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Characteristics of Low and High Density Lipoprotein Binding and Lipoprotein-Induced Signaling in Quiescent Human Vascular Smooth Muscle Cells

VALERY N. BOCHKOV, VSEVOLOD A. TKACHUK, YELENA S. KUZMENKO, YULIA L. BORISOVA, FRITZ R. BÜHLER, and THÉRÈSE J. RESINK

Department of Research, Basel University Hospital, CH 4031 Basel, Switzerland (F.R.B., T.J.R.), and Laboratory of Molecular Endocrinology, Institute of Experimental Cardiology, Cardiology Research Center, Moscow, Russia (V.N.B., V.A.T., Y.S.K., Y.L.B.)

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SUMMARY

Low density lipoprotein (LDL) and high density lipoprotein (HDL) have been shown to stimulate signal transduction events in a number of cell types, including cultured vascular smooth muscle cells (VSMC), but it is not known whether these events are mediated through distinct lipoprotein receptors for transmembrane signaling. This study has used confluent quiescent cultures of human microarteriolar VSMC to investigate the relationship between the characteristics of ^{125}I-LDL and ^{125}I-HDL$_3$ binding and those of LDL- and HDL$_3$-stimulated cell signaling. Two distinct binding sites for LDL ($K_{d1} \approx 2$ μg/ml and $K_{d2} \approx 40$ μg/ml) and a single class of sites for HDL$_3$ ($K_d \approx 30$ μg/ml) were identified. The K_{d1} for high affinity ^{125}I-LDL binding in quiescent VSMC was comparable to the value for heparin-sensitive binding of ^{125}I-LDL to apolipoprotein B/E receptors in fibroblasts ($K_d \approx 1$ μg/ml). Concentrations of lipoproteins required for half-maximal stimulation (EC_{50}) of phosphoinositide catabolism and intracellular calcium mobilization in VSMC were ≈35 μg/ml for HDL$_3$ and ≈40 μg/ml for LDL. Both LDL- and HDL$_3$-stimulated signaling responses in VSMC, as well as ^{125}I-HDL$_3$ binding and low affinity ^{125}I-LDL binding to VSMC, were insensitive to heparin. Competition binding studies (with unlabeled lipoproteins at 2.5–200 μg/ml) showed partial displacement of ^{125}I-LDL by HDL$_3$ and of ^{125}I-HDL$_3$ by LDL, whereas complete displacement of ^{125}I-LDL or ^{125}I-HDL$_3$ by their homologous lipoproteins was achieved. Thus, the binding sites for HDL$_3$ are distinct from those for LDL. Because the response of VSMC to combinations of LDL and HDL$_3$ was additive, LDL and HDL$_3$ also exert their signaling effects through distinct sites. Further investigation is required to unequivocally demonstrate that the heparin-insensitive HDL$_3$ and low affinity LDL binding sites in VSMC are those through which LDL and HDL$_3$ stimulate transmembrane signaling.

LDL and HDL play pivotal roles in cholesterol homeostasis (1–3). In addition to the known effects on lipid metabolism, LDL and HDL can regulate a number of apparently related processes, such as expression of β-hydroxy-β-methylglutaryl coenzyme A reductase, expression of LDL receptor, and cell growth (4–6). These effects indicate that lipoproteins can exert some action at the level of regulation of gene expression, for which intracellular levels of cholesterol, or its oxygenated derivatives, have been proposed to be responsible (7). LDL, which increases cellular cholesterol content, and HDL, which decreases cholesterol levels, generally have opposite effects on these processes (2, 3). Another set of regulatory properties of lipoproteins cannot be explained only by their effects on lipid metabolic pathways or gene expression. These include the promotion of platelet shape change, proaggregatory effects in platelets, and an influence on blood vessel tone (8, 9). It is not clear how lipoproteins mediate these effects, but the rapidity with which they occur (within seconds or minutes after exposure of cells to lipoproteins) suggests a mode of action that is distinct from that related to cholesterol homeostasis.

The stimulation of phosphoinositide catabolism, intracellular Ca^{2+} mobilization, and protein kinase C activation by LDL has been described for a number of cell types, including platelets, cultured VSMC, endothelial cells, and fibroblasts (9–14). The rapidity and saturability of the effects suggest that they probably occur through specific receptor-mediated processes. Similar cell signaling responses to HDL have been described in fibroblasts (15) and VSMC (14, 16). In endothelial cells, the stable guanine nucleotide analogs guanosine-5′-*O*-(3-thiotriphosphate) and guanosine-5′-*O*-(2-thiodiphosphate) have been shown to potentiate and inhibit LDL-stimulated phosphoinositide turnover, respectively (12). Therefore, lipoproteins may

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ABBREVIATIONS: LDL, low density lipoprotein; HDL, high density lipoprotein; VSMC, vascular smooth muscle cells; BSA, bovine serum albumin; $[Ca^{2+}]_i$, intracellular calcium concentration; apo E, apolipoprotein E; apo B, apolipoprotein B.

stimulate cell signaling, either directly or indirectly, through G protein-coupled receptor systems. The existence of lipoprotein receptors (or recognition sites) different from those involved in lipid transport is also implied.

The ability of lipoproteins to stimulate phosphoinositide and calcium signaling in VSMC is dependent on culturing conditions, being markedly reduced under mitogen-containing medium conditions (16). Therefore, to investigate whether VSMC may indeed express distinct lipoprotein receptors (recognition sites) for transmembrane signaling, the present study has investigated LDL and HDL_3 binding in cultures of human microarteriolar VSMC maintained under conditions (serum-deprived and quiescent) that are optimal for stimulation of signaling by LDL and HDL_3. The characteristics of LDL and HDL_3 binding in these cultures have been compared with those of LDL- and HDL_3-stimulated transmembrane signaling.

Materials and Methods

Cell culture. The isolation, phenotypic characterization, and propagation of human VSMC from microarterioles associated with omental tissue was performed according to previously described procedures (11). Immunohistochemical characterization of all VSMC isolates (positive for smooth muscle specific α-actin and negative for factor VIII) was performed on primary cultures as described (11). The isolation, characterization, and propagation of human skin fibroblasts was performed as described (17). For the studies herein, VSMC and fibroblasts were used between passages 10 and 18; cell cultures were grown to confluence and were rendered quiescent by serum deprivation and maintenance in serum-free medium containing 0.1% (w/v) BSA, for 48 hr before experimentation. Cell numbers were determined using a Coulter counter after trypsinization of cell layers as described previously (11); for cultures grown in 24-well dishes, cell numbers at confluence were ≈2.2–2.5 × 10^5 cells/well. The Lowry method was used for determination of cell protein concentrations.

