent studies with 6,6′-azo-5α-cholestan-3β-ol ("photocholesterol") showed the practicality of this approach in identifying membrane sterol−protein complexes (*38*−*40*). A key requirement is that the sterol analogue, prior to photoactivation, be without effect on the structure of native biological complexes. The novel photoactivable sterol, FCBP, was used in this research to monitor changes within caveolae during FC efflux which lead to the genesis of HDL. The benzophenone photophore was selected because this group is stable, readily activated by UV light, and efficient and selective for labeling amino acid α-carbon atoms (*19*, *41*−*43*). A 4-methyl-4-azasteroid containing a benzophenone photophore with exactly the same geometric relationship to the steroid nucleus has been used successfully to label steroid 5α-reductase (*44*). Studies of the temperature and apo A-I dependence of FCBP efflux, and its response to vanadate, were very similar to those of FC in each case, indicating that FCBP is an appropriate surrogate for FC in studies of FC homeostasis.

The primary sequence of caveolin contains multiple trypsin cleavage sites within the N- and C-terminal regions. A central hydrophobic domain (∼4.2 kDa), the only extended sequence without trypsin hydrolysis sites, extends between K_{101} and K_{135}. Trypsinization of covalent complexes of FCBP and caveolin generated a labeled peptide of 4−5 kDa. The data presented here are consistent with previous models (*4*, *6*, *45*) in which the aliphatic chain of FC (and, by analogy, the benzophenone group of FCBP) extends parallel to caveolin into the hydrophobic core of the membrane bilayer. Finally, FCBP formed cross-links with caveolin that were resistant to proteolysis, organic solvents, and detergent extraction, confirming that FCBP could be an effective monitor of changes in the association of sterols with caveolin in response to physiological changes.

Cells equilibrated with ^{3}H-FCBP were used to determine if changes in the sterol bound to caveolin accompanied sterol efflux to apo A-I. We took advantage of a previous observation that transport of FC to the cell surface was inhibited at 18 °C (*13*, *22*). If FC lost from the cell originated from caveolae under these conditions, when they cannot be refilled from intracellular pools, the decrease in caveolin-associated FC could be expected to parallel FC total efflux. Sterol efflux and sterol distribution within FCBP- and FC-labeled cells were compared. Studies in the presence of apo A-I with cells equilibrated with either FCBP or FC showed significant decreases in caveolin-associated sterol. Both cross-linking and immunoprecipitation indicated that caveolin-associated sterol was a preferential source of sterol contributed to apo A-I. At 18 °C, nearly 80% of FC efflux was balanced by a comparable reduction in caveolin-associated FC, indicating that the caveolin-associated FC pool is the major contributor to total FC efflux under these conditions. The reduction at 37 °C, while smaller, was still significantly greater than would be predicted if FC had been drawn nonselectively from the plasma membrane.

There has been controversy, recently reviewed (*45*), concerning both the origin of FC reaching HDL and the mechanism of FC efflux to apo A-I. Some studies concluded that the movement of both FC and PL from the membrane bilayer was directly catalyzed by the ABCA1 transporter. This finding was mainly based on the observation that both FC and PL effluxes were deficient in human Tangier disease (congenital ABCA1 deficiency) (*46*, *47*). In contrast, other investigators suggested that the direct product of ABCA1 activity might be a FC-depleted, apo A-I−PL complex, to which FC was added in a second, ABCAI-independent step (*11*, *40*, *48*). Vanadate selectively inhibited FC efflux from SMC without effect on PL; the apo A-I−PL complexes accepted FC from caveolae in the absence of ABCA1 activity (*11*). Caveolin antisense DNA strongly inhibited FC efflux, but not PL efflux, from activated THP-1 human monocytic cells, consistent with the origin of HDL FC from caveolae (*48*). Finally, PL efflux from ABCA1-deficient HEK293 cells was ABCA1-dependent, while FC transfer to preformed apo A-I−PL complexes was ABCA1-independent (*40*).

One study, using immortalized fibroblasts, reported no contribution of caveolae to FC efflux at 37 °C (*49*). The present studies appear to explain these results. At 37 °C, most FC lost from caveolae was rapidly replaced from intracellular pools. In addition, transformed cells, unlike the primary cells used in this study, are generally recognized to contain reduced levels of caveolin and few if any caveolae; caveolin in transformed cells may be largely confined to intracellular vesicles (*50*−*52*). While further research is needed to define the origin of the FC utilized for efflux in different cell models, the data in the present study are fully consistent with the conclusion drawn previously (*11*, *48*) that caveolae can be a significant source of the FC transferred from caveolin binding sites to apo A-I, possibly via scavenger proteins within caveolae, such as SR-BI (*45*). The remaining major component of cellular FC efflux probably reflects simple diffusion (*32*).

Recruitment of PDGF receptor protein to caveolae, and ligand binding, is followed by autophosphorylation and signal transduction and, finally, by dissociation of the PDGF receptor from caveolae (*33*, *53*). The stimulation of protein kinases by FC efflux in the course of sperm capacitation was recently reported (*54*); sperm have been shown to contain significant levels of caveolin (*55*). Cyclodextrin, which nonspecifically depleted cell membrane FC, increased PDGF-mediated signaling (*56*). FC was also recently shown

to be lost from PDGF complexes during signal transduction (*18*), though it was unclear if this decrease reflected displacement of the PDGF receptor from caveolae or delipidation of caveolae as a component of signal transduction.

The present studies indicate that apo A-I can significantly amplify PDGF-dependent protein kinase activity. This effect was coincident with the stimulation of FC efflux from the caveolin-associated pool. Indeed, caveolin-associated sterol levels were lower after 10 min incubation with PDGF plus apo A-I than after 5 h with apo A-I alone. It seems likely that the induction of transient changes in plasma membrane cholesterol content has its physiological basis directly in the stimulation of signal transduction when membrane cholesterol is reduced (*2*, *3*, *12*). In contrast, the increase in cholesterol efflux seen after 24 h exposure to this growth factor (*34*) may be related to the onset of mitosis (*33*).

The present study thus describes for the first time an acute and rapid stimulation of sterol efflux in the presence of PDGF that is associated with an enhanced physiological response. Further research should indicate if lipid-poor HDL, which are present at relatively high concentrations in interstitial fluids (*57*, *58*), play a broader role as general activators of cell surface receptor kinases.

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