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Atypical Low Density Lipoprotein Binding Site That May Mediate Lipoprotein-Induced Signal Transduction

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SUMMARY

The characteristics of low density lipoprotein (LDL) binding in quiescent cultures of human vascular smooth muscle cells (VSMC) have been further investigated and compared with the characteristics of high affinity LDL binding in human fibroblasts [via the apolipoprotein (apo) B/E receptor] and with the properties of LDL-induced phosphoinositide catabolism in VSMC. In VSMC the bulk of specific ^{125}I-LDL binding occurs at a low affinity site, several characteristics of which are distinct from those of ^{125}I-LDL binding to the apo B/E receptor in fibroblasts. (a) The affinity of LDL binding in VSMC is 25–50 times lower than that in fibroblasts ($K_d \approx 50$ μg/ml versus $K_d \approx 2$ μg/ml). (b) The kinetics of LDL association and dissociation in VSMC are more rapid than those in fibroblasts. (c) In contrast to apo B/E receptor-mediated binding of LDL in fibroblasts, binding of LDL to VSMC is insensitive to heparin, chemical modification of lysine residues, and chelation (with EDTA) of divalent cations. (d) Apo E-free high density lipoprotein$_3$ displaces labeled LDL more effectively in VSMC than in fibroblasts. (e) The ratio of bound/internalized LDL to degraded LDL differs markedly between fibroblasts and VSMC. LDL-stimulated phosphoinositide catabolism in VSMC, which occurs with an activation constant similar to the K_d for low affinity LDL binding, is insensitive to heparin, modification of lysine and arginine residues in LDL, and chelation of divalent cations. Thus, the atypical low affinity receptor in these cells may mediate the effects of LDL on signal transduction.

The importance of the LDL receptor in supplying cells with cholesterol and regulating plasma LDL levels has been established (1, 2). This receptor, via recognition of apo B-100, binds LDL with an affinity of 1–2 μg/ml (3) and delivers LDL cholesterol into hepatic and extrahepatic cells in mammalian species. Normally, two thirds of LDL clearance from plasma is mediated through this apo B/E receptor. Heritable mutations of the receptor (as in human familial hypercholesterolemia or in Watanabe hyperlipidemic rabbits) result in slower degradation of LDL (2, 3). The remainder of LDL clearance from plasma is mediated via a nonreceptor pathway (1, 2) or via receptors that are different from the apo B/E receptor. Other lipoprotein receptor types identified include the acetyl-LDL (scavenger) receptor, the apo E receptor, the HDL receptor, and the immunoregulatory receptor (2–4). However, the roles, structures, and mechanisms of action of these receptors are much less clearly detailed than those of the apo B/E receptor.

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There is much evidence indicating that LDL is capable of influencing cellular functions independently of its lipid-transporting pathway. For example, LDL can induce shape change in platelets (5), secretion of β-glucoronidase and prostaglandin E by monocytes (6), inhibition of relaxation and potentiation of constriction in arterial rings (7, 8), and exocytosis of phospholipid components of pulmonary surfactant by alveolar type II cells (9). Such rapid (within seconds to minutes) functional responses to LDL occur concomitantly with a number of intracellular events, including a decrease in phosphatidylinositol-4,5-bisphosphate levels, increases in inositol-1,4,5-trisphosphate levels, diacylglycerol levels, and $[Ca^{2+}]_i$, cytoplasmic alkalinization, and PKC activation (5, 7, 9–13). Importantly, intracellular signaling responses to LDL can occur even in the presence of antibodies against apo B/E receptor, as well as in cells lacking functional apo B/E receptors (14, 15). Thus, one can hypothesize the existence of a receptor for LDL that is distinct from the "classical" apo B/E receptor and that is coupled to signal transduction pathways. In fibroblasts, endothelial smooth muscle cells, and VSMC, HDL also stimulates

ABBREVIATIONS: LDL, low density lipoprotein; HDL, high density lipoprotein; IP, inositol monophosphate; apo B, apolipoprotein B; apo E, apolipoprotein E; apo B-100, apolipoprotein B-100; LPDS, lipoprotein-deficient serum; PKC, protein kinase C; PI, phosphoinositide; $[Ca^{2+}]_i$, intracellular calcium concentration; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; VSMC, vascular smooth muscle cell(s); BSA, bovine serum albumin; Ac-LDL, acetylated low density lipoprotein; Carb-LDL, carbamylated low density lipoprotein; MEM, minimal essential medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

