LDL receptor family-dependent and -independent pathways for the internalization and digestion of lipoprotein lipase-associated β-VLDL by rat vascular smooth muscle cells

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Abstract Lipoprotein lipase (LPL) promotes the binding and internalization of β-VLDL (very low density lipoprotein) by many cell types. We examined the function of receptors in the LDL receptor family (LRF) and heparan sulfate proteoglycans (HSPG) in the metabolism of LPL-associated β-VLDL by rat vascular smooth muscle cells (VSMCs) in culture. These cells express LDL receptor-related protein and the VLDL receptor, but not the LDL receptor. LPL greatly increased the binding of ^{125}I-labeled β-VLDL to VSMCs at 4°C. Binding was almost entirely inhibited by heparin, but essentially unaffected by the potent LRF-antagonist, receptor-associated protein (RAP), indicating that LRFs do not contribute significantly to the VSMC binding capacity for LPL-associated β-VLDL. At 37°C, RAP inhibited the rapid internalization of LPL-associated ^{125}I-labeled β-VLDL and the digestion of the β-VLDL into trichloroacetic acid soluble radioactivity; these processes still occurred, but at a decreased rate. RAP did not inhibit the ability of β-VLDL–LPL complex to stimulate VSMC ACAT activity. Furthermore, in Oil red-O histochemistry experiments, which model foam cell transformation in vitro, RAP paradoxically increased cholesteryl ester storage in VSMCs treated with β-VLDL and LPL under specific cell culture conditions. These results support a model in which the internalization of LPL-associated β-VLDL by VSMCs is mediated by two pathways, one involving LRFs and a second that is independent of LRFs, probably involving direct uptake by HSPG. The LRF-dependent pathway leads to less cellular storage of cholesteryl ester and thus may be antiatherogenic under certain conditions.—**Weaver, A. M., J. J. Lysiak, and S. L. Gonias.** LDL receptor family-dependent and -independent pathways for the internalization and digestion of lipoprotein lipase-associated β-VLDL by rat vascular smooth muscle cells. *J. Lipid Res.* 1997. **38:** 1841–1850.

Supplementary key words low density lipoprotein receptor-related protein • VLDL receptor • α_2-macroglobulin • cholesterol • chylomicron remnants • VLDL • heparan sulfate proteoglycans • ACAT • atherosclerosis

Lipoprotein lipase (LPL) is a 110 kDa heparin-binding enzyme that hydrolyzes core triglycerides in circulating chylomicrons and very low density lipoproteins (VLDL) to generate cholesterol-enriched remnants (1). LPL is synthesized by many cell types, including vascular smooth muscle cells (VSMCs) and macrophages, and associates with the vascular endothelium. Increased LPL activity in the arterial wall correlates with increased areas of lipid deposition and increased atherosclerotic lesion formation (2). The activity of LPL in promoting atherosclerosis may depend on its dual role as an enzyme and as a bridging protein that increases the binding of lipoproteins to cell surfaces (1).

The enzymatic activity of LPL is localized within its N-terminal domain. The C-terminal domain of LPL contains binding sites for lipoproteins and for specific cell-surface receptors, including heparan sulfate proteoglycans (HSPG) and members of the LDL receptor family (collectively referred to as LRFs) (3–7). By bridging lipoproteins to a greatly increased number of cell surface binding sites, LPL promotes the internalization of lipoproteins by cells; this LPL function is independent of its enzymatic activity (4, 5).

LRFs which may be involved in the binding and uptake of LPL-associated lipoproteins include the LDL receptor, LDL receptor-related protein (LRP), glycoprotein 330 (gp330), and the VLDL receptor (8, 9). As this is a growing superfamily, other unrecognized LRFs may be involved as well. Internalization of LPL-associated lipoproteins by LRFs may require that the LPL–lipopro-

Abbreviations: LPL, lipoprotein lipase; VLDL, very low density lipoprotein; β-VLDL, chylomicron and VLDL remnants isolated from cholesterol-fed rabbits; HSPG, heparan sulfate proteoglycans; LRFs, receptors in the LDL receptor family; VSMCs, vascular smooth muscle cells; RAP, receptor-associated protein; LRP, low density lipoprotein receptor-related protein; α_2M, α_2-macroglobulin; SFM, serum-free medium; ACAT, acyl-CoA:cholesterol acyltransferase.

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tein complex first bind to cell-surface HSPG (9–12). The LPL–lipoprotein complex is then transferred to the LRF and undergoes rapid endocytosis in clathrin-coated pits and intracellular transport to lysosomes (9). LPL–lipoprotein complexes may also be internalized directly by HSPG without requiring transfer to LRFs (12, 13). The slow endocytosis of cell-surface HSPG and the ability of HSPG to mediate the internalization of associated proteins, such as basic fibroblast growth factor, are well documented (14–17).

The relative activities of LRFs and HSPG in the cellular uptake of LPL-associated lipoproteins are incompletely understood and may depend on the cell type under investigation (11–13, 18). Distinguishing between LRF-dependent and -independent pathways may be important as there is evidence to suggest that the intracellular processing of the LPL–lipoprotein complex depends on the pathway followed. In endothelial cells, LPL that is internalized in association with HSPG is metabolized at a slow rate and may be recycled back to the cell surface and reutilized (19). Presumably, the high affinity of LPL for HSPG at low pH prevents dissociation in endosomes. By contrast, LPL that is internalized by LRFs is degraded in lysosomes (5, 9, 11).

