Apolipoprotein D and Platelet-Derived Growth Factor-BB Synergism Mediates Vascular Smooth Muscle Cell Migration

Wesley C.Y. Leung, Allan Lawrie, Sandra Demaries, Andrea Burry, Shmuel Yablonsky, Jennifer M. Sarjeant, Euridiki Fera, Eric Rassart, J. Geoffrey Pickering, Marlene Rabinovitch

Abstract—We identified apolipoprotein (apo)D in a search for proteins upregulated in a posttranscriptional manner similar to fibronectin in motile smooth muscle cells (SMCs). To address the function of apoD in SMCs, we cloned a partial apoD cDNA from ovine aortic (Ao) SMCs using RT-PCR. We documented a 2.5-fold increase in apoD protein but no increase in apoD mRNA in Ao SMCs 48 hours after a multiwound migration assay (P<0.01). Confocal microscopy revealed prominent perinuclear and trailing edge expression of apoD in migrating SMCs but not in the confluent monolayer. Stimulation of Ao SMCs with 10 ng/mL platelet-derived growth factor (PDGF)-BB increased apoD protein expression (P<0.05). Moreover, PDGF-BB–stimulated migration of human pulmonary artery SMCs was suppressed by knock-down of apoD using RNAi. Stable overexpression of apoD in Ao SMCs cultured in 10% fetal bovine serum promoted random migration by 62% compared with vector-transfected cells (P<0.01). Overexpression of apoD or addition of exogenous apoD to a rat aortic SMC line (A10) stimulated their migration in response to a subthreshold dose of PDGF-BB (P<0.05). This was unrelated to increased phosphorylation of ERK1/2 or of phospholipase C-γ1, but correlated with enhanced Rac1 activation. This study shows that apoD can be expressed or taken up by SMCs and can regulate their motility in response to growth factors. (Circ Res. 2004;95:000-000.)

Key Words: vascular smooth muscle □ apolipoprotein D □ cell migration □ platelet-derived growth factor □ Rac1

The ductus arteriosus (DA) is a fetal vessel that develops a neointima in late gestation and closes on constriction after birth. Our previous studies related formation of the DA neointima to heightened vascular smooth muscle cell (SMC) migration, which is linked to increased fibronectin synthesis relative to that in SMCs from the aorta (Ao).1–4 Subsequent studies showed that the increase in DA fibronectin synthesis is regulated by enhanced mRNA translation involving an interaction between an mRNA binding protein, identified as light chain 3 (LC3) of microtubule-associated proteins 1A and 1B1 with an AU-rich element (ARE) in the 3′-untranslated region (3′UTR) of fibronectin mRNA. An LC3 protein affinity column was then used to identify mRNAs in which enhanced translation might be similarly regulated in motile SMCs. One of the bound transcripts encoded the 3′UTR of apoD (unpublished data, 2004); therefore, the present study was undertaken to establish whether apoD has a role in SMC motility.

ApoD is a 29- to 30-kDa glycoprotein identified in plasma.5–6 It has a lipocalin structure predicting that it binds small hydrophobic ligands.7 Subsequently, apoD was identified as a carrier molecule with high affinity for steroids such as progesterone, as well as arachidonic acid8 and metabolites that enhance SMC migration.9 ApoD, also a component of HDL, is present in human serum at concentrations of 47 to 155 μg/mL.10 It has been localized to pericytes in developing blood vessels11 and is seen in association with mature blood vessels in a variety of animal tissues,12,13 most recently by our group in human atherosclerotic plaques.14 Induction of apoD occurs in neuronal,15–18 as well as nonneuronal cells19 after injuries that act as stimuli for cell migration. ApoD interacts weakly with the long form of the leptin receptor,20 whose ligand, leptin, promotes SMC migration.21 Moreover, other lipoproteins such as apoJ22 and other lipocalins are associated with cell migration and are prevalent in tissues where active remodeling is taking place.23 It was, however, unknown whether apoD is promigratory either alone or in association with motogenic factors such as platelet-derived growth factor (PDGF).24
We now demonstrate that apoD is associated with SMC migration in fetal lamb DA and aortic (Ao) SMCs and that PDGF-BB can regulate its expression and localization. Moreover, apoD appears necessary for human pulmonary artery (hPA) SMC migration in response to PDGF-BB. We also report in A10 cells, an apod-null SMC line derived from rat aorta, that uptake of apoD or transfection of cells with a plasmid expressing apoD stimulates cell motility in response to a subthreshold dose of PDGF-BB. This synergism is associated with activation of Rac1\(^{25}\) and appears independent of phosphorylation of MAP kinase\(^{26}\) or of phospholipase (PLC)–γ.\(^{27}\)