Purification of LDL and HDL_3. LDL (density, 1.019–1.063 g/ml) and HDL_3 (density, 1.125–1.20 g/ml) were isolated from the plasma of healthy men using sequential buoyant density centrifugation techniques, with the use of potassium bromide solutions for density adjustments (18). LDL and HDL_3 were exhaustively dialyzed against 0.15 M NaCl before Lowry determination of protein concentrations and sterilization by filtration through 0.45-μm Gelman filters. Apo E-free HDL_3 was prepared from the total HDL fraction (density, 1.063–1.20 g/ml) by heparin-agarose affinity chromatography (19). EDTA (1 mM) and butylated hydroxytoluene (1 μM) were present during all isolation and dialysis procedures. Gradient sodium dodecyl sulfate-polyacrylamide (5–19%) gel electrophoresis revealed that HDL_3 preparations were free of apo B-100 protein and albumin and that LDL preparations contained only apo B-100 protein and were free of protein fragments known to be generated during LDL oxidation (20). Lipoproteins were stored in sterile plastic tubes at 4° and were used within 3 weeks of isolation.

Iodination of LDL and HDL_3 and binding studies. LDL and HDL_3 were iodinated using the iodine monochloride method as described (21). More than 95% of the radioactivity in iodinated lipoprotein preparations was precipitable by trichloroacetic acid and <2% was extractable into chloroform/methanol. Binding of ^{125}I-LDL and ^{125}I-HDL_3 was studied in confluent quiescent cultures of VSMC and fibroblasts essentially as described previously (11, 21). Saturation binding of ^{125}I-LDL and ^{125}I-HDL_3 (0.5–100 μg/ml, 50–150 cpm/ng) was performed at 4° in Dulbecco's modified Eagle's medium containing 0.1% BSA. Parallel series of dishes contained excess (1 mg/ml) unlabeled lipoprotein for determination of nonspecific binding. After 4 hr, cell layers were washed (6 × 3 ml) with ice-cold Hanks' solution containing 0.1% BSA. Cell-bound ^{125}I-lipoprotein and cell protein content per well were determined after solubilization in 0.1 M NaOH. The equilibrium dissociation constants (K_d) and maximum binding capacities (B_{max}) of lipoprotein binding sites were estimated by both linear regression analysis and nonlinear regression analysis (LIGAND) of saturation binding data.

Measurements of phosphoinositide catabolism and intracellular $[Ca^{2+}]_i$. Measurements of inositol phosphates were performed on confluent cultures of VSMC (in 24-well plates) that had been rendered quiescent in serum- and inositol-free medium, with inclusion of *myo*-[2-^{3}H]inositol (2.0 μCi/ml) to prelabel inositol phospholipids. Experimental procedures for stimulation of cells (at 37° and in the presence of 15 mM LiCl), termination of reactions, and resolution of inositol phosphates in cell lysates on Dowex AG 1-X8 anion exchange columns have been described previously (11, 14, 22). Measurements of diacylglycerol were performed in VSMC that had been prelabeled with [^{3}H]arachidonic acid (2 μCi/ml) during the last 24 hr of the quiescence period. Experimental procedures for stimulation of cells (at 37°), lipid extraction, and determination of [^{3}H]diacylglycerol by thin layer chromatography have been detailed (22). The cell-permeant acetoxymethyl ester form of the Ca^{2+}-sensitive probe fura-2 was used to determine $[Ca^{2+}]_i$, and measurements were performed at 37° on monolayers of VSMC grown on glass coverslips as detailed before (14).

Results

Binding of ^{125}I-LDL and ^{125}I-HDL_3 to VSMC. Binding experiments were performed at 4° to minimize the process of internalization (21, 23), and the 4-hr incubation period was sufficient to allow binding to proceed to equilibrium (data not shown). As shown in Fig. 1, human VSMC expressed specific binding sites for LDL and HDL_3 (Fig. 1, A and C, respectively). The major part of cell-associated ^{125}I-lipoproteins was surface bound, because about 90% of radioactivity could be removed from cell monolayers by trypsin treatment (24) (data not shown). The Scatchard plot of binding data for ^{125}I-LDL was curvilinear, suggesting the existence of more than one binding site. Assessment of binding parameters by nonlinear regression analysis using the LIGAND program (25) indicated a statistically improved fit of ^{125}I-LDL binding data to a two-site model, compared with a one-site model (F test, $p = 0.008$). For ^{125}I-LDL, LIGAND-calculated binding affinities ($K_{d1} = 0.88 \pm 0.67$ μg/ml, $K_{d2} = 47.3 \pm 15.1$ μg/ml) and B_{max} values ($B_{max1} = 13 \pm 6.2$ ng/mg, $B_{max2} = 145 \pm 79$ ng/mg) were comparable to those obtained by linear regression analysis of the two linear portions of Scatchard-transformed data (Fig. 1B). For ^{125}I-HDL_3, the F test did not indicate a significantly better fit of the data to a two-site model than to a one-site model ($p = 0.48$). The LIGAND-calculated binding affinity ($K_d = 18 \pm 7.5$ μg/ml) and B_{max} (32 ± 18.7 ng/ml) for ^{125}I-HDL_3 correlated reasonably well with those obtained by linear regression analysis of the Scatchard plot (Fig. 1D).

In two other experiments, comparable K_d values for LDL binding were obtained but B_{max} values were 2–4-fold greater (data not shown), suggesting a variability of the latter parameter. Variability of B_{max} values for both the apo B/E receptor and the HDL receptor is well known and occurs via up- or down-regulation according to cellular demand for cholesterol (26, 27). In an additional experiment, saturation binding of ^{125}I-labeled apo E-free HDL to human VSMC was characterized (Scatchard analysis) by a K_d of 17 μg of protein/ml and a B_{max} of 250 ng/mg of cell protein (data not shown).