In the liver, the LDL receptor and LRP are the major known LRFs involved in remnant lipoprotein uptake (9, 20). However, in the arterial wall, LRP and the VLDL receptor are more likely to be involved in remnant lipoprotein catabolism. Unlike the LDL receptor, LRP and the VLDL receptor are not down-regulated by cholesterol and have been shown to be present in VSMCs within atherosclerotic lesions from humans and cholesterol-fed rabbits (9, 21–24). Gp330 also functions as a lipoprotein receptor (25); however, this LRF is expressed almost exclusively by epithelial cells and not by VSMCs (26, 27).

Receptor-associated protein (RAP) is a 39 kDa protein which was originally copurified with LRP and serves as an intracellular chaperone for LRP (28–30). RAP binds with high affinity to LRP (K_D of 5–14 nM), gp330 (K_D of 8 nM), and the VLDL receptor (K_D of 0.7 nM) and with low affinity (K_D 250 nM) to the LDL receptor (27, 31–35). When associated with LRFs, RAP blocks the binding of all other known ligands, including LPL, and has been shown to inhibit chylomicron remnant uptake by the liver (8, 9, 28, 36).

In the present study, we utilized RAP to evaluate the function of LRFs and HSPG in the metabolism of LPL-associated β-VLDL by rat VSMCs. β-VLDL is a preparation of cholesterol-enriched chylomicron and VLDL remnants isolated from cholesterol-fed rabbits (37). We considered the analysis of VSMCs to be particularly important because VSMCs, like macrophages, transform into foam cells in atherosclerotic lesions (38, 39). Our results demonstrate that HSPGs contribute the majority of the VSMC binding sites for LPL-associated β-VLDL. RAP partially inhibits internalization and digestion of LPL-associated β-VLDL; however, RAP has no effect on the resulting development of ACAT activity. Furthermore, RAP either has no effect or paradoxically promotes the formation of cholesteryl ester-containing lipid droplets, as determined by Oil red-O histochemistry. These studies demonstrate that two pathways are operational in the metabolism of LPL-associated β-VLDL by VSMCs. The LRF-dependent pathway causes rapid ligand digestion and promotes less cholesteryl ester storage and thus may be antiatherogenic under some circumstances.

MATERIALS AND METHODS

Reagents

Rat RAP was expressed in bacteria as a glutathione *S*-transferase (GST) fusion protein, using a construct kindly provided by Dr. Joachim Herz (Southwestern Medical Center, Dallas). The intact fusion protein was purified as previously described and used without further modification (40). LPL was purified to homogeneity from bovine milk and kindly provided by Dr. Jonathan LaMarre (University of Guelph, Canada). α_2-Macroglobulin (α_2M) was purified from human plasma by the method of Imber and Pizzo (41) and converted into the "activated" or receptor-recognized conformation by reaction with methylamine (42). Activated α_2M was radioiodinated with Iodobeads, as described by the manufacturer (Pierce). Heparin (Grade I-A, purified from porcine intestinal mucosa) was purchased from Sigma (St. Louis, MO).

Vascular smooth muscle cell culture

VSMCs were isolated from Sprague-Dawley rat aortas by enzymatic digestion as previously described (43). Cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's (DME) medium and Ham's F12 medium, supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT) and L-glutamine (0.68 mM). At subconfluence, cells were passaged with 0.05% trypsin, 0.02% EDTA. Cultures were maintained at 37°C in 5% CO_2, 95% air. For experiments, VSMCs were plated at 3×10^3 cells/cm^2 and grown to confluence. The cells were then growth-arrested by culturing for 4 days in defined serum-free medium (SFM) containing a 1:1 mixture of DME and Ham's F12 supplemented with glutamine (0.68 mM), insulin (500 nM), transferrin (5 μg/ml), ascorbic acid (0.2 mM), and selenium (38 nM). This medium has been shown to maintain VSMCs in a quiescent, noncatabolic state and to

promote expression of VSMC-specific contractile proteins (44).

Preparation of β-VLDL

β-VLDL (d < 1.006) was isolated by ultracentrifugation of plasma obtained from rabbits fed a diet containing 2% cholesterol and 10% coconut oil (45) and then radioiodinated by the iodine monochloride method, as previously described (46). Free $Na^{125}I$ was separated from ^{125}I-labeled β-VLDL by chromatography on Sepharose G-25. The protein concentration of the ^{125}I-labeled β-VLDL was determined by the bicinchonic acid (BCA) assay (Sigma). Radioactivity in the β-VLDL preparations was <10% chloroform-extractable.

Western blot analysis

Hybridomas producing monoclonal antibody C7, which recognizes the LDL receptor, and 11H4, which recognizes the 85-kDa LRP light chain, were obtained from the ATCC. The antibodies were raised in ascites fluid and purified by Protein G-Sepharose chromatography. Purified polyclonal antibody which is specific for the VLDL receptor was a kind gift from Dr. Dudley Strickland (American Red Cross, Rockville, MD). Growth-arrested VSMCs were extracted in 1.0% Nonidet P-40, 0.25% sodium deoxycholate, and a cocktail of proteinase inhibitors as previously described (47). As a control, rat hepatocytes were isolated by collagenase perfusion (48) and extracted with the same solution of detergents and proteinase inhibitors. The two preparations were then subjected to SDS-PAGE on 10% slabs and electrotransferred to nitrocellulose membranes. Identical blots were probed with each of the three antibodies. Enhanced chemiluminescence was used to detect antibody binding.