**Materials and Methods**

**Cell Culture**

Fetal Rambouillet lambs were delivered by Caesarian section on day 100 of a 145-day timed gestation. Ductus arteriosus and Ao were removed en bloc, and SMCs cultured from explants as previously described\(^{24}\) (see expanded Materials and Methods section in the online data supplement available at http://circres.ahajournals.org). In some experiments, cells were maintained in differentiation medium (DM) containing 0.1 μM insulin, 1 nmol/L selenium, 0.1 mmol/L sodium pyruvate, 200 μmol/L ascorbic acid, and 5 mg/mL transferrin with 0.1% BSA.

**Wounding Assays**

A multilayer culture model was used to induce cell migration. Confluent monolayers of ovine Ao, DA, and a rat aortic SMC line (A10) grown on cover slips were wounded with a multitooth scratch comb with 1-mm teeth. Ovine SMCs were cultured in M199/10% fetal bovine serum (FBS) and A10 cells in DMEM/10% FBS for 12, 24, or 48 hours. Preparation of whole cell lysates and cytosolic fractions is described in the online data supplement.

**Immunocytochemistry**

Ductus arteriosus and Ao SMCs at passage 2 were plated on 2-chamber slides at a density of 1.5 × 10⁵ cells/well. To induce migration, half the cells were scraped with a rubber policeman, and the remainder grown for 12, 24, or 48 hours in 10% FBS. For the PDGF-BB studies, subconfluent Ao SMCs were starved in DM for 48 hours and then treated with 10 ng/mL PDGF-BB for 4 hours. At each specified time point, cells were fixed with 100% ice-cold methanol at –20°C and dried at RT. After blocking in 1% BSA/PBS for 30 minutes at 37°C, cells were probed with a monoclonal mouse anti-human apoD antibody (B29) (1:25) for 1 hour. Cells were then washed and incubated with a fluorescein isothiocyanate (FITC)–conjugated goat anti-mouse IgG (1:50) (Jackson Immunoresearch Laboratories) and counterstained with 300 μmol/L 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma) nuclear stain. Confocal microscopy (Zeiss LSM 510 confocal microscope) was performed on 0.35-μm sections of cells using 63× magnification, and representative sections were acquired. Slow, intermediate, and fast migrating cells were classified as being 1, 2, and 3 microscopic fields of view, respectively. Intensity of apoD was assessed semiquantitatively in 10 migrating cells in each field.

**Chemokinetic Migration Assays**

To quantify the effect on rates of migration of apoD overexpression in Ao SMCs, cells were tracked in M199/10%FBS or M199/0.1% BSA using time-lapse video microscopy. As a control, Ao SMCs transfected with pAP2 vector were used. Locomotion was monitored for 6 hours using a ×10 objective attached to a Zeiss Axiosvert 100 inverted microscope equipped with Hoffman Modulation contrast optical filters and a 37°C heated stage. Images were captured with a CCD video camera module attached to a controller. Motility was assessed with Northern Eclipse software. Migration speed was determined as the sum of hourly distances divided by the total time.

**ApoD Expression in Response to PDGF-BB**

Aortic SMCs at passage 2 were grown to 60% to 80% confluence, and then starved in defined media for 48 hours to deplete cells of apoD. The time course of PDGF-BB–mediated apoD induction was determined by treating the cells with 10 ng/mL PDGF-BB for 4, 24, or 48 hours and assessing apoD expression by western immunoblot. The optimal dose of PDGF-BB required to induce apoD expression was determined by treating the cells for 4 hours at 1, 5, 10, and 50 ng/mL PDGF-BB and measuring apoD by Western immunoblot.