inositol-1,4,5-trisphosphate and diacylglycerol formation and a transient cytosol-to-membrane translocation of PKC (12, 13, 16–18). Several characteristics of the intracellular signaling responses to LDL and HDL resemble those established for Ca^{2+}-mobilizing hormones that activate G protein-coupled phospholipase C. Firstly, a single $[Ca^{2+}]_i$ transient is observed in VSMC monolayers, whereas oscillatory changes of $[Ca^{2+}]_i$ occur in single VSMC (12, 13). Secondly, lipoprotein-stimulated PI catabolism is attenuated by activators of PKC and of cAMP- and cGMP-dependent protein kinases (12). Thirdly, induction of PI catabolism by lipoproteins in VSMC is pertussis toxin sensitive (13), thus invoking the participation of G proteins. Finally, PI catabolism and increases in $[Ca^{2+}]_i$ occur within seconds after addition of lipoproteins and the responses are both dose dependent and saturable (12, 13, 18). Such data strongly suggest that LDL and HDL possess "hormone-like" properties, and it thus seems pertinent to search for membrane receptor(s) mediating lipoprotein-stimulated signal transduction. To this end we have further examined the characteristics of LDL binding in cultures of quiescent human VSMC and compared the properties of LDL binding with those of LDL-induced PI catabolism.

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 (10). 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 (10). The isolation, characterization, and propagation of human skin fibroblasts was performed as described (19). For the studies described herein, VSMC and fibroblasts were used between passages 10 and 18; cell cultures were grown to confluence and rendered quiescent by serum deprivation and maintenance in serum-free medium containing 0.1% (w/v) BSA for 48 hr before experimentation. 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 male humans using sequential buoyant density centrifugation techniques, with the use of potassium bromide solutions for density adjustments (20) and with inclusion of 1 mM EDTA and 1 μM butylated hydroxytoluene during all isolation and dialysis procedures. Apo E-free HDL_3 was prepared from the total HDL fraction (density, 1.063–1.20 g/ml) by heparin-agarose affinity chromatography (21). 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 (22). LDL was subjected to different chemical treatments that modify lysine residues in apo B, namely acetylation (Ac-LDL) (23) and carbamylation (Carb-LDL) (24). Arginine residues of apo B were modified by 1,2-cyclohexanedione treatment (25). Complete chemical modification of LDL was confirmed by enhanced anodic electrophoretic mobility under nondenaturing conditions (data not shown). Protein concentrations of lipoprotein preparations were determined using the Lowry method.

Iodination of LDL and HDL_3 and binding studies. LDL and HDL_3 were ^{125}I-labeled on tyrosine residues (specific radioactivity, 150–450 cpm/ng of protein) using the iodine monochloride method, as described (26). Binding of ^{125}I-LDL and ^{125}I-HDL_3 was studied in confluent quiescent cultures of VSMC and fibroblasts essentially as described previously (10, 13, 26). Saturation binding of ^{125}I-LDL and ^{125}I-HDL_3 (1.0–100 μg/ml) was performed at 4° either in TES/HEPES-buffered (each at 10 mM, pH 7.3) MEM containing 0.1% BSA (MEM/BSA) (in the case of VSMC) or in the same medium supplemented with 5% LPDS (MEM/BSA/LPDS) (in the case of human skin fibroblasts). Parallel series of dishes contained excess (1 mg/ml) unlabeled lipoprotein for determination of nonspecific binding. After incubation for selected intervals (indicated in the figure legends), cell layers were washed (6 × 3 ml; total washing time, 10 min) 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. Competition binding studies were performed under the same conditions as described above, except that all dishes contained a fixed concentration of tracer (usually 5 μg/ml for fibroblasts and 30 μg/ml for VSMC) and increasing concentrations of unlabeled competing lipoprotein. Where indicated, the proportion of ^{125}I-LDL selectively bound to apo B/E receptors of fibroblasts was measured as described (26). Briefly, after binding protocols cell monolayers were washed free of unbound tracer and then further incubated for 1 hr at 4° in medium (MEM/BSA/LPDS) containing 10 mg/ml heparin. Radioactivity released into the medium represents that previously bound to apo B/E receptor (26). Equilibrium dissociation constants (K_d) and maximum binding capacities (B_{max}) were estimated by analysis of binding data using the nonlinear least-square fitting program LIGAND (27).