Binding of ^{125}I-labeled β-VLDL to VSMCs

Confluent cultures of VSMCs were washed twice with ice-cold DME, 25 mM HEPES, 10 mg/ml bovine serum albumin, pH 7.4 (DHB buffer) and then incubated with 3 μg/ml ^{125}I-labeled β-VLDL in the same buffer for 4 h at 4°C. In some wells, other reagents added simultaneously with the ^{125}I-labeled β-VLDL, including 5 nM LPL, 180 nM RAP, a 50-fold molar excess of nonradiolabeled β-VLDL, or 100 U/ml heparin. Incubations were terminated by washing the cells four times with ice-cold DME, 25 mM HEPES, pH 7.4. Cell-associated radioactivity was recovered in 1.0 M NaOH and quantitated in a γ-counter. Cellular protein was determined by the BCA assay. Specific and nonspecific binding were distinguished by determining radioligand binding in the presence of a 50-fold molar excess of non-radiolabeled β-VLDL. In some experiments, ^{125}I-labeled β-VLDL–LPL complexes were pre-formed, before addition to cultures, by incubation for 20 min at 37°C. Pre-formed ^{125}I-labeled β-VLDL–LPL complex bound to VSMCs identically to complex that was allowed to form in the culture wells, as expected.

Internalization of LPL-associated ^{125}I-labeled β-VLDL

VSMCs were incubated with 5 nM LPL and 3μg/ml ^{125}I-labeled β-VLDL in EBSS, 25 mM HEPES, 10 mg/ml bovine serum albumin, pH 7.4 (EHB buffer) at 37°C for the indicated periods of time. The cultures were then chilled to 4°C and washed twice with ice-cold EHB buffer. To dissociate cell surface radioligand, the cultures were treated with 10 mg/ml dextran sulfate in EHB for 20 min, washed twice with 2 M NaCl, 20 nM HEPES, pH 7.5, and twice with 2 M NaCl, 20 mM sodium acetate, pH 4.0. Internalized radioligand was then recovered in 1 M NaOH. In control studies, ^{125}I-labeled β-VLDL–LPL complex was incubated with VSMCs for 4 h at 4°C so that cell-associated radioactivity would be restricted to the cell surface. The dextran sulfate/high salt/acid wash protocol was then executed, releasing about 90% of the cell-associated radioactivity.

Digestion of LPL-associated ^{125}I-labeled β-VLDL

VSMCs were incubated with 5 nM LPL and 3 μg/ml ^{125}I-labeled β-VLDL, with or without 180 nM RAP, in EHB for 4 h at 4°C. After washing the cells, fresh EHB was added to each culture. RAP was added back to the cultures that had been pre-incubated with RAP. All of the cultures were then warmed to 37°C. At the specified times, conditioned medium was separated from the cells and cell-associated radioactivity (surface + internalized) was recovered in 1 M NaOH. Conditioned media samples were incubated with 10% trichloroacetic acid (TCA) (final concentration) on ice for 15 min. TCA-soluble and precipitable radioactivity were separated by centrifugation at 9,000 *g* and measured in a gamma counter. All incubations were conducted in the presence and absence of a 50-fold excess of nonradiolabeled β-VLDL. Specific cell-associated and TCA-soluble radioactivity were determined by subtracting that detected in the presence of nonradiolabeled β-VLDL. Specific radioactivity was always greater than 90% of the total.

Cholesteryl oleate formation

Cholesteryl oleate formation was measured as previously described (46). Briefly, quiescent VSMCs were incubated with 5 nM LPL and 3 μg/ml β-VLDL in EHB at 37°C. After 5 h, sodium [^{3}H]oleate (0.2 mM), in complex with albumin, was added to each well and incubated at 37°C for an additional 2 h. Cellular lipids were extracted with hexane–isopropanol 3:2 and recovered by evaporation of the solvent. Cholesteryl esters were separated from other cellular lipids by thin-layer chromatography, identified with iodine vapor, and cut from

the chromatogram. Cholesteryl [^{3}H]oleate formation was quantitated in a scintillation counter.

Histochemical staining with Oil red-O

VSMCs were cultured on chamber slides and incubated with β-VLDL, in the presence and absence of LPL, RAP, or heparin in SFM at 37°C for 24 h. Cells were then washed with PBS, fixed in paraformaldehyde (4% in PBS), and stained with 1.25% Oil red-O in 80% acetic acid and Harris' hematoxylin. Oil red-O stains exclusively triglycerides and cholesteryl esters (49).

RESULTS

LRF expression by cultured VSMCs

We previously demonstrated that rat VSMCs in culture express LRP by Western blot analysis using a polyclonal antibody that recognizes the 515-kDa heavy chain and the 85-kDa light chain (47). **Figure 1** confirms this result and shows that rat hepatocytes also express LRP, as expected (50). The monoclonal antibody, 11H4, which was used to probe the blot shown in Fig. 1, recognizes only the LRP light chain. Interestingly, the level of LRP detected in the VSMC extracts was greater than that detected in an equivalent amount of cellular protein extracted from hepatocytes.