The K_d (approximately 1 μg/ml) of the high affinity binding site for LDL is very close to the affinity of the "classical" apo B/E receptor for LDL (1, 21, 23), and also correlates well with the presently obtained K_d (1.1 μg/ml) for binding of ^{125}I-LDL to quiescent human fibroblasts (Fig. 2). The low affinity LDL

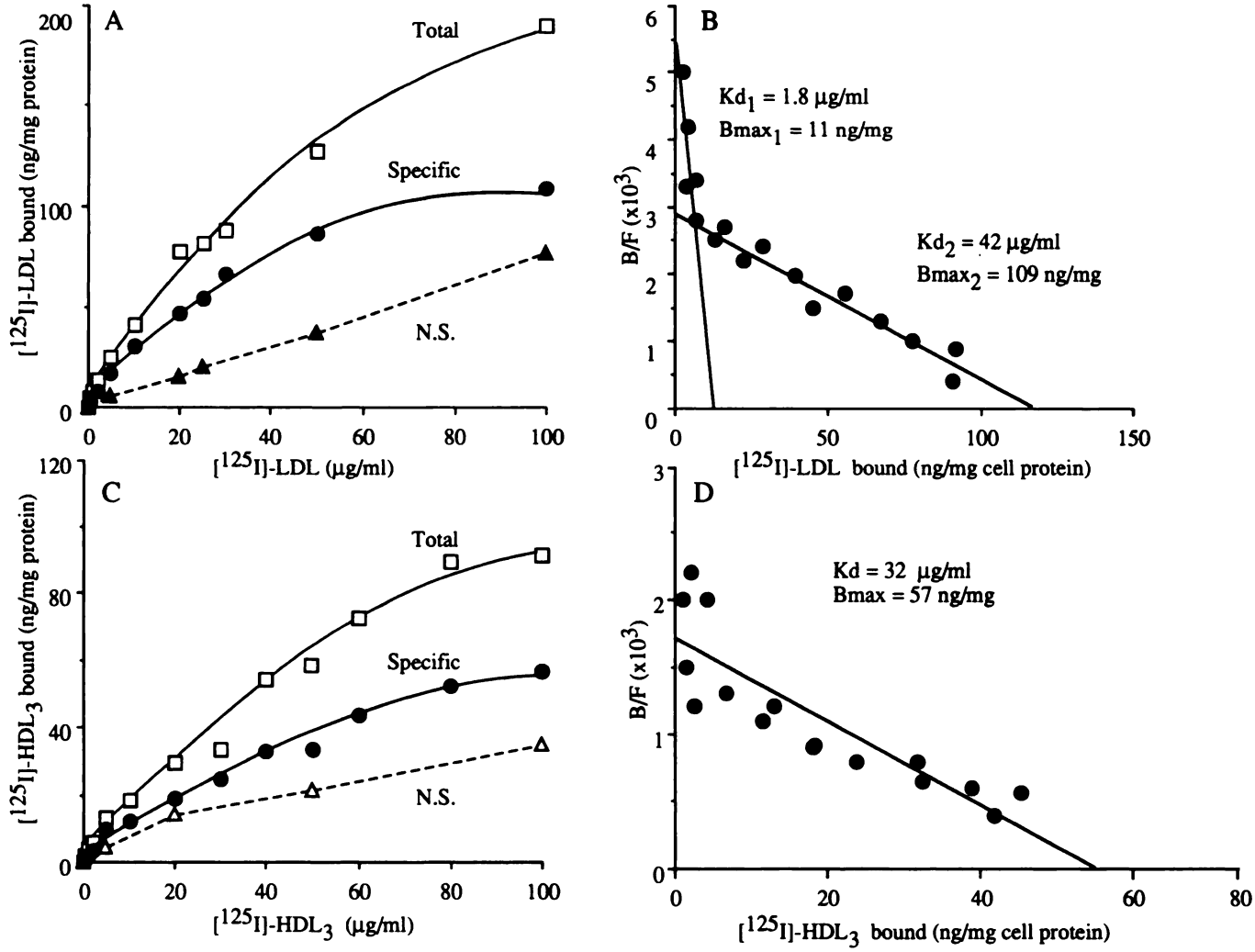

Fig. 1. Binding of LDL and HDL_3 to quiescent human VSMC. Saturation binding of ^{125}I-LDL (A) and ^{125}I-HDL_3 (C) in confluent quiescent cultures of human VSMC was performed at 4° as described in Materials and Methods. Specific ^{125}I-lipoprotein binding was determined after correction for nonspecific binding (*N.S.*) (^{125}I-LDL or ^{125}I-HDL_3 bound in the presence of an excess of the corresponding unlabeled lipoprotein). The data are from two independent experiments. Scatchard-transformed data from these experiments are presented in B ([^{125}I-LDL) and D (^{125}I-HDL_3). In A and C, mean values are given when the same concentrations of ^{125}I-LDL or ^{125}I-HDL_3 were used in both experiments. Binding parameters (K_d and B_{max}) presented were obtained by linear regression analysis. Values for these parameters, as estimated by nonlinear regression analysis (LIGAND), are given in the text.

binding site in VSMC has not been previously described, and this may be due to a number of methodological distinctions. Firstly, in contrast to the present use of serum-deprived cell cultures, LDL binding studies have usually been performed on cultures maintained in the presence of either complete serum or lipoprotein-deficient serum. Secondly, the majority of past reports on LDL binding in VSMC and other cell types have focused on high affinity binding only; in particular, either saturation binding was performed at concentrations of LDL that did not exceed 50 μg/ml or only the portion of high affinity binding was carefully analyzed by Scatchard transformation. Finally, rather than the present use of excess unlabeled lipoprotein to determine nonspecific binding, many studies have taken heparin-releasable LDL as the index of specifically surface-bound LDL (21, 23), and our data indicate that low affinity binding is heparin insensitive (see Fig. 7A).

Lipoprotein-stimulated signaling in VSMC. Fig. 3 demonstrates that, in human microarteriolar VSMC, LDL and HDL_3 stimulate phosphoinositide-specific phospholipase C and elevate $[Ca^{2+}]_i$ within the same concentration range as for binding. The concentrations of lipoproteins required for half-maximal stimulation (EC_{50}) of inositol phosphate accumulation were 35 ± 10 μg/ml for HDL_3 and 45 ± 12 μg/ml for LDL (mean ± standard deviation, five experiments) (Fig. 3A). EC_{50} values for both HDL_3- and LDL-stimulated elevation in $[Ca^{2+}]_i$ were ≈40 μg/ml (mean values from two experiments) (Fig. 3B).