For measurement of association kinetics, cells were incubated for different times at 4° in MEM/BSA containing ^{125}I-LDL (50 μg/ml) (Fig. 1) and were rapidly washed (6 × 3 ml within 5 min), and cell-associated radioactivity was quantitated after solubilization in 0.1 M NaOH. The kinetics of dissociation were measured after incubation of monolayers with ^{125}I-LDL (50 μg/ml, for 4 hr at 4°) and rapid washing procedures. Labeled cultures were incubated at 4° in MEM/BSA, and at selected times (see Fig. 1) the medium was aspirated and cell-associated radioactivity was quantitated after solubilization in 0.1 M NaOH. Parallel series of dishes contained 1 mg/ml unlabeled LDL, to determine nonspecific binding at corresponding times points. Rate constants were calculated according to classical formulations (28). The dissociation rate constant (k_{-1}) was determined as the negative slope of the plot of log (RL^*) versus t, where RL^* is the concentration of bound ligand and t is time. The association rate constant (k_{+1}) was calculated using the formula $k_{obs} = (k_{+1} \times L^*) + k_{-1}$, where L^* is the free ligand concentration and k_{obs} is the "observed" association rate constant. The k_{obs} value was determined as the slope of the plot of log $[1 - (RL^*/RL^*_{eq})]$ versus t, where RL^*_{eq} is bound ligand at equilibrium. Duplicate or triplicate determinations were made for each data point in each independent binding experiment, and n indicates the number of independent experiments.

Measurements of PI catabolism and $[Ca^{2+}]_i$. 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-1X8 anion exchange columns have been described previously (10, 12, 13). 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 smooth muscle cells grown on glass coverslips, as detailed before (12, 13).

Results

Kinetics of ^{125}I-LDL binding to VSMC. To characterize LDL-recognizing sites on the surface of quiescent VSMC, we have applied a widely used procedure utilizing LDL labeled with ^{125}I on tyrosine residues by the iodine monochloride method (26). We previously observed that stimulation of phospholipase C by hormones and lipoproteins is impaired in actively growing cells (18). Thus, to make valid associations between binding and cell signaling, we have necessarily chosen

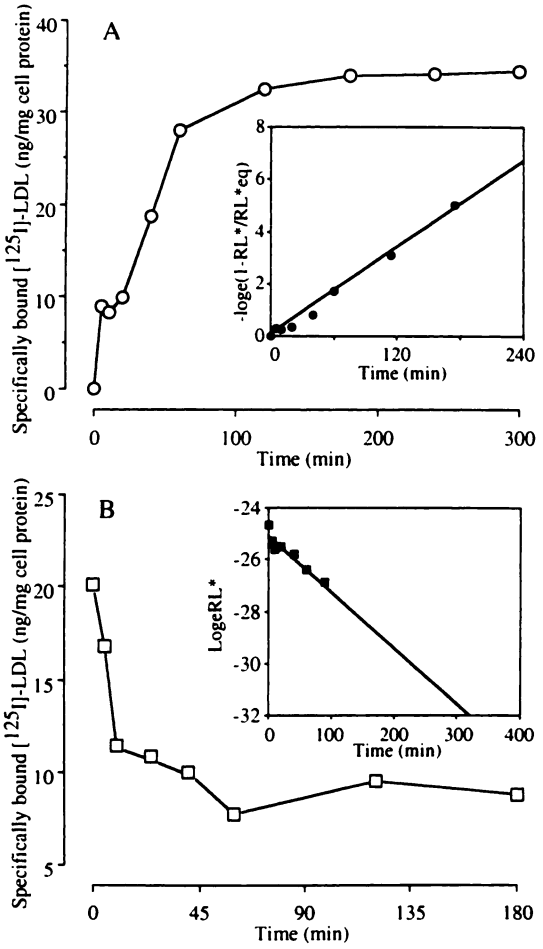

Fig. 1. Kinetics of ^{125}I-LDL interaction with quiescent human VSMC. A, VSMC were incubated with 30 μg/ml ^{125}I-LDL in the absence or presence of 1 mg/ml unlabeled LDL (for nonspecific binding) for the indicated times at 4°. After rapid washing (five washes within 5 min) and solubilization of cell monolayers in 0.1 M NaOH, cell-bound radioactivity was determined. Specific binding was calculated by subtraction of radioactivity bound in the presence of unlabeled LDL from radioactivity bound in the absence of unlabeled LDL. B, VSMC were preincubated for 3 hr at 4° in the presence of 30 μg/ml ^{125}I-LDL, without or with inclusion of excess unlabeled LDL. After washing, the dishes were incubated at 4° for the indicated times to allow dissociation and, after aspiration of supernatants, cell monolayers were solubilized in 0.1 M NaOH and radioactivity was quantitated. The plotted data represent specific binding, calculated as described for A. Direct and reverse rate constants were determined after linear transformation of binding data (*insets*). The data presented (mean values of triplicate determinations in a single experiment) are representative of three individual experiments.

to perform all binding studies on monolayer cultures of quiescent VSMC. The binding of ^{125}I-LDL to cultured fibroblasts was previously studied in culture medium supplemented with either BSA or LPDS, and LDL receptor activity was found to be maximally expressed under the latter condition (26). We found that the two culture conditions yielded equal estimates of specific binding of ^{125}I-LDL to VSMC, although the nonspecific binding was slightly lower in the presence of LPDS (data not shown). In spite of the sensitivity advantage gained with the latter, the use of BSA-containing medium (MEM/BSA) was considered preferable, not only because it is precisely defined (with respect to LPDS) but also because it is the medium utilized for signaling experiments in VSMC.