The VLDL receptor was detected as a 115-kDa band in the VSMC extracts but not in the hepatocyte extracts,

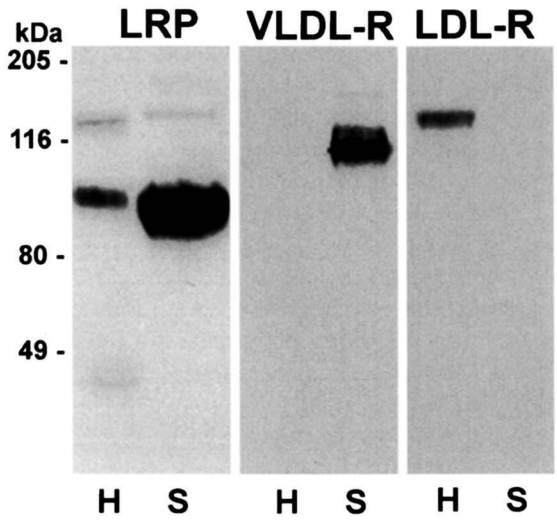

Fig. 1. Western blot analysis to detect receptors in the LDL receptor family. Equal amounts of cellular protein (100 μg) from rat hepatocyte extracts (H) and rat VSMC extracts (S) were subjected to SDS-PAGE and transferred to nitrocellulose membranes. LRP was detected with monoclonal antibody 11H4. LDL receptor (LDL-R) was detected with monoclonal antibody C7. VLDL receptor (VLDL-R) was detected with a polyclonal antibody. Molecular mass markers are shown to the left.

consistent with the reported tissue distribution for the VLDL receptor (24, 51). The LDL receptor was detected in the hepatocyte extracts; however, significant amounts of this receptor were not expressed by the VSMCs. Thus, the pattern of expression of LRFs in the growth-arrested VSMCs provided an appropriate model of LRF expression by VSMCs in atherosclerotic lesions (9, 21–24).

Activity of GST-RAP in VSMC cultures

In the β-VLDL binding and internalization experiments to be presented, RAP was used as an intact GST-fusion protein to antagonize LRF activity. To confirm that the RAP was active, we tested its ability to inhibit the specific binding of activated ^{125}I-labeled α_2M to VSMCs. Activated α_2M was chosen instead of LPL because activated α_2M binds to LRP and not HSPG. Furthermore, we previously demonstrated that RAP inhibits greater than 90% of the specific binding of activated α_2M to VSMCs (40). Activated ^{125}I-labeled α_2M (1.0 nM) was incubated in VSMC cultures with increasing concentrations of RAP for 4 h at 4°C. The cultures were then washed and specific radioligand binding was determined. RAP inhibited the binding of activated α_2M as expected; IC_{50} values ranged from 1.0 to 2.0 nM, indicating K_I values for RAP binding to LRP of about 0.5–1.0 nM (results not shown). In experiments with β-VLDL and LPL, we standardly utilized RAP at a concentration of 180 nM. Our experiments demonstrating high-affinity binding of RAP to LRP and the reported K_D for RAP binding to the VLDL receptor (K_D = 0.7 nM) (34) suggest that our standard concentration of RAP (180 nM) was sufficient to neutralize the ligand-binding activities of LRP and the VLDL receptor by greater than 99%.

Binding of ^{125}I-labeled β-VLDL to VSMCs

When ^{125}I-labeled β-VLDL was incubated with VSMCs at 4°C, minimal binding was detected; however, co-incubation with LPL caused a 9-fold increase in β-VLDL binding (**Fig. 2**). Binding of LPL-associated ^{125}I-labeled β-VLDL was inhibited by 89 ± 2% when a 50-fold molar excess of nonradiolabeled β-VLDL was included. Heparin decreased the binding of LPL-associated β-VLDL by 82 ± 4%. By contrast, 180 nM RAP did not significantly affect the binding of ^{125}I-labeled β-VLDL–LPL complex ($P > 0.4$). Thus, the majority of the binding of β-VLDL–LPL complex to VSMCs may be attributed to HSPG and not to LRFs that bind RAP with high affinity (LRP and the VLDL receptor).

Internalization of LPL-associated ^{125}I-labeled β-VLDL by VSMCs

To assess the role of LRFs in mediating the internalization of LPL-associated β-VLDL, ^{125}I-labeled β-VLDL and LPL were incubated with VSMCs at 37°C, in the