Lipoprotein-mediated phospholipase C activation occurred rapidly, with maxium levels of [^{3}H]inositol bis- and trisphosphate being obtained within ≈1 min, and the kinetics of inositol phosphate generation were similar for LDL (Fig. 4A) and HDL_3 (Fig. 4B). Using anion exchange high performance liquid chromatography, we previously demonstrated that, for both LDL and HDL_3, the inositol phosphate isomers generated include inositol-1,3,4,5-tetrakisphosphate, inositol-1,4,5-trisphosphate, inositol-1,3,4-trisphosphate, inositol-1,4-bisphosphate, and

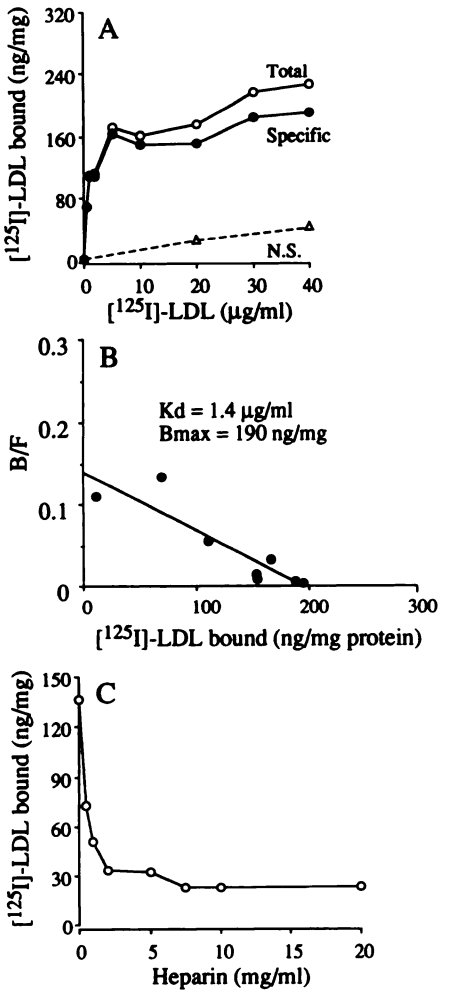

Fig. 2. LDL binding in quiescent fibroblasts. A, Saturation binding of ^{125}I-LDL to confluent quiescent cultures of human skin fibroblasts was performed at 4° as described in Materials and Methods. Specific ^{125}I-LDL binding was determined after correction for nonspecific binding (*N.S.*) (^{125}I-LDL bound in the presence of excess unlabeled LDL). B, Scatchard transformation of the data. C, The indicated concentrations of heparin were included during incubation (4 hr at 4°) of fibroblasts with 5 μg/ml ^{125}I-LDL. Data for specifically bound ^{125}I-LDL are presented.

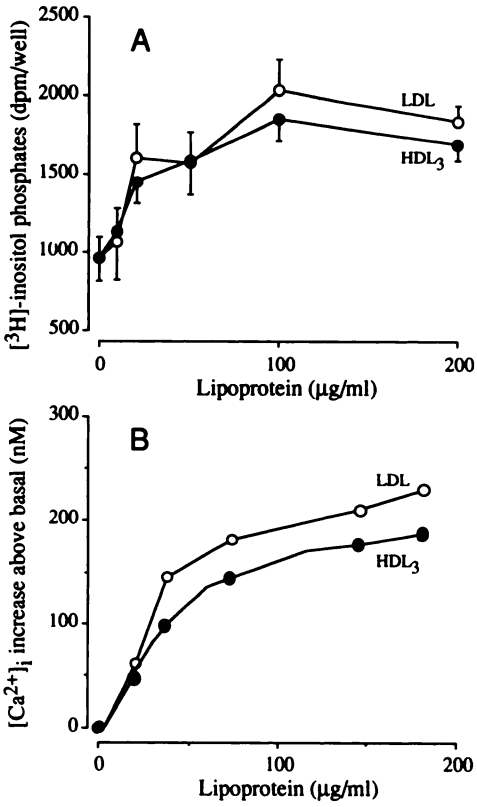

Fig. 3. Dose-dependent signaling responses of human VSMC to LDL and HDL$_3$. A, The content of [^{3}H]inositol phosphates (inositol mono-, bis-, and trisphosphates) in cell lysates was determined after exposure (5 min) of confluent, quiescent, *myo*-[2-^{3}H]inositol-prelabeled VSMC to the indicated concentrations of LDL and HDL$_3$. Values given are means ± standard deviations of data obtained from five separate experiments. B, The $[Ca^{2+}]_i$ response to the indicated concentrations of LDL and HDL$_3$ was determined in fura-2-loaded VSMC, and results (mean of data from two separate experiments) express the difference between the peak elevation in $[Ca^{2+}]_i$ and the corresponding basal value (≈40–60 nM). Kinetic profiles for $[Ca^{2+}]_i$ responses to LDL and HDL$_3$ are presented in Fig. 4D. All methodological procedures are given in Materials and Methods.

inositol-4-monophosphate (14). Biphasic kinetics were observed for generation of diacylglycerol in response to LDL and HDL$_3$, with the first and second phases peaking at ≈15 sec and ≈3 min, respectively (Fig. 4C). Fluorescence recordings presented in Fig. 4D illustrate the rapid and reversible kinetics of the $[Ca^{2+}]_i$ response to LDL and HDL$_3$.

Evidence that LDL and HDL$_3$ have different recognition sites on VSMC. From the data in Figs. 3 and 4 it is evident that LDL and HDL$_3$ exert qualitatively comparable effects on $[Ca^{2+}]_i$, inositol phosphate accumulation, and diacylglycerol generation. Theoretically, this may be due to recognition of both lipoproteins by a common binding site coupled to cell signaling systems. In support of this proposal, we found in competition binding experiments that ^{125}I-LDL could be displaced by HDL$_3$ (Fig. 5A) and that ^{125}I-HDL$_3$ could be displaced by LDL (Fig. 5B). Nevertheless, the concentration characteristics of this competition were markedly different from what might have been expected with respect to the apparent binding affinities of LDL and HDL$_3$. Data in Fig. 1 indicate that LDL (at its lower affinity site) and HDL$_3$ have similar binding affinities (42 and 32 μg of protein/ml, respectively). Thus, if LDL and HDL$_3$ were to compete for the same receptor, displacement of labeled lipoproteins by homo- and heterologous unlabeled competitors should occur at equivalent concentrations. However, our results (Fig. 5) clearly indicate that the homologous lipoproteins were more efficacious in displacing ^{125}I-LDL and ^{125}I-HDL$_3$. We found that 8.3-fold (for ^{125}I-LDL displacement) (Fig. 5A) and 12-fold (for ^{125}I-HDL displacement) (Fig. 5B) higher concentrations of unlabeled heterologous lipoproteins, compared with those of the homologous lipoproteins, were required to achieve 50% displacement of labeled lipoproteins. Such data suggest that LDL and HDL$_3$ bind to distinct sites on VSMC.