The binding of ^{125}I-LDL to VSMC at 4° approached equilibrium within 60 min of incubation (Fig. 1A). This contrasts with the 5–6-hr incubation period required to achieve equilibrium binding of ^{125}I-LDL to apo B/E receptors in fibroblasts (4). Specific ^{125}I-LDL binding to VSMC was reversible, with an estimated half-life of ≈20 min (Fig. 1B). Linear transformation of kinetic binding data (see Fig. 1, *insets*) revealed rate constants of $7.8 \pm 1.2 \times 10^3$ M^{-1} sec^{-1} (mean ± standard deviation, $n = 3$) for association (k_{+1}) and $2.8 \pm 0.7 \times 10^{-4}$ sec^{-1} (mean ± standard deviation, $n = 3$) for dissociation (k_{-1}). These values differ markedly from the kinetic characteristics of LDL binding to the classical apo B/E receptor, where $k_{+1} = 5.5 \times 10^4$ M^{-1} sec^{-1} and $k_{-1} = 6.3 \times 10^{-5}$ sec^{-1} (4). Inclusion of excess unlabeled LDL (1 mg/ml) in the dissociation medium did not significantly accelerate the dissociation process in VSMC ($k_{-1} = 6.4 \times 10^{-4}$ sec^{-1}) (data not shown), suggesting a lack of or weak negatively cooperative interactions between binding sites. The rapid dissociation of specifically bound ^{125}I-LDL from VSMC might explain why these atypical sites have not been detected previously on VSMC. In the most commonly used method for analysis of LDL binding (26), the duration of the washing procedure is about 30–60 min, a period sufficient to permit extensive dissociation of LDL bound to the atypical site in VSMC (Fig. 1B). Therefore, in all subsequent binding experiments the total washing time did not exeed 10 min.

Dose dependence and heparin insensitivity of ^{125}I-LDL binding to VSMC. The interaction of LDL with the apo B/E receptor of fibroblasts is characterized by a K_d of 3×10^{-9} M (4) and can be inhibited by heparin (26, 29). In good correlation with these data, we observed high affinity binding of ^{125}I-LDL to fibroblasts [$K_d = 2 \pm 1.2$ μg/ml (4×10^{-9} M), $B_{max} = 203 \pm 97$ ng/mg of cell protein, $n = 3$], which was inhibited by heparin (Fig. 2A). In VSMC, specific binding of ^{125}I-LDL was characterized by lower affinity [$K_d = 53 \pm 19.9$ μg/ml (1×10^{-7} M), $B_{max} = 86 \pm 16.5$ ng/mg of cell protein, $n = 9$] and insensitivity to inhibition by heparin (Fig. 2B). From data obtained in the study of the kinetics of LDL binding to quiescent VSMC (Fig. 1), a K_d value of $0.36 \pm 0.1 \times 10^{-7}$ M ($n = 3$) was calculated (as the ratio of the dissociation rate constant to the association rate constant). This value is close to the K_d values obtained in saturation (Fig. 2) and competition (Fig. 3) binding studies.

Although the Scatchard plots of binding data tended to be nonlinear in all experiments on VSMC, assessment of binding parameters by nonlinear regression using the LIGAND program (27) did not consistently indicate a statistically significant improvement of fit of the data to a two-site model, compared with a one-site model (data not shown). The present difficulty in reproducibly resolving high affinity LDL binding sites probably reflects a down-regulation of the high affinity apo B/E receptor in cells maintained at quiescence (2) and is in accordance with our previous observation (13) that quiescent VSMC express only very small amounts of high affinity LDL receptors (<10% of total ^{125}I-LDL specifically bound).

In competition binding studies, cells were incubated in the presence of a fixed concentration of ^{125}I-LDL and increasing amounts of unlabeled LDL. Binding parameters estimated (using LIGAND) from competition binding studies were comparable to those estimated in saturation binding studies. In fibroblasts, binding of LDL was characterized by a K_d of 2.5 ± 0.4 μg/ml (4.5×10^{-9} M, $n = 2$) (Fig. 3A). LDL binding in