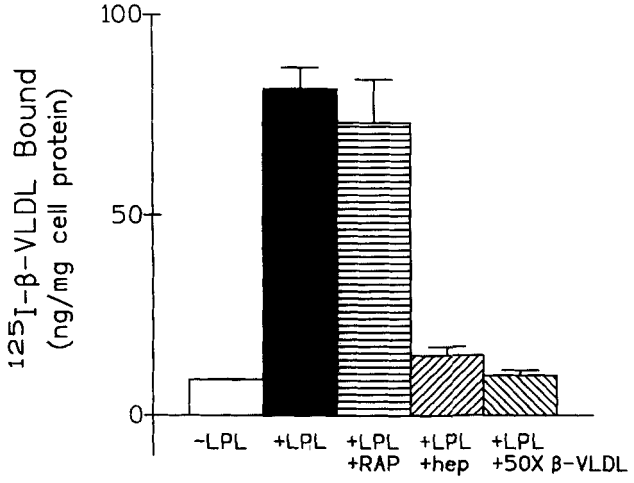

Fig. 2. Binding of β-VLDL to VSMCs. ^{125}I-labeled β-VLDL (3 μg/ml) was allowed to bind to VSMCs in the presence (+LPL) or absence (−LPL) of 5 nM LPL for 4 h at 4°C. In some wells, 180 nM RAP (+RAP) or 100 U/ml of heparin (+hep) was added. Nonspecific binding of ^{125}I-labeled β-VLDL in the presence of LPL was determined by adding a 50-fold molar excess of nonradiolabeled β-VLDL (+50× β-VLDL). Values represent the mean ± SEM (n = 6).

presence and absence of heparin and/or RAP. At various times, cell-surface radioligand was dissociated so that internalized β-VLDL could be measured. In cultures that were not treated with heparin or RAP, the level of internalized radioactivity increased rapidly for 60 min and then began to plateau, probably reflecting release of digested radioligand (**Fig. 3**). RAP partially

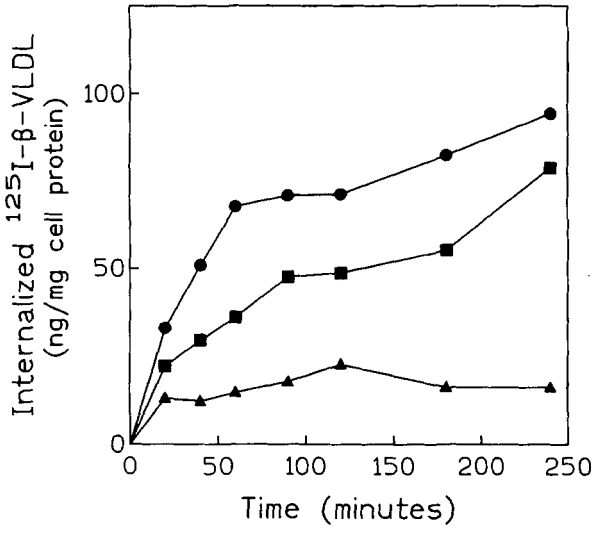

Fig. 3. Internalization of LPL-associated ^{125}I-labeled β-VLDL by VSMCs. In this representative study, LPL (5 nM) and ^{125}I-labeled β-VLDL (3 μg/ml) were incubated with VSMCs for various periods of time at 37°C. The cultures were then chilled to 4°C. Cell surface radioactivity was dissociated, allowing the determination of internalized radioactivity. Internalized ^{125}I-labeled β-VLDL is shown for control cultures (●), VSMCs treated with 180 nM RAP (■), and VSMCs treated with 100 U/ml heparin (▲).

inhibited internalization of LPL-associated β-VLDL; however, heparin was substantially more effective, decreasing internalization to near-background levels. The percentage of the heparin-sensitive internalization that was inhibited by RAP was 62 ± 3% at 40 min and 23 ± 3% at 120 min (n = 3). The combination of heparin + RAP was no more effective than heparin alone at inhibiting radioligand internalization (results not shown). Thus, internalization of LPL-associated β-VLDL by VSMCs is partially mediated by LRFs, but also by a RAP-resistant, heparin-sensitive pathway.

Degradation of LPL-associated ^{125}I-labeled β-VLDL by VSMCs

In order to evaluate the effects of RAP on the degradation of LPL-associated ^{125}I-labeled β-VLDL, ^{125}I-labeled β-VLDL and LPL were pre-incubated with VSMCs, in the presence and absence of RAP, at 4°C. After washing the cultures, fresh medium containing the same concentration of RAP, without β-VLDL and LPL, was added back to each well. The cells were then warmed to 37°C and radioligand processing was studied. In cultures that were not RAP-treated, cell-associated radioactivity decreased, beginning at about 60 min, accompanied by an increase in TCA-soluble radioactivity in the medium (**Fig. 4**). TCA-insoluble radioactivity did not collect in the medium as a function of time, indicating that the LPL-associated β-VLDL remained cell-associated and was only released after degradation. By 8 h, 40% of the initial radioactivity was still cell-associated, suggesting that a portion of the LPL-associated ^{125}I-labeled β-VLDL was turned over at a slow rate. In control experiments, chloroquine (0.2 mM) completely prevented the accumulation of TCA-soluble radioactivity in the medium, indicating that the digestion of β-VLDL–LPL complex occurred in acidified endosomes and/or lysosomes (results not shown).

In cultures that contained 180 nM RAP, cell-associated ^{125}I-labeled β-VLDL–LPL complex was converted into TCA-soluble radioactivity and released into the medium; however, the kinetics of radioligand digestion were altered. The initial rapid turnover phase was eliminated; cell-associated radioactivity remained near 100% and release of degraded ^{125}I-labeled β-VLDL into the medium was undetectable before 120 min. After the initial lag-phase, digestion occurred in the presence of the RAP and the rate of conversion of cell-associated radioligand into TCA-soluble radioactivity paralleled that observed in the absence of RAP.