Fig. 6 presents data from a functional test for different recognition sites, whereby VSMC were exposed to LDL or HDL$_3$ either alone or in combination with the heterologous lipoprotein. Regardless of the lipoprotein concentrations used, the response of VSMC (accumulation of inositol phosphates) to combinations of LDL and HDL$_3$ approximated a summation of the responses elicited by each lipoprotein individually (Fig. 6). This additive response to combinations of LDL and HDL$_3$ indicates that one lipoprotein does not imitate the effect of the

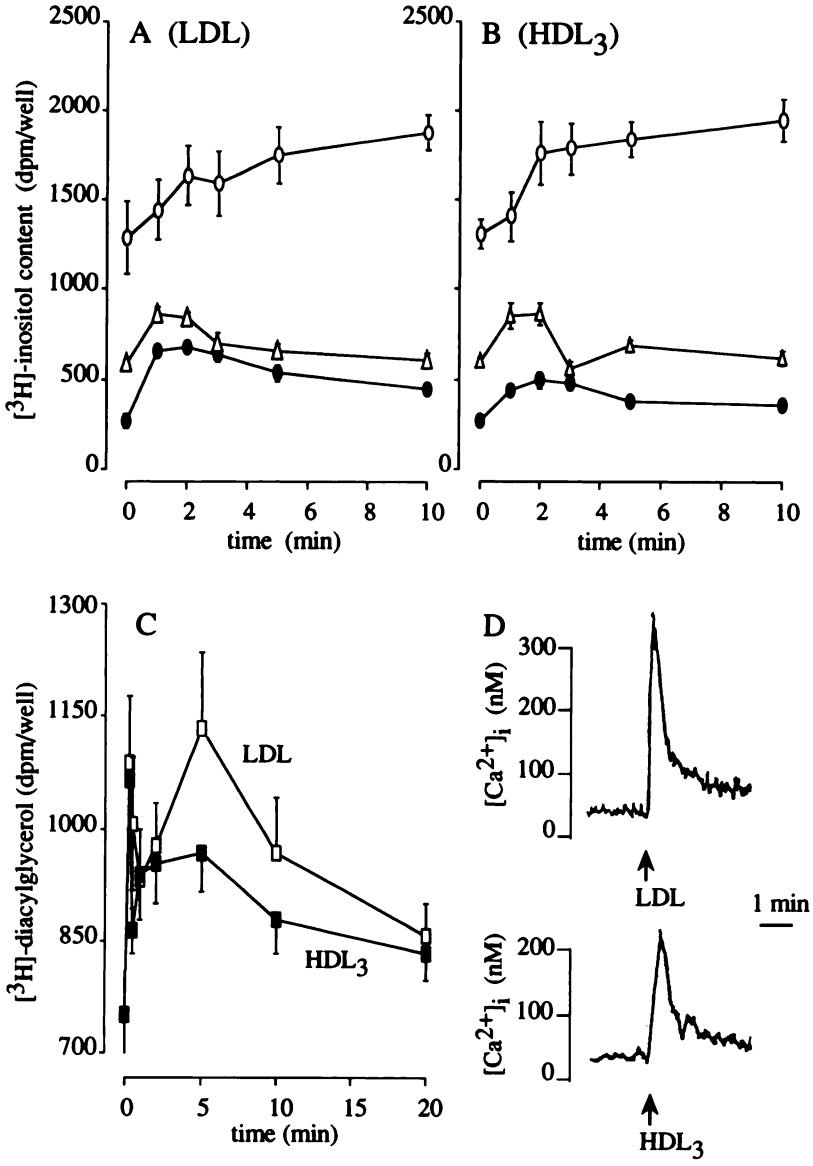

Fig. 4. Kinetics of lipoprotein-induced cellular signaling in human VSMC. A and B, *myo*-[2-^{3}H]inositol-prelabeled VSMC were exposed to 50 μg/ml LDL (A) or HDL_3 (B) for the indicated times. The ^{3}H content in inositol monophosphates (○), bisphosphates (△), and trisphosphates (●) was determined after resolution of cell lysates on Dowex anion exchange columns. Means ± standard errors of data from six separate experiments are given. C, Diacylglycerol production was measured after the indicated periods of exposure of [^{3}H]arachidonic acid-prelabeled VSMC to 50 μg/ml LDL (□) or HDL_3 (■) and analysis of cell extracts by thin layer chromatography. Means ± standard deviations of data from four separate experiments are presented. D, The $[Ca^{2+}]_i$ response of VSMC to LDL or HDL_3 (each at 100 μg/ml) was measured in fura-2-loaded VSMC, and representative fluorescence signal recordings from the entire monolayer culture are presented.

other through recognition of the same site. Rather, each lipoprotein would appear to exert its effects through distinct recognition sites, each of which is coupled to a common signal transduction pathway. Furthermore, previous control experiments on fura-2-loaded VSMC have consistently shown that "single" cells (compared with fluoresence recordings from the cell monolayer as a whole) respond to both LDL and HDL_3 (16). Thus, we can exclude the possibility that the additive response of VSMC to LDL and HDL_3 might be due to different populations of VSMC that express different complements of lipoprotein binding sites.