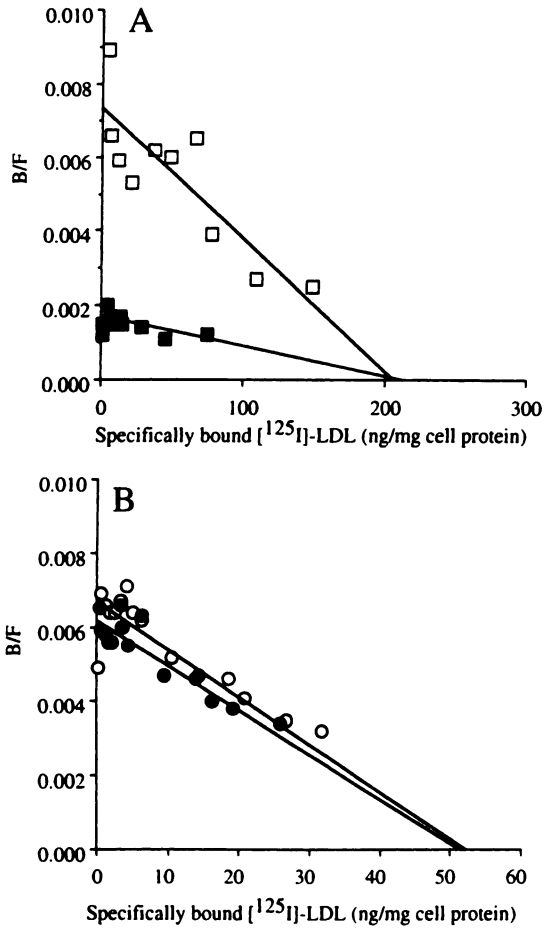

Fig. 2. Scatchard plots of specific ^{125}I-LDL binding to human fibroblasts and VSMC. Fibroblasts (A) and VSMC (B) were incubated for 4 hr at 4° in the presence of increasing concentrations of ^{125}I-LDL (1–100 μg/ml), either without (*open symbols*) or with (*closed symbols*) inclusion of 10 mg/ml heparin. The data (mean values of triplicate determinations in a single experiment) are representative of five independent experiments. Quantitative estimates of binding parameters are presented in the text.

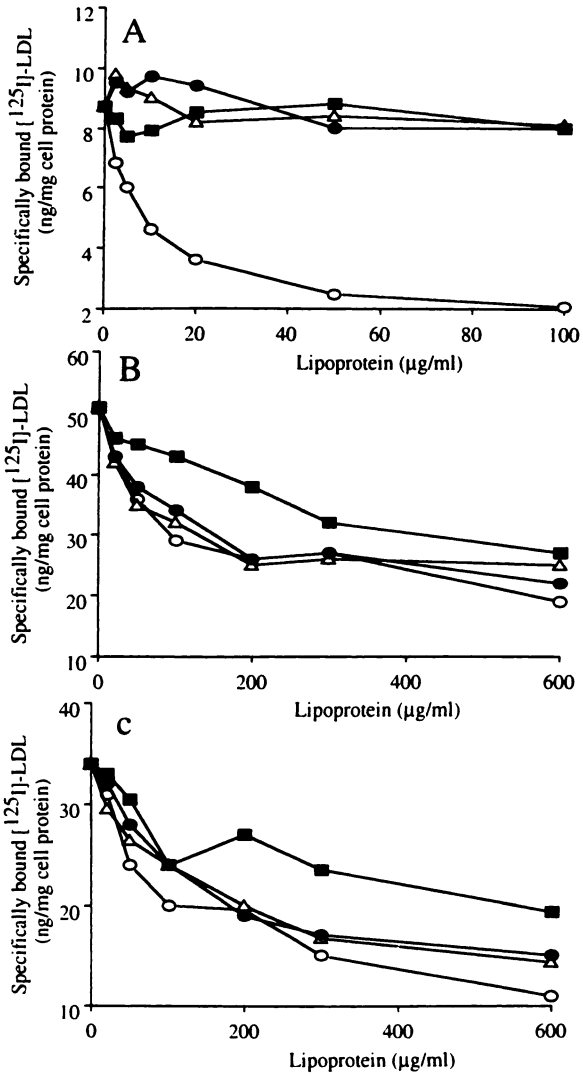

Fig. 3. Lipoprotein specificity of apo B/E receptors of fibroblasts and atypical LDL binding sites on VSMC. A, Fibroblasts were incubated for 4 hr at 4° in the presence of 5 μg/ml ^{125}I-LDL, with the indicated concentrations of unlabeled LDL (○), HDL_3 (■), Ac-LDL (△), or Carb-LDL (●). ^{125}I-LDL specifically bound to the apo B/E receptor was determined as heparin-releasable radioactivity (26). B and C, VSMC were incubated for 4 hr at 4° in the presence of 30 μg/ml ^{125}I-LDL, with the indicated concentrations of unlabeled LDL (○), HDL_3 (■), Ac-LDL (△), or Carb-LDL (●) and either without (B) or with (C) inclusion of 10 mg/ml heparin. ^{125}I-LDL specifically bound to VSMC was determined. Data represent mean values of triplicate determinations from a single experiment. Comparable results were obtained in at least two additional independent experiments.