The affinity of RAP for the LDL receptor is low (K_D = 250 nM) (35). Furthermore, the Western blot analysis shown in Fig. 1 demonstrated that rat VSMCs do not express significant levels of LDL receptor when cultured under our conditions. Nevertheless, to determine whether the LDL receptor or other uncharacterized

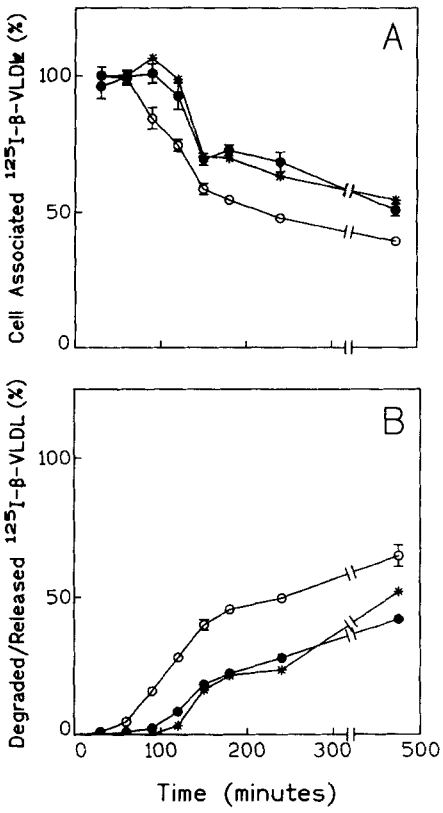

Fig. 4. Internalization and degradation of LPL-associated ^{125}I-labeled β-VLDL. ^{125}I-labeled β-VLDL (3 μg/ml) and LPL (5 nM) were incubated for 4 h at 4°C with VSMCs in the absence of RAP (○) (n = 3) in the presence of 180 nM RAP (●) (n = 3) or 2 μM (*) RAP (representative study, n = 2). The cultures were then washed and RAP was added back at the same concentration to the wells that originally had the antagonist. All of the cultures were simultaneously warmed to 37°C (at time 0). After incubation for the specified times, the medium was separated from the cells. (A) Cell-associated ^{125}I radioactivity; (B) ^{125}I-labeled β-VLDL converted into trichloroacetic acid-soluble radioactivity and released into the medium.

LRFs with low affinity for RAP might contribute to the processing of LPL-associated ^{125}I-labeled β-VLDL, degradation experiments were repeated in the presence of 2 μM RAP. As shown in Fig. 4, the higher concentration of RAP was no more effective than 180 nM RAP at inhibiting the processing of the ^{125}I-labeled β-VLDL. Thus, the slow digestion of LPL-associated β-VLDL, observed in the presence of 180 nM RAP, was probably due to HSPG-mediated cellular uptake and not to incomplete antagonism of LRFs.

Synthesis of cholesteryl oleate

Cholesteryl esters contained within internalized lipoproteins are hydrolyzed by cellular acid lipases (46). Excess cholesterol may be reesterified and stored in lipid droplets in the cytoplasm. The rate-limiting enzyme in cholesterol esterification is acyl-CoA:cholesterol *O*-acyltransferase (ACAT) (52). To test for ACAT activity,

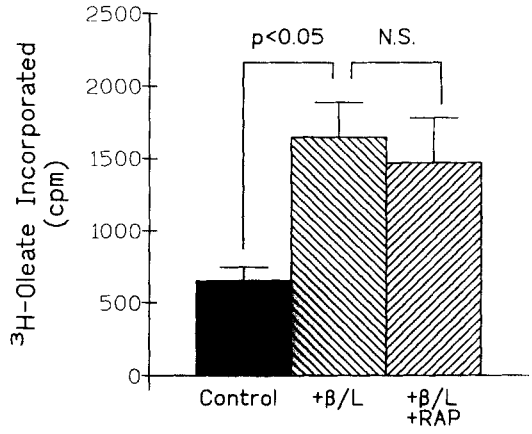

Fig. 5. Cholesteryl [^{3}H]oleate formation in VSMCs treated with LPL and β-VLDL. VSMCs were incubated with 5 nM LPL and 3 μg/ml β-VLDL (+β/L) in the presence of (+RAP) or absence of 180 nM RAP for 5 h at 37°C. Cultures that did not receive LPL and β-VLDL are also shown (Control). At 5 h, sodium [^{3}H]oleate was added and the incubation continued for an additional 2 h. Cholesteryl esters were extracted and analyzed by thin-layer chromatography. Cholesteryl [^{3}H]oleate formation was quantitated by β-scintillation and is graphed as the amount of [^{3}H]oleic acid incorporated into cholesteryl esters.

VSMCs were treated with β-VLDL and LPL or with vehicle for 5 h and then exposed to sodium [^{3}H]oleate for 2 h. LPL increased [^{3}H]oleate incorporation into choleteryl esters by 2.5-fold compared with control cultures (**Fig. 5**). RAP (180 nM) did not significantly affect cholesteryl oleate synthesis in the presence of LPL and β-VLDL.