Effects of heparin on lipoprotein binding and lipoprotein-stimulated signaling. Heparin has been used as a powerful tool to study the interaction of LDL with the LDL receptor mediating endocytosis (2, 21, 23). This polyanionic compound interacts with apo B, which has a polycationic structure, and by changing the charge on LDL particles heparin renders these particles unrecognizable to the LDL receptor. We previously found that the effect of LDL on phosphoinositide catabolism in human microarteriolar VSMC can be partially blocked by polyclonal antibodies to apo B (11), and thus we expected that heparin would prevent binding of LDL to VSMC, as well as LDL-stimulated signal transduction. However, using the same human VSMC isolate we found that heparin had no effect on either the binding of ^{125}I-LDL (Fig. 7A) or LDL-stimulated production of inositol phosphates (Fig. 7B). The lack of effect of heparin on ^{125}I-LDL binding in VSMC is apparently discrepant with the known effects of heparin on LDL binding to apo B/E receptors in other cell types (2, 21, 23). However, in parallel experiments on human skin fibroblasts using the same methods and the same batch of heparin, we observed complete inhibition of specific binding of ^{125}I-LDL (Fig. 2C). Thus, our inability to detect heparin-sensitive ^{125}I-LDL binding in VSMC might be attributed to the high proportion (≈90%) of low affinity ^{125}I-LDL binding sites in these cells (Fig. 1B). Heparin had no effect on either ^{125}I-HDL_3 binding (Fig. 7A) or HDL_3-stimulated production of inositol phosphates (Fig. 7B). The heparin analogue pentosan polysulfate, which is a highly sulfated homopolymer of β-D-xylopyranosyl units substituted in the 2′-position by 4-*O*-methylglucomonate (28), also had no effect (dose

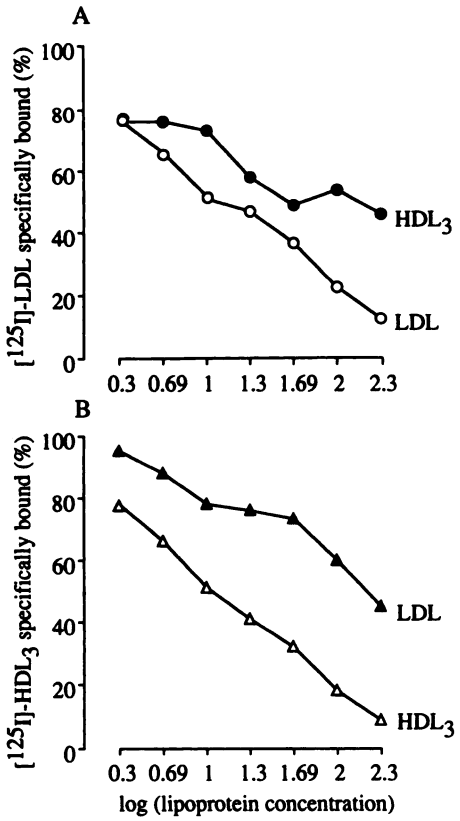

Fig. 5. LDL and HDL_3 partially displace each other on human VSMC. Confluent quiescent cultures of VSMC were incubated for 4 hr at 4° in the presence of 5 μg/ml concentrations of either ^{125}I-LDL (A) or ^{125}I-HDL_3 (B), with inclusion of different concentrations (2.5–200 μg/ml) of either homologous (*open symbols*) or heterologous (*closed symbols*) unlabeled lipoproteins. Nonspecific ^{125}I-LDL and ^{125}I-HDL_3 binding was determined by including excess (1 mg/ml) unlabeled corresponding lipoprotein in parallel series of dishes. Results for specific binding of ^{125}I-LDL and ^{125}I-HDL_3 in the presence of competitors are expressed relative to the amount bound specifically (100%) in the absence of either homolgous or heterologous competitor. Absolute values for 100% specific binding were 16.5 ng/mg of protein for ^{125}I-LDL and 12.2 ng/mg of protein for ^{125}I-HDL_3. The data represent mean values from two independently conducted experiments.

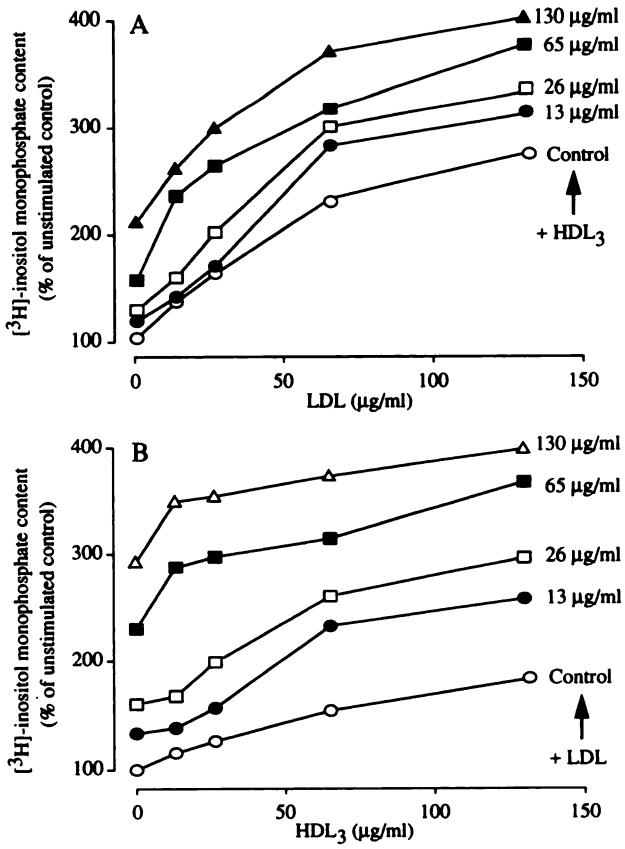

Fig. 6. Additive effects of LDL and HDL_3 on phosphoinositide catabolism. *myo*-[2-^{3}H]Inositol-prelabeled VSMC were exposed for 5 min to various concentrations (0–150 μg/ml) of LDL without or with inclusion of different concentrations (13–130 μg/ml) of HDL_3 (A) or various concentrations of HDL_3 (0–150 μg/ml) without or with inclusion of different concentrations (13–130 μg/ml) of LDL (B). After resolution of cell lysates on Dowex anion exchange columns, radioactivity in the inositol monophosphate fraction was determined. Changes in [^{3}H]inositol monophosphate content are expressed relative to the content (100%) in control VSMC (i.e., not exposed to either of the lipoproteins). Mean values (standard deviation, <10%; error bars not shown) from three different experiments are given.

range of 1–20 mg/ml) on LDL- or HDL_3-stimulated inositol phosphate accumulation (data not shown).