**ApoD siRNA SMARTpool Transfection**

Human pulmonary artery smooth muscle cells (hPASMCs) (Cascade Biologics) were seeded at a density of 3.25 × 10⁴ cells in T25 flasks. Transfections were performed using a custom SMARTpool of short interfering RNA oligonucleotides (siRNAs) targeted against apoD mRNA (Dharmacon). As controls, luciferase RNAi as well as cyclophilin RNAi were used (Dharmacon), all in concentrations of 100 nmol/L of siRNA complexed with 5 μL of Lipofectamine 2000 (Invitrogen) in 500 μL of Opti-MEM I (Invitrogen). Knockdown of ApoD was assessed at 24 and 48 hours for both mRNA by TaqMan Q-PCR and protein by Western immunoblotting.

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**Figure 1.** ApoD protein and mRNA levels in response to wounding of SMCs. A, Representative Western immunoblot of apoD protein monitored 48 hours after wounding of Ao and DA SMCs in 10% FBS and in serum free media (0.1% BSA). Graph depicts densitometric analysis with bars representing mean ± SEM of 3 experiments. *P<0.05 vs nonwounded. B, Representative Northern blots of ovine Ao SMCs wounded or nonwounded for 48 hours in the presence of 10% FBS using the [32P]c-CTP labeled ovine apoD cDNA and GAPDH as a control. Densitometric analysis is depicted with bars representing mean ± SEM of 3 experiments. On the RHS, ethidium bromide stained 28S and 18S ribosomal RNA on an agarose gel indicates RNA loading and quality. C, Densitometric analysis of apoD protein by Western immunoblot in wounded vs nonwounded A10 cells in 10% FBS and in serum-free media (0.1% BSA). Bars represent mean ± SEM of 3 experiments. *P<0.05 vs nonwounded.
Boyden Chamber Assays

Boyden chambers (fibronectin-coated PET track-etched membranes with 8-μm pore size) (Falcon) were used for the migration assays. The transfected hPASMCs (3×10^4 cells) were added to the upper chamber, and the lower chamber was filled with 750 μL of DMEM containing 10 ng/mL PDGF-BB. The chambers were incubated for 6 hours at 37°C, 5% CO₂. Cells at the top of the filter were then scraped off with a Q-tip to remove nonmigrating cells, and the remaining cells that migrated to the bottom of the filter were fixed and stained with Diff-Quik (Baxter Scientific Products). The cells were counted in 6 separate fields in each experiment using a light microscope (40× objective) and average values were obtained. In other experiments, apoD-transfected A10 SMCs were grown to 60% to 80% confluence and starved in defined media for 72 hours. PDGF-BB (1, 2, 5, and 10 ng/mL) was plated in the bottom chamber and then 2.5×10^4 A10 cells were added to each upper Boyden chamber, and migration was assessed after a 5-hour incubation. To determine the influence of apoD uptake on SMC migration, untransfected cells were grown to 60% to 80% confluence, and starved in defined media for 72 hours. Then, 10, 100, or 1000 ng/mL human apoD purified from breast fluid form patients with gross cystic disease (supplied by E.R.) were added for 18 hours, and the cells harvested for Boyden chamber assays or for Western immunoblotting to confirm uptake of apoD.

Intracellular Signaling Mechanism

PDGF-BB and ERK1/2, p38, and PLCγ-1

To assess signaling pathways that might be influenced by the interaction between PDGF-BB and apoD, A10 SMCs were pretreated with 100 ng/mL human apoD for 18 hours and then with 1 and 10 ng/mL PDGF-BB for 5, 10, and 30 minutes. Expression of pERK1/2, p38, or PLCγ-1 was assessed by Western immunoblot as detailed in the online data supplement.

Rac 1 Activity Assay

To assay the activity of Rac1, the SMC lysate was affinity purified using a fragment of p21-activated kinase 1 (PAK1) expressed as a fusion protein with glutathione S-transferase (GST). Affinity purified proteins were separated by 15% SDS-PAGE, transferred to a nitrocellulose membrane, immunoblotted with a Rac1 monoclonal antibody (Transduction Laboratories), and detected with anti-mouse peroxidase-conjugated IgG secondary antibody and enhanced chemiluminescence (ECL) detection reagents (Boehringer Mannheim). Rac1 activity was expressed...
the amount of apoD in the cell lysates was reduced and not influenced by wounding (Figure 1A). This suggests that serum is required for increased apoD synthesis or stability in response to wounding of Ao cells or that apoD, which is a component of FBS, is being taken up by the cells in response to wounding. The higher levels of apoD in DA cells imply heightened synthesis by a transcriptional or posttranscriptional mechanism, greater stability of the protein, or greater uptake that is not influenced by wounding. To further address whether induction of synthesis may explain the differences in wounded and nonwounded DA and Ao cells, we monitored apoD mRNA levels by Northern blot. ApoD steady-state mRNA levels were similar and not significantly changed after wounding in the presence of serum in Ao and DA SMCs (Figure 1B).