Lipid accumulation in VSMCs

Oil red-O histochemistry was used to detect cholesteryl ester-containing lipid droplets in the cytoplasm of VSMCs, as an in vitro model of VSMC foam cell formation. In initial experiments, nearly confluent cultures of VSMCs were treated with β-VLDL, in the presence or absence of LPL, for 24 h. The concentrations of β-VLDL (3 μg/ml) and LPL (5 nM) were the same as those used in the previously described experiments. In the absence of LPL, β-VLDL did not induce detectable lipid droplet formation (results not shown). By contrast, when LPL was added with β-VLDL, substantial staining was observed (**Fig. 6**).

The effects of RAP (180 nM) on Oil red-O staining were initially evaluated using confluent cultures of VSMCs treated with β-VLDL (3 μg/ml) and LPL (5 nM). In eight separate experiments, lipid droplet formation in the presence of RAP was at least as great as that observed in the absence of RAP (Fig. 6). Thus, RAP did not inhibit storage of cholesteryl esters and/or triglycerides in this long-term assay.

Under our standard conditions, RAP occasionally appeared to enhance lipid droplet formation; however,

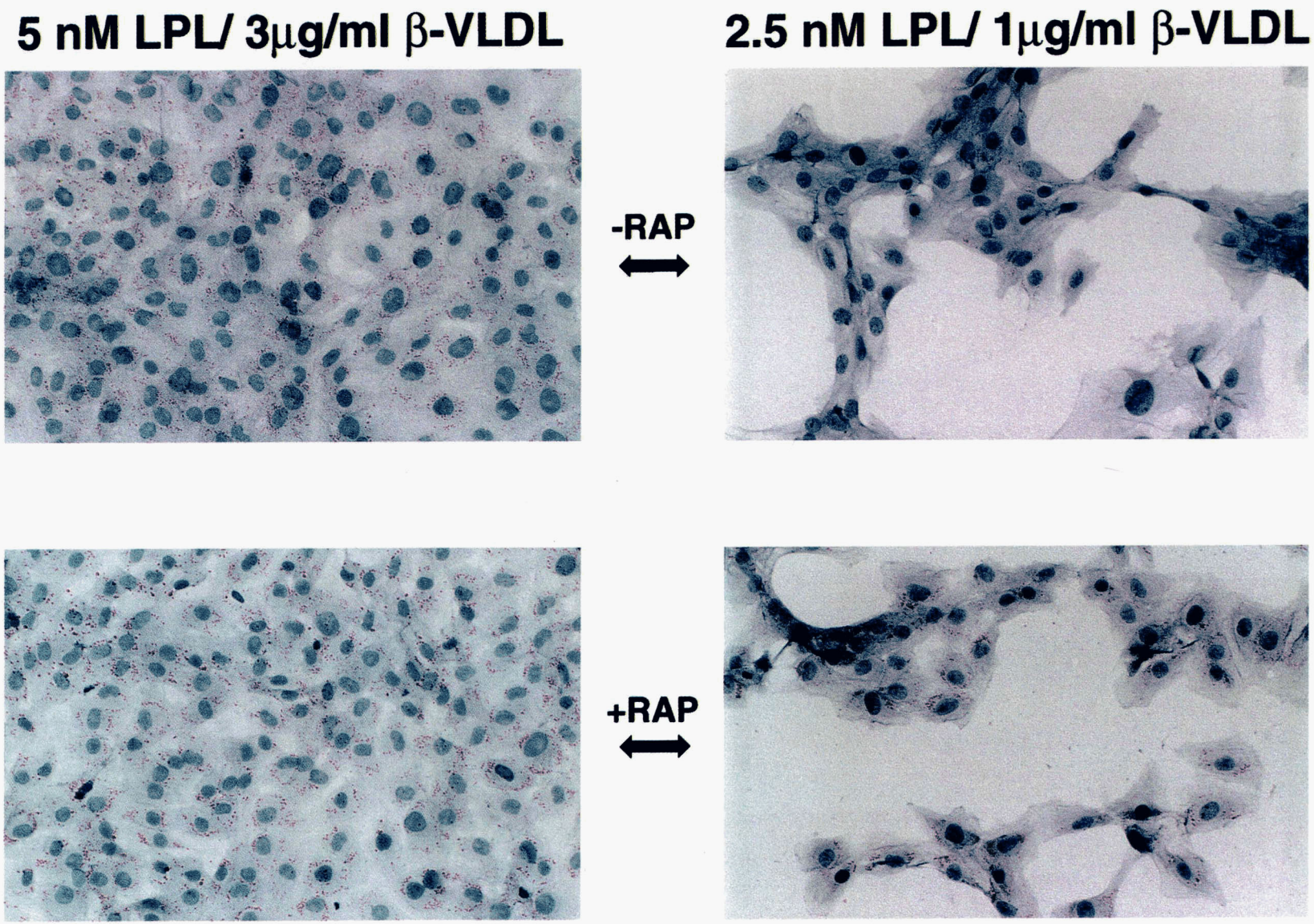

Fig. 6. Oil red-O-staining of VSMCs treated with LPL and β-VLDL. The concentrations of LPL and β-VLDL are shown in the figure. Note that the cultures shown in the right-hand panels were uniformly of lower confluency. The cells shown in the top two panels (−RAP) were not treated with RAP. The cells shown in the bottom two panels (+RAP) were treated with 180 nM RAP. Incubations were allowed to proceed for 24 h at 37°C before processing the cells. The cultures were fixed and then stained with Oil red-O and Harris' hematoxylin.