Pertussis toxin sensitivity of lipoprotein-stimulated signaling. A diverse array of agonists mediate their effects on calcium mobilization and phosphoinositide catabolism via receptors that couple to G proteins (29, 30). To test whether the signaling-responsive lipoprotein recognition sites might also involve G proteins, we tested the effect of pertussis toxin on lipoprotein-stimulated signaling. Pertussis toxin causes ADP-ribosylation of G_i and G_o α subunits and thereby prevents the coupling of membrane receptors to phospholipase C (29). Under conditions in which complete ADP-ribosylation of pertussis toxin-sensitive G proteins (α subunits of G_{i2} and G_{i3}) in VSMC is achieved (31), both calcium and phosphoinositide signaling responses of VSMC to LDL and HDL_3 were significantly inhibited (Table 1). In spite of the pertussis toxin sensitivity of lipoprotein-induced signaling in VSMC, we cannot conclude that the LDL and HDL_3 recognition sites mediating cell signaling events are directly coupled to G proteins. It is conceivable that the involvement of G proteins in LDL- and HDL_3-stimulated signaling arises not through a direct coupling of their specific receptors to G proteins but through indirect effects of LDL and HDL_3 on precoupled G protein-phospholipase C complexes.

Discussion

Our data support the existence of at least three different lipoprotein binding sites on quiescent VSMC, namely high ($K_d \approx 1$ μg/ml) and low ($K_d \approx 40$ μg/ml) affinity sites for LDL and a single class of sites ($K_d \approx 30$ μg/ml) preferentially recognizing HDL_3. High affinity binding of LDL in VSMC probably occurs via the classical apo B/E receptor that is expressed in a number of cell types including VSMC (1). Our observation that the B_{max} for this high affinity site on VSMC is much lower than that for the site on human fibroblasts (this study and Ref. 21) is consistent with a previous report that the binding capacity of LDL receptors in nonhuman primate VSMC was only 20% of that observed in homologous fibroblasts (32). A low B_{max} for high affinity LDL receptor binding (10 ng of LDL/mg of cell protein) has also been reported for human microvascular endothelial cells (33). Thus, for certain cell types expression of the high affinity apo B/E receptor may be characteristically less prominent than in fibroblasts. The number of LDL (apo B/E)

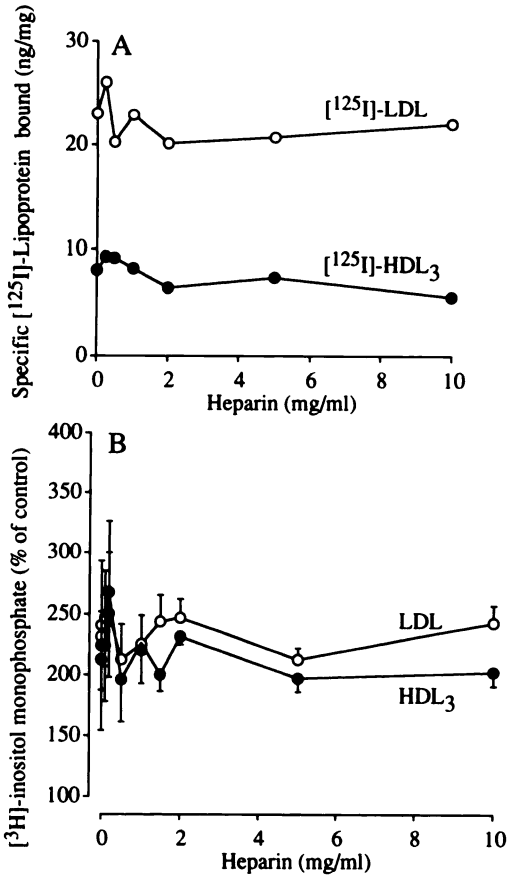

Fig. 7. Heparin does not inhibit either LDL and HDL_3 binding or signaling responses in VSMC. A, VSMC were incubated for 4 hr at 4° with 5 μg/ml ^{125}I-LDL or ^{125}I-HDL_3, in the absence or presence of the indicated concentrations of heparin. Nonspecific ^{125}I-LDL and ^{125}I-HDL_3 binding was determined by including excess (1 mg/ml) unlabeled corresponding lipoprotein in parallel series of dishes. Mean values from two separate experiments are presented. B, *myo*-[2-^{3}H]Inositol-prelabeled VSMC were pretreated with the indicated concentrations of heparin for 30 min and then exposed for 5 min to LDL or HDL_3 (each at 100 μg/ml). After resolution of cell lysates on Dowex anion exchange columns, radioactivity in the inositol monophosphate fraction was determined. Values (mean ± standard deviation, four experiments) express changes in [^{3}H]inositol monophosphate content relative to the content (100%) in control VSMC that had been pretreated with heparin but not exposed to lipoproteins. Under the given experimental conditions, heparin by itself did not alter the content of [^{3}H]inositol monophosphate.

receptors per cell depends on the rate of cell division, the age of the cells, and cellular requirements for cholesterol (1) and, at least in fibroblasts, expression of apo B/E receptors is maximal in actively proliferating cells (21). In VSMC, up-regulation of expression of the apo B/E receptor gene has been shown to be stimulated by growth factors (34). Therefore, the present observation of a low capacity for high affinity LDL binding in VSMC may also be due to our standard procedure of rendering VSMC cultures quiescent (48-hr serum deprivation) before all experiments.

The identity of the second recognition site/receptor for LDL in quiescent VSMC is unknown. Binding of LDL to this site occurs with low affinity ($K_d \approx 40$ μg/ml) and is heparin insensitive, which contrasts sharply with the high affinity ($K_d \approx 1$ μg/ml), heparin-sensitive characteristics of LDL binding to the apo B/E receptor. An additional difference between the two sites concerns ligand (lipoprotein) specificity. High affinity LDL binding to intact cells, membrane preparations, or purified apo B/E receptors cannot be inhibited by HDL (35, 36), whereas the specificity of the low affinity LDL recognition site is relatively relaxed, inasmuch as we observed a high degree of ^{125}I-LDL displacement by HDL_3 in VSMC. Although antioxidants were present throughout lipoprotein isolation procedures and LDL preparations were free of protein fragments known to be generated during LDL oxidation (20), we did consider the possibility that the low affinity LDL binding observed may represent the binding to "scavenger" receptors of a small proportion of oxidized ^{125}I-LDL present in our preparations. However, the present LDL binding experiments were performed under conditions (i.e., VSMC cultures at quiescence) in which expression of the acetyl-LDL (scavenger) receptor in VSMC is negligible (37). Even after up-regulation of scavenger receptors in VSMC by preincubation of cells with serum or phorbol ester, the B_{max} of ^{125}I-acetyl-LDL is only 10% of that in macrophages (37) and at least 1 order of magnitude less than the presently observed B_{max} for low affinity LDL binding. Such major quantitative differences in binding characteristics render it very unlikely that our observed low affinity binding of LDL to VSMC may be due to partial oxidation of LDL. Therefore, the properties of low affinity LDL binding are quite different from those of the known (apo B/E and scavenger) cell surface LDL receptors.