Western immunoblot analysis revealed that similar to primary ovine Ao SMCs, there was a 2- to 3-fold increase in apoD in wounded versus nonwounded A10 SMCs in 10% FBS (Figure 1C), whereas in 0.1% BSA, apoD levels were very low and no increase occurred after wounding. Because we failed to detect an apoD mRNA hybridization signal using either murine or human apoD cDNA probes (88% and 78% homologous to rat apoD, respectively; unpublished observations, 2004), we speculated that A10 SMCs might take apoD up directly from serum.

ApoD Localization in Migrating Ao SMCs in Response to Wounding
We next investigated where apoD might be localized in migrating versus quiescent cells. Confocal microscopy re-
revealed that in Ao SMCs associated with the confluent monolayer or located close to the wound edge, there was minimal perinuclear staining of apoD (Figure 2A and 2B). However, with increasing distance of migration from the wound edge, immunostaining for apoD was more intense in the perinuclear region and extending toward the rear of the cell, but was relatively absent in the leading lamellipodia (Figure 2C and 2D).

**ApoD Overexpressing Ao SMCs and Chemokinesis**

We next investigated whether apoD overexpression was sufficient to induce chemokinesis. Retroviral induction of stable transfection of apoD in fetal lamb Ao SMCs increased random locomotion by 62% in the presence, but not in the absence of serum as assessed by time-lapse video microscopy (Figure 2, bottom).

**PDGF-BB Induction of apoD Expression in Ao SMCs**

To exclude the possibility that a prochemokinetic or promigratory serum factor might further elevate apoD levels, we measured apoD protein in response to PDGF-BB at different time points after a 48-hour starvation period. A dose of 10 ng/mL PDGF-BB increased apoD at 4 hours in AoSMCs and values returned to basal levels at 48 hours as judged by Western immunoblotting of cytoplasmic extracts (Figure 3A). We also observed a dose-dependent increase in apoD in response to PDGF-BB (1 to 50 ng/mL) at 4 hours (Figure 3B).

**ApoD siRNA and PDGF-BB Mediated hPASMC Migration**

To determine whether induction of elevated apoD was necessary for the promigratory response to PDGF-BB, we transfected hPASMCs with apoD siRNA and produced a 75% and 90% knockdown of apoD mRNA transcripts by Q-PCR at 24 and 48 hours, respectively, relative to control conditions with either luciferase siRNA or cyclophilin siRNA (Figure 4A); no detectable apoD was observed on Western immunoblotting at 48 hour in cells transfected with apoDsi, whereas strong bands were seen under control conditions (not shown). Migration in Boyden chamber assays in response to PDGF (10 ng/mL) was suppressed in cells transfected with apoD siRNA below levels of untransfected control cells, and control cells transfected with luciferase siRNA or cyclophilin siRNA (Figure 4B).

In A10 cells, an increase in apoD was not observed in response to PDGF-BB (data not shown). Thus, it seemed that A10 cells might be useful in determining how PDGF-BB interacts with apoD in inducing motility, independent of influencing endogenous apoD levels. We confirmed apoD overexpression in A10 transfected SMCs by Western immunoblotting and determined the subthreshold (1 ng/mL), submaximal (2 ng/mL), and maximal (10 ng/mL) doses of PDGF-BB that would induce migration (Figure 5A). ApoD-overexpressing A10 SMCs migrated faster at a subthreshold dose of 1 ng/mL PDGF-BB, but not at 2, 5, and 10 ng/mL PDGF-BB, compared with vector-transfected cells (Figure 5B).