VSMC was characterized by a K_d of 50 ± 1.0 μg/ml (1×10^{-7} M, $n = 5$) (Fig. 3B) and insensitivity to heparin (Fig. 3C).

The data thus far presented indicate that in fibroblasts the majority of specific LDL binding is mediated by a high affinity, heparin-sensitive site, whereas in quiescent VSMC high affinity binding represents only a very small proportion of specific binding, with the major portion being due to interaction of LDL with a low affinity, heparin-insensitive site. It should be stated, however, that we do not exclude the presence of low affinity binding sites for LDL in fibroblasts. Although we can presume that the high affinity binding site for LDL in VSMC represents the apo B/E receptor, the identity of the low affinity LDL binding site is unknown. The following experiments were aimed at further distinguishing the properties of high affinity LDL binding sites (using fibroblasts) and low affinity LDL binding sites (using quiescent VSMC).

Effects of EDTA and HDL_3 on ^{125}I-LDL binding to VSMC. An absolute requirement for the presence of divalent cations is characteristic of LDL binding to the apo B/E receptor (1, 26). As expected, we observed complete inhibition of ^{125}I-LDL binding to the high affinity receptors of fibroblasts in the presence of EDTA (Fig. 4). Binding of LDL to VSMC was minimally reduced by EDTA, indicating a different (compared with the apo B/E receptor) divalent cation requirement for low affinity LDL binding (Fig. 4). This partial inhibition may reflect the presence of a small proportion of EDTA-sensitive apo B/E receptors.

The high affinity LDL receptor recognizes apo B/E- and apo E-containing lipoproteins and does not interact with apo E-free HDL (1, 26). We have found that HDL_3, additionally purified from apo E by adsorbtion on heparin-agarose (21), did not compete with ^{125}I-LDL for binding to high affinity receptors on fibroblasts (Fig. 3A). In contrast, HDL_3 displaced ^{125}I-LDL in VSMC, albeit much less potently [K_i = 200 ± 113 μg/ml (2×10^{-6} M), $n = 3$] than did unlabeled LDL (Fig. 3B). A very

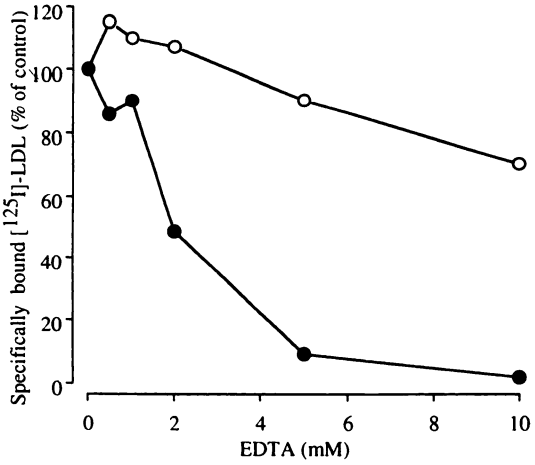

Fig. 4. Effect of EDTA on the ability of ^{125}I-LDL to interact with high affinity receptors on fibroblasts and atypical binding sites on VSMC. VSMC (○) and fibroblasts (●) were incubated for 4 hr at 4° in the presence of the indicated concentrations of EDTA and either 5 μg/ml (fibroblasts) or 30 μg/ml (VSMC) ^{125}I-LDL. ^{125}I-LDL specifically bound in the presence of EDTA is expressed as a percentage of that bound (100%) in the absence of EDTA. Absolute values for 100% specific binding were 10 and 48 ng/mg of cell protein for fibroblasts and VSMC, respectively. The data (mean values of duplicate determinations in a single experiment) are representative of three independent experiments.

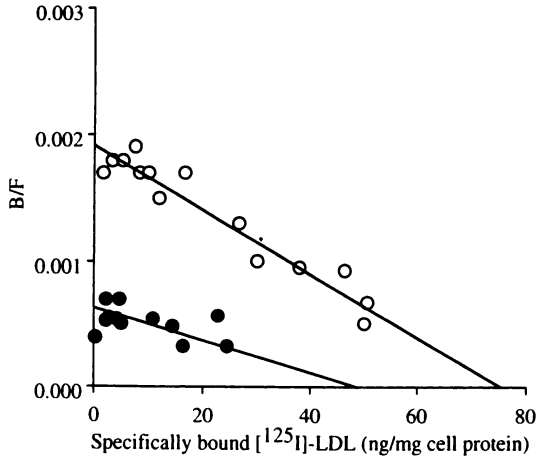

Fig. 5. Binding of ^{125}I-LDL to VSMC in the presence of HDL. VSMC were incubated for 4 hr at 4° with increasing concentrations of ^{125}I-LDL (1–100 μg/ml), either in the absence (○) or in the presence (●) of 200 μg/ml HDL_3. Data for specific binding of ^{125}I-LDL represent mean values of duplicate determinations.