LDL and other apo B-100-containing lipoproteins such as intermediate density lipoprotein and the lipoprotein Lp(a) can interact with sulfated proteoglycans (38, 39). Such proteoglycans are normal constituents of the extracellular matrix elaborated by VSMC in culture (40) and could explain our observations of low affinity LDL binding to VSMC. Heparin is structurally similar to those glycosaminoglycan moieties (e.g., chondroitin/dermatan sulfate) present in sulfated proteoglycans and therefore competitively inhibits binding of LDL to proteoglycans (38, 39, 41). However, the presently described

TABLE 1

Pertussis toxin sensitivity of LDL- and HDL_3-induced signaling

Fura-2-loaded VSMC and *myo*-[^{3}H]inositol-prelabeled VSMC were preincubated for 4 hr in the absence or presence of pertussis toxin (1 μg/ml). Thereafter, $[Ca^{2+}]_i$ and inositol phosphate production responses to LDL or HDL_3 (each at 100 μg/ml) were measured. All experimental procedures are described in Materials and Methods. The increase in $[Ca^{2+}]_i$ represents the peak change above basal $[Ca^{2+}]_i$ (≈40–60 nM), and [^{3}H]inositol content in total inositol phosphates was determined after a 5-min incubation in the absence (basal) or presence of lipoproteins. Data given are mean ± standard deviation values from at least four separately performed experiments.

	Increase in $[Ca^{2+}]_i$		[^{3}H]Inositol phosphate content		
	LDL	HDL_3	Basal	LDL	HDL_3
	nM			*dpm*	
Control	239 ± 80	240 ± 35	1313 ± 35	2297 ± 55	2199 ± 45
Pertussis toxin	98 ± 6[a]	75 ± 17[a]	1400 ± 50	1478 ± 65[a]	1596 ± 62[a]

[a] Significant differences ($p < 0.01$, at least) between control and pertussis toxin-treated VSMC.

heparin insensitivity of LDL binding by quiescent VSMC does not support the idea that LDL-proteoglycan interactions contribute to low affinity LDL binding.

The affinity and binding capacity of HDL_3 (and apo E-free HDL_3) binding in human microarteriolar VSMC correlate well with the properties of apo E-free HDL_3 binding previously described for fibroblasts and arterial smooth muscle cells (19). Evidence that the binding of HDL does not involve the classical (apo B/E) LDL receptor was previously obtained by the demonstration that LDL (apo B/E) receptor-negative fibroblasts exhibit almost identical saturation binding profiles for ^{125}I-HDL_3 and apo E-free ^{125}I-HDL (19). Our finding that HDL_3 is capable of stimulating diacylglycerol formation in VSMC is consistent with the observation that in fibroblasts binding of HDL_3 to the HDL receptor is accompanied by activation of protein kinase C (15). In fibroblasts, activation of protein kinase C has been proposed to mediate HDL receptor-dependent efflux of intracellular cholesterol by facilitating the translocation of cholesterol from intracellular pools to the plasma membrane (15, 42, 43).

Competition binding studies in VSMC demonstrated that LDL and HDL_3 are capable of displacing one another. Such displacement either may represent very low affinity competition (albeit at sites not resolvable on the Scatchard plots) or may reflect a mixed-type noncompetitive mechanism. The latter interaction has been described previously for LDL and HDL binding sites on blood platelets (44). In support of this mechanism we have obtained preliminary results (data not shown) from Scatchard analysis of ^{125}I-LDL saturation binding (at 1–100 μg/ml, without or with inclusion of 200 μg/ml unlabeled HDL_3) to indicate that inclusion of HDL_3 produces both a decreased B_{max} (from 76 to 48 ng/mg) and decreased affinity (K_{d2} changes from 39 to 74 μg/ml).

Half-maximal stimulation of phosphoinositide catabolism and elevation of $[Ca^{2+}]_i$ in quiescent human microarteriolar VSMC were observed at LDL or HDL_3 concentrations of ≈35–45 μg/ml. These EC_{50} values closely approximate K_d values obtained for HDL_3 and low affinity LDL binding in VSMC, raising the possibility that these lipoprotein recognition sites (receptors) may be coupled to activation of second messenger systems. As found in lipoprotein binding experiments, cell signaling effects of LDL and HDL_3 were also insensitive to inhibition by heparin. When LDL and HDL_3 were added to VSMC in combination, additive effects on inositol phosphate production were observed, suggesting that LDL and HDL_3 exert their effects on cell signaling through LDL-specific and HDL_3-specific binding proteins, respectively. The additive response of VSMC to LDL and HDL_3 is consistent with the results from competition binding studies, in which LDL and HDL_3 were determined to bind to distinct sites.

Because LDL and HDL have opposite effects on cholesterol transport (1, 3) but similar effects on transmembrane signaling, the effects of LDL and HDL on signaling events in VSMC are unlikely to be related to lipid metabolism. Furthermore, the rapidity and transience with which lipoprotein-induced cell signaling occurs do not support the idea that cell signaling is due to net changes in cell cholesterol content. The kinetics of inositol phosphate generation, diacylglycerol production, and elevation of $[Ca^{2+}]_i$ in response to LDL and HDL_3 are comparable to those established for a diverse array of Ca^{2+}-mobilizing hormones. The pertussis toxin sensitivity of LDL- and HDL_3-stimulated Ca^{2+} mobilization and phosphoinositide catabolism further accentuates the resemblance between lipoprotein-stimulated cell signaling and receptor-coupled transmembrane signal transduction by Ca^{2+}-mobilizing hormones.

This study has demonstrated a correspondence between lipoprotein binding and lipoprotein activation of cell signaling in quiescent VSMC, with respect to concentration- and heparin-insensitive characteristics. However, at present we are unable to unequivocally conclude that HDL_3 and LDL couple to second messenger systems in VSMC via the HDL_3 and low affinity LDL binding sites described herein. Further investigation is necessary to substantiate the proposed correlation between binding and cell activation and to determine the physiological function(s) of lipoprotein-mediated signal transduction.

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Send reprint requests to: T. J. Resink, Department of Research ZLF 320, University Hospitals, CH 4031 Basel, Switzerland.