Administration of apoD also stimulated migration in response to a subthreshold dose of PDGF-BB (Figure 6A). In the absence of PDGF-BB, there was no migration regardless of the amount of apoD added. At a low dose of PDGF-BB (1 ng/mL), addition of 100 or 1000 ng/mL apoD promoted migration (Figure 6B). At higher doses of PDGF-BB (5 and 10 ng/mL), a lower dose of 10 ng/mL apoD stimulated migration, indicating a dose response synergistic relationship (Figure 6C and 6D).

**ApoD and PDGF Activation of ERK 1/2, PLCγ1, and Rac1**

In different cell types, PDGF-BB–directed chemotaxis has been related to stimulation of activity of MAP kinases (ERK 1/2 and p38), PLCγ1, and Rac1. Application of a dose of apoD (100 ng/mL), which stimulated migration in the presence of a subthreshold dose of PDGF-BB (1 ng/mL) did not, however, influence phosphorylation of ERK 1/2 levels (P44/P42) (Figure 7A) or of PLCγ1. There was greater induction of phosphorylated PLCγ1 with 10 ng/mL when compared with 1 ng/mL PDGF-BB±apoD (100 ng/mL) (Figure 7B). We could not relate apoD potentiation of migration to the induction of p38 MAPK because we found no activated p38 in the presence of PDGF-BB, apoD, or both, whereas unphosphorylated p38 was unchanged (data not shown). Rac1 activation was increased with 10 ng/mL or with 1 ng/mL PDGF-BB+apoD (100 ng/mL) compared with 1 ng/mL PDGF-BB alone (Figure 8), suggesting that cooperative activation of this pathway might account for the apoD-PDGF synergism-inducing motility.
This study focused on the novel observations that apoD, a major component of HDL, is produced and can also be taken up by vascular SMCs and positively influences their migratory behavior. We showed that apoD is present in high concentrations in motile cells such as DA SMCs, and increases in Ao SMCs after stimulation of migration by wounding in association with growth factors such as PDGF-BB. In hPASMCs, apoD is necessary for PDGF-BB–mediated migration. In A10 cells, apoD works in concert with PDGF-BB to stimulate SMC motility by a mechanism that could involve activation of Rac1.

Ovine DA SMCs, which show increased migration in culture in association with DA intimal cushion formation,1–3 have higher levels of apoD protein, but not mRNA levels, when compared with cells from the Ao. ApoD protein levels also increase in Ao SMCs in response to wounding, but steady-state mRNA levels are similar.31 The discrepancy between high protein and unchanged mRNA levels could be related to heightened mRNA translation after transcription of apoD or to greater stability of the protein. Increased efficiency of translation of apoD mRNA is a possibility because LC3 binds to the 3′UTR of apoD mRNA, and because binding of LC3 to the 3′UTR of fibronectin mRNA increases fibronectin mRNA translation and SMC motility.3 The increase in apoD protein with wounding is also observed in neurons in response to injury.17,18 Interestingly, another apolipoprotein, apoJ, is induced in medial and neointimal SMCs in response to balloon injury of rabbit aorta, and stimulates SMC migration and proliferation.22 A similar role was postulated for another lipocalin, extracellular fatty-acid binding protein.23

In data not shown, we confirmed increased immunostaining for apoD in fetal lamb DA versus Ao vascular tissues, and intense apoD immunostaining is observed in atherosclerotic plaques relative to normal coronary artery tissues.14 In some studies, we used A10 cells, a SMC line derived from rat thoracic aorta. A10 cells resemble neointimal cells12 in that they are less differentiated and more migratory than primary SMCs,33 and the signaling mechanisms involved are relatively well characterized in these cells.34 We could not detect apoD mRNA or apoD protein induction in A10 cells, perhaps because of their less differentiated phenotype, consistent with studies in which poorly differentiated carcinomas express low levels of apoD.35

We showed that PDGF-BB, which is released by wounding24 and is a potent inducer of SMC motility,36 is associated with an increase in apoD protein in ovine SMCs. The transient nature of this effect is consistent with the short half-life of PDGF-BB.37 It was of interest then, that we were able to link PDGF-BB–mediated synthesis or stability of apoD in primary hPASMCs to motility, by showing that knock-down of apoD with RNAi suppressed PDGF-BB–mediated motility.