similar competition profile was observed in the presence of heparin (K_i = 510 μg/ml) (Fig. 3C). Although it is thus evident that HDL_3 can displace ^{125}I-LDL bound to the low affinity, heparin-insensitive, binding sites, the data nevertheless indicate that the atypical, low affinity, LDL binding sites on VSMC preferentially recognize apo B-containing lipoproteins. To further investigate the mechanisms whereby HDL_3 displaces LDL, we compared the concentration-dependent binding of ^{125}I-LDL to VSMC in the absence and presence of excess (200 μg/ml) HDL_3. Scatchard transformation of the binding data (Fig. 5) revealed that HDL_3 decreases the B_{max} (78 ng/mg of cell protein versus 47 ng/mg of cell protein in the absence and presence of HDL_3, respectively) and increases the K_d [43 μg/ml (0.86×10^{-7} M) versus 98 μg/ml (2×10^{-7} M) in the absence and presence of HDL_3, respectively]. Displacement of ^{125}I-LDL by HDL_3 appears not to be truly competitive (both K_d and B_{max} were altered), and the data instead support a noncompetitive mixed type of interaction. This may result from the interaction of HDL_3 either with a separate receptor or with a site on the low affinity LDL receptor that is distinct from that recognized by LDL. This view is supported by our previous demonstration that VSMC exhibit additive signaling responses to LDL and HDL_3 (13).

Effect of modification of lysine residues on ^{125}I-LDL binding. Positively charged lysine and arginine residues of apo B play a central role in the recognition of LDL by the apo B/E receptor (3). Anionization (4, 23, 24) or blockade of these residues with heparin (29) attenuates the interaction of LDL with the high affinity receptor. As expected for fibroblasts, chemically anionized (by acetylation or carbamylation) preparations of LDL did not not compete with ^{125}I-LDL for binding to high affinity receptors (Fig. 3A). In contrast, in VSMC both Ac-LDL and Carb-LDL displaced specifically bound ^{125}I-LDL, and this displacement occurred both in the absence [K_i values of 33 μg/ml (0.66×10^{-7} M) and 50 μg/ml (1.0×10^{-7} M) for Ac-LDL and Carb-LDL, respectively] (Fig. 3B) and in the presence of heparin (Fig. 3C). In the presence of heparin, K_i values for Ac-LDL and Carb-LDL were 75 μg/ml (1.5×10^{-7} M) and 65 μg/ml (1.3×10^{-7} M), respectively (Fig. 3C). The interaction of LDL with low affinity receptors on VSMC therefore does not depend upon the polycationic structure of apo B. Ac-LDL, but not native LDL, is a recognized ligand for the "scavenger" receptor (1–3). Therefore, because native and Ac-LDL exhibited equal potencies in displacing ^{125}I-LDL from VSMC, it is possible to conclude that the low affinity binding of LDL in these cells is not mediated by the Ac-LDL (scavenger) receptor. Furthermore, the scavenger receptor is negligibly expressed in quiescent VSMC (30).

Binding, internalization, and degradation of ^{125}I-LDL at 37°. The apo B/E receptor functions to deliver lipoproteins to cells and thus to maintain cholesterol homeostasis (1–3). This is achieved by internalization and degradation of bound LDL, which can be measured at 37° (26). We investigated these processes using trypsin-releasable radioactivity as a measure of surface-bound ^{125}I-LDL (31). After prolonged incubation of fibroblasts, the major portion of cell-associated ^{125}I-LDL resides within the cells (26). In contrast, after a 5-hr incubation at 37° the major portion of cell-associated ^{125}I-LDL in quiescent VSMC remained on the cell surface (Fig. 6). Both surface binding and uptake in VSMC were only marginally inhibited by heparin (Fig. 6B), suggesting that the major portion of ^{125}I-LDL binding is mediated by the atypical LDL receptor. The degradation of lipoproteins by VSMC was, however, completely blocked by inclusion of heparin in the incubation medium. The data are consistent with the hypothesized existence of two LDL binding sites in VSMC, namely a heparin-sensitive site, which mediates internalization and degradation of ^{125}I-LDL, and a heparin-insensitive site, which is not (or is very slowly) internalized. The data in Fig. 6 also illustrate that the ratio of cell-associated LDL to degraded LDL in VSMC is very different from that known for fibroblasts. Another distinction between the apo B/E receptor and the low affinity LDL binding sites on VSMC is evident from comparison of surface binding at 4° and 37°. Although, as was the case for the apo B/E receptor, the LDL binding capacity of VSMC increased with temperature (B_{max} = 86 ± 16.5 ng/mg of cell protein at 4°, n = 9, versus 351