the effect was variable from field to field. Cells in less confluent areas typically showed increased staining compared with cells in more confluent regions, whether or not RAP was present. Thus, we varied the concentrations of LPL and β-VLDL, as well as culture density, and re-examined the effects of RAP on lipid droplet formation. As anticipated, the amount of Oil red-O staining increased with increasing concentrations of β-VLDL and LPL. The study shown in Fig. 6 (representative of four separate experiments) shows that RAP most clearly promoted Oil red-O staining when the cultures were subconfluent and when low concentrations of β-VLDL and LPL were used. Under these conditions, lipid droplets were rarely present in the absence of RAP but clearly visible in the presence of RAP. When heparin was added, Oil red-O staining was reduced to near-background levels in the presence or absence of RAP (results not shown). Thus, a heparin-sensitive, RAP-insensitive pathway promotes lipid storage when LRF activity is blocked. The effect of culture confluency on the activity of RAP in the Oil red-O histochemistry experiments has not been explained; however, LRF expression may be increased in low-density cultures (46).

DISCUSSION

This study provides evidence that LRFs and HSPG are both involved in the catabolism of LPL-associated β-VLDL by rat VSMCs. LRFs contributed minimally to the total binding capacity for LPL-associated β-VLDL, as suggested by the inadequacy of RAP as a competitor in the radioligand binding experiments. By contrast, heparin was highly effective as an inhibitor of the specific binding of LPL-associated β-VLDL. While this, in all likelihood, reflects inhibition of LPL binding to HSPG, it should be noted that heparin also inhibits the binding of LPL to purified LRP (6).

The function of HSPG in the binding and internalization of LPL and LPL-associated lipoproteins has been demonstrated in other cell types (12, 13). Most studies indicate that chondroitin sulfate is not active in the binding of LPL (53, 54), although an oversulfated form of chondroitin sulfate with LPL-binding activity has been described in macrophages (55). Whether oversulfated chondroitin sulfate is expressed by VSMCs in culture has not been determined; however, it is feasible that this type of macromolecule may contribute to the activity which we have attributed to HSPG in this study.

Our studies demonstrated that LRFs are involved in the internalization and degradation of LPL-associated β-VLDL. As the LRFs did not contribute significantly to the cell-surface binding capacity for LPL-associated β-VLDL, the β-VLDL–LPL complex was probably transferred from HSPGs to the LRFs on the VSMC cell surface, as has been proposed for other cell types (10, 11). When the LRFs were blocked with RAP, LPL-associated β-VLDL was still internalized and subsequently digested into TCA-soluble peptides which were released into the medium. Thus, cellular processing of β-VLDL–LPL complex by VSMCs is still possible with the major LRFs inhibited. The primary effect of RAP in the β-VLDL digestion experiments (Fig. 4) was to block the early phase of β-VLDL digestion, consistent with the known ability of LRFs to mediate rapid ligand endocytosis in clathrin-coated pits and target ligands for catabolism in lysosomes (9). The RAP-resistant pathway demonstrated properties that are characteristic of direct HSPG-mediated internalization, including the inhibition by heparin and slower kinetics of β-VLDL digestion and release (12, 13).

Despite its effects on the uptake and digestion of LPL-associated ^{125}I-labeled β-VLDL, RAP did not affect ACAT activity in VSMCs exposed to β-VLDL and LPL. RAP also did not inhibit intracellular lipid droplet formation in cells treated with 3 μg/ml β-VLDL and 5 nM LPL, as determined by histochemistry with Oil red-O. These results suggest that LRF-dependent pathways do not contribute in a substantial way to the accumulation of intracellular cholesterol ester in VSMCs treated with β-VLDL and LPL. An extension of this hypothesis would be the ability of RAP to promote cholesteryl ester storage under conditions in which LRFs compete with HSPG for ligand internalization. By lowering the concentrations of β-VLDL and LPL, we identified conditions under which RAP increased Oil-red O-stained droplet formation. Thus, we propose that the fate of internalized β-VLDL–LPL complex depends on the pathway followed. LRFs shuttle LPL-associated β-VLDL through a pathway that leads to more rapid degradation and less cholesteryl ester formation. If the β-VLDL–LPL complex is internalized directly by HSPG, processing occurs more slowly and cholesterol storage is favored. According to this model, VSMC LRFs play an antiatherogenic role by preventing catabolism via HSPG.

Other investigators have shown that receptors in clathrin-coated pits and HSPGs follow different intracellular pathways after internalization (14). Our studies suggest that, in VSMCs, relative levels of expression of HSPG, LRP, and the VLDL receptor may be critical in determining the cellular processing of a ligand (LPL-associated β-VLDL) that is common to all three. Atherosclerotic arteries may contain higher levels of proteoglycans compared with normal arteries (56, 57). Furthermore, atherogenic cytokines, such as PDGF-BB, alter the kinetics of LRP recycling in VSMCs so that more of this receptor is present on the cell surface (47). Thus, it is conceivable that the balance between LRFs and HSPG

may be altered in VSMCs in the neointima compared with cells in the media.

In summary, we have demonstrated that LRFs and HSPG function in the internalization and digestion of LPL-associated β-VLDL by VSMCs in culture. These two classes of macromolecules may play antagonistic roles in the transformation of VSMCs into foam cells.

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