The apparent absence of endogenous apoD made A10 cells useful in assessing the effect on migration of either exogenous apoD or “endogenous” apoD produced after transfection of the cDNA. Transfection of apoD allowed the A10 SMCs to migrate in response to a subthreshold dose of PDGF.38 Because apoD is prevalent in very high concentrations in the plasma, a physiologically relevant question, especially under conditions when there is endothelial injury, is whether adding apoD to the A10 cells in combination with PDGF-BB would have a similar effect to transfecting apoD. Indeed, by titrating exogenous apoD, we were able to show a dose-dependent synergism with a subthreshold dose of PDGF-BB. It is of interest that apoD binds arachidonic acid, because arachidonic acid pretreatment of SMCs enhances FBS- and PDGF-BB–induced migration.9 PDGF-BB also mediates release of arachidonic acid.9

The synergism between apoD and PDGF-BB in inducing SMC motility could not be related to phosphorylation of p38,
ERK 1/2,40 or PLC-γ,41 PLC-γ1 overexpression leads to an enhanced chemotactic response to PDGF-BB in endothelial cells, phosphorylation of p38 was demonstrated in migrating porcine aortic endothelial cells,42 and ERK 1/2 is phosphorylated in response to PDGF-induced chemotaxis in human mesangial cells.43 In colonic epithelial cells, leptin promotes invasiveness via rho- and rac-dependent signaling pathways,44 and overexpression of wild-type Rac in endothelial cells led to increased motility in response to PDGF-BB.45 We did in fact show that activation of Rac1 could be achieved with maximal doses of PDGF-BB, as well as with subthreshold PDGF in combination with apoD. Immunolocalization indicated that apoD is present in the perinuclear region and extending toward the rear end of migrating cells, whereas Rac1 activity is associated with extension of lamellipodia.46 It is possible that apoD is important in translocating a protein that directly influences Rac1 activity. ApoD could also influence retraction of the rear of the cell previously associated with cytoskeletal rearrangement47 related to regulatory molecules such as rhoA, calcineurin, and tyrosine kinases.48 In addition, apoD is associated with protease activity,49 required in breaking cell-matrix attachments in motile cells. This novel functional relationship between apoD and SMC motility, may be relevant to developmental as well as disease processes.

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References


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Cell Culture Conditions

Cells were maintained in Medium 199 (M199; GIBCO) containing 10% heat-inactivated fetal bovine serum (FBS) (Wisent, St. Bruno, QC) and 1% antibiotics/antimycotics (Ab-Am), grown for 4 weeks, and passaged initially by scraping and subsequently using 0.05% trypsin/EDTA. Experiments were conducted with SMC at passages 2 and 3. The rat aortic SMC line A10 (ATCC) was cultured with Dulbecco’s Modified Eagle’s Medium (DMEM) (Cellgro, Hearndon, VA) containing 10% FBS and 1% Ab-Am.

Cloning of ovine apolipoprotein D cDNA

RNA was isolated from Ao SMC lysates using Trizol reagent (GIBCO-BRL), purified according to the Trizol manufacturer’s instructions, and reverse transcribed to create cDNA. Degenerate primers were designed based on a homology search for apo D nucleotide sequences in other species using ClustalW multiple alignment program. The forward (5’-GATGGTACGAAATTGAGAAGATCCC-3’), and reverse (5’-TGGGTGGCCAGGACCCAGTA-3’) primers were used to amplify and internal fragment of apoD gene from the cDNA obtained by RT-PCR. A band of the correct estimated size, ~250 base pair (bp) was excised from the gel, cloned into the pGEM-T vector (Promega) and positive clones were selected. The alignment of DNA and amino acid sequences was performed using ClustalW multiple-alignment software (http://www.ebi.ac.uk/clustalw/) and protein structure predictions were performed using Meta PredictProtein program (http://www.embl-heidelberg.de/predictprotein/submit_meta.html)

Northern blot analysis

RNA was harvested from nonwounded and wounded monolayers of Ao and DA SMC by the Trizol method. RNA samples (15µg) were then loaded and electrophoresed on 1.5% formaldehyde agarose gels. Gels were stained with ethidium bromide to determine the position of the 28S and 18S ribosomal RNA, then transferred overnight to a hybond membrane using capillary action. After transfer, the membrane was ultraviolet (UV)-crosslinked and northern hybridization was performed using a [32P]-dCTP (10^6 cpm/ml, Perkin Elmer Life Sciences) labeled ovine cDNA probe (238 bp) using the Quikhybe method according to manufacturer’s instructions. The membrane was then exposed to phosphoimager or film. A
similarly radiolabeled ‘housekeeping’ glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (1100bp, Clontech, Palo Alto, CA) was used to confirm integrity of RNA and equal RNA loading.