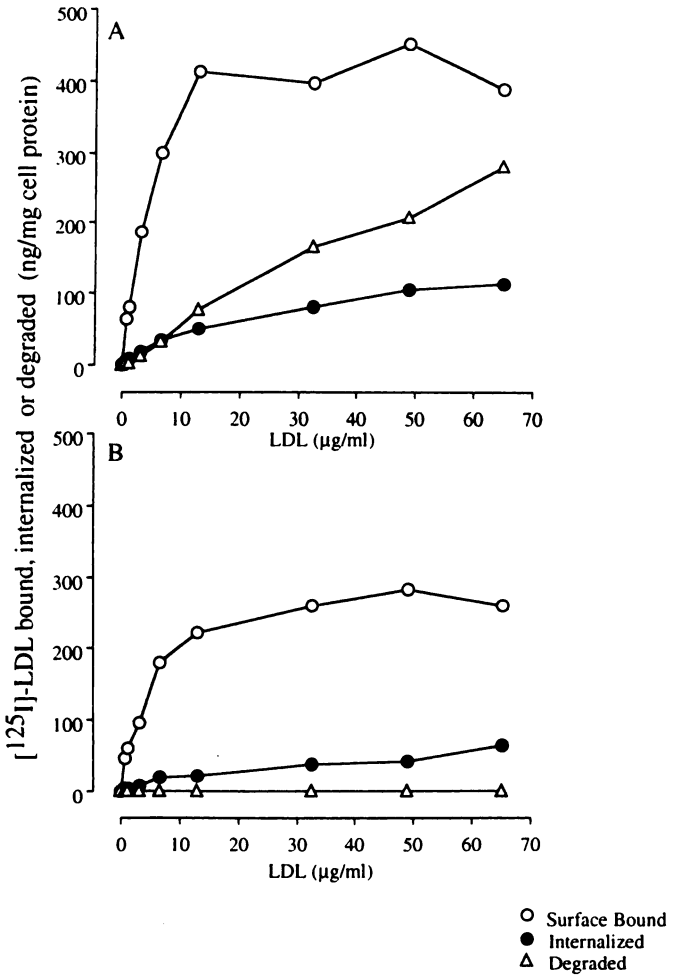

Fig. 6. Surface binding, internalization, and degradation of ^{125}I-LDL by VSMC. VSMC were incubated at 37° with the indicated concentrations of ^{125}I-LDL, without (A) or with (B) inclusion of 10 mg/ml heparin. Parallel series of dishes included 1 mg/ml unlabeled LDL for determination of nonspecific binding. After 5 hr, the dishes were transferred onto ice. The medium was collected for determination of degraded ^{125}I-LDL (△) by measurement of ^{125}I-tyrosine (26). After washing of monolayers, surface binding of ^{125}I-LDL (○) was determined as radioactivity released by trypsin (31). The cells were then solubilized in 0.1 M NaOH for determination of internalized (trypsin-insensitive) radioactivity (●). The data (mean values of triplicate determinations in a single experiment) are representative of two independent experiments.

± 194 ng/mg of cell protein at 37°, $n = 2$), the affinity of binding to the atypical receptor did not display the 10-fold decrease known for the classical LDL receptor (26). In contrast, the affinity of the atypical binding site tended to be higher at higher temperatures [$K_d = 53 \pm 19.9$ μg/ml (1×10^{-7} M) at 4°, $n = 9$, versus 18 ± 6.5 μg/ml (0.35×10^{-7} M) at 37°, $n = 2$].

Characteristics of LDL-stimulated PI catabolism in VSMC. To identify candidate membrane receptors through which LDL activates second messenger systems, we studied the action of LDL on PI catabolism (assessed as accumulation of IP) in VSMC under conditions known to block the interaction of LDL with apo B/E receptors. The binding of LDL to cultured fibroblasts, a classical cell system for investigation of the high affinity (apo B/E) LDL receptor, shows an absolute requirement for divalent cations (1–3), as illustrated in the present study by complete inhibition of ^{125}I-LDL binding in the presence of EDTA (Fig. 4). In VSMC, which marginally express high affinity binding sites for LDL, the chelation of divalent cations produced only a marginal effect on ^{125}I-LDL binding (Fig. 4) and no effect on the ability of lipoprotein to stimulate PI catabolism (Fig. 7A). It is well recognized that divalent cation chelation eliminates the contribution of the Ca^{2+} influx component to agonist-induced increases in $[Ca^{2+}]_i$. Thus, predictably, the $[Ca^