**Western immunoblot analysis**

Protein concentration in cell lysates was determined using bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL) followed by spectrophotometry at 562 nm. Protein extracts from cell lysates (10-20ug) were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 8-16% polyacrylamide tris-glycine gels (Novex, Invitrogen, Carlsbad, CA) and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore, Mississauga, ON). Membranes were washed three times in tris buffered saline-0.1% tween-20 (TBS-T) and blocked overnight in 5% powdered milk/TBS-T at 4°C. Membranes were washed again and incubated with the designated primary antibody for 1h at RT. These included mouse monoclonal anti-human apoD (1:1000-1500), (kindly supplied by Drs. Eric Rassart and Ross Milne, University of Ottawa), mouse monoclonal GFP (Clontech, Palo Alto, CA), rabbit polyclonal antihuman phospho-p44/p42 kinase (1:1000), p44/p42 kinase (1:1000), phospho-p38 kinase (1:1000), p38 kinase (1:1000), phospho-PLC-γ1 (1:1000), PLC-γ1 (1:1000). Membranes were washed and incubated in sheep anti-mouse IgG secondary antibody conjugated to horseradish peroxidase (HRP) at 1:2000-3000 (Amersham Biosciences) (for apoD and GFP) or donkey anti-rabbit (1:2000) (Amersham Biosciences) (for the polyclonal antibodies). The targeted protein was then detected and visualized by enhanced chemiluminescence western blotting detection reagent (PerkinElmer Life Sciences, Woodbridge, ON) according to manufacturer’s instructions.

**Preparation of cell lysates:**

Media was removed and plates were rinsed in PBS. About 100 µl RIPA buffer (1XPBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS with aprotinin and leupeptin) were added to the plates and cells were scraped and transferred to a microcentrifuge tube to which PMSF (0.6 µl of 100 mM) was added. Samples were incubated on ice for 30 min, then centrifuged at 10000g for 10 min at 4°C and the supernatant kept as total cell lysate.
Preparation of cytosolic fraction:
Cells were harvested as described above, spun at 500g at 4°C, resuspended in lysis buffer, freeze-thawed three times, and spun at 10000g for 20 min at 4°C. Supernatants were collected for cytosolic fractions.

Retroviral transfection of smooth muscle cells
The 293GPG retroviral packaging cell line was a generous gift from Dr. Richard Mulligan (Children’s Hospital, Boston, MA). These cells were maintained in 293 media. This packaging cell line is pseudotyped with the vesicular stomatitis virus G (VSVG) envelope protein and synthesizes retroviruses when transfected with the AP2 plasmid (provided by Dr. Gerald Batist, Lady Davis Institute for Medical Research, Montreal, Quebec) which contains a signal to package the plasmid into the retrovirus. The AP2 plasmid was designed to coexpress an inserted cDNA as well as the enhanced green fluorescent protein (EGFP) reporter within a bicistronic framework. To make the apoD construct, the human apoD sequence was isolated from bluescript SK plasmid using EcoRI and BamHI and inserted into the pAP2 plasmid so that it was linked with EGFP expression, generating a pAA plasmid. This plasmid was transfected into the 293 GPG retroviral packaging cell line using Lipofectamine (Gibco). Viral supernatant was collected daily for one week starting three days after transfection, filtered with 0.45 um syringe-mounted filters, and stored at –80°C. Aortic SMC were plated at 4x104 cells per well in a 12-well dish and allowed to adhere. Medium was removed and replaced with 800 µl thawed retrovirus-conditioned medium (containing polybrene at 6 ug/ml final concentration) collected from transiently transfected 293GPG cells. This procedure was repeated daily for three consecutive days. Flow cytometric analysis was performed within two weeks after transduction (typically 10 days after) to ascertain gene transfer efficiency as measured by GFP fluorescence. Live cells were gated based on forward/side scatter profile and analyzed for GFP fluorescence, and GFP positive cells were plated and maintained in DMEM/10% FBS. Western immunoblot was performed to confirm endogenous apoD and GFP expression after incubation in defined media for 48 hours.
**Rac 1 activity assay**

To assay the activity of Rac1, the SMC lysate was affinity purified using a fragment of p21-activated kinase 1 (PAK1) expressed as a fusion protein with glutathione S-transferase (GST) (Bagrodia, 1998). The assay exploits the fact that PAK1, a downstream effector of Rac1, binds Rac1 in only its activated, GTP-bound form (Bagrodia et al, 1998). cDNA from the protein binding domain of PAK1 (PBD, corresponding to amino acids 67-150) was amplified by PCR from human placental cDNA and cloned into the bacterial expression vector pGEX-2T. This was expressed in DH5a E. coli which were lysed by passing through a French pressure cell at 20,000 psi followed by mixing with 1% Triton X-100. Glutathione-Sepharose 4B beads (Sigma) were added and the PBD-GST-bead mixture was suspended in interaction buffer (20 mM Hepes pH 7.4, 150 mM NaCl, 0.05% Nonidet P-40, 10% glycerol, 1mM PMSF, 1 µg/ml leupeptin). After stimulating SMC with the designated treatment, they were washed in ice-cold PBS and lysed with cold interaction buffer. Cell lysate containing 200 µg of protein was incubated with 20 µl of the PBD-GST-bead mixture overnight at 4°C.

**Figure 1: The ovine apoD cDNA cloned ovine cDNA and human apoD cDNA.**

A. A 238 bp ovine cDNA fragment generated using an RT-PCR approach aligned with the human apoD cDNA and identified to be 86% homologous at the nucleotide level using ClustalW multiple-alignment software.

B. The amino acid sequence of the ovine fragment is 80% homologous to the human apoD sequence using ClustalW multiple-alignment software.
Figure S1

A. Apolipoprotein D DNA Sequence

<table>
<thead>
<tr>
<th>Human</th>
<th>Sheep</th>
<th>Start</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAAGTATCTCGAGAGTGGTACGAAATTGAGAAGATCCCAACAACCTTTGAGAATGGACG</td>
<td>--------------GATGGTACGAAATTGAGAAGATCCCAACAACCTTTGAGAATGGACG</td>
<td>240</td>
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<tr>
<td>CTCGATGCCAGGCCAATCTACTCACTAAGAAAAGAGATCTCAAATGTGAACCCAGGA</td>
<td>--------------GATGGTACGAAATTGAGAAGATCCCAACAACCTTTGAGAATGGACG</td>
<td>46</td>
</tr>
<tr>
<td>GTTGAGAGCTGATGGAACTGTGAATCAAATCGAAGGTGAAAGCCACCCAGTTAACCTCAC</td>
<td>--------------GATGGTACGAAATTGAGAAGATCCCAACAACCTTTGAGAATGGACG</td>
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<tr>
<td>GCTGAGAGCTGATGGAACTGTGAATCAAATCGAAGGTGAAAGCCACCCAGTTAACCTCAC</td>
<td>--------------GATGGTACGAAATTGAGAAGATCCCAACAACCTTTGAGAATGGACG</td>
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<tr>
<td>AGAGCCTGCCAAGCTGGGAAGTTAAGTTTTCCTGGTTTATGCCATCGGCACCGTACTGGAT</td>
<td>--------------GATGGTACGAAATTGAGAAGATCCCAACAACCTTTGAGAATGGACG</td>
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<tr>
<td>CCTGGGCACCAAGCTGGGAAGTTAAGTTTTCCTGGTTTATGCCATCGGCACCGTACTGGAT</td>
<td>--------------GATGGTACGAAATTGAGAAGATCCCAACAACCTTTGAGAATGGACG</td>
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</table>

B. Apolipoprotein D Amino Acid Sequence

<table>
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<tr>
<th>Sheep</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>WYEIEKIPVSFEKSCQANYSLKENGNVKVINELRAD</td>
<td><em>TT**N</em>R<em><strong><strong><em><strong>M</strong></em>KI</strong>L</strong></em>**</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sheep</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTVNQIEGEATQENITEPAKLGVKFWF</td>
<td><em>PV</em>L<strong><strong>G</strong></strong>S***</td>
</tr>
</tbody>
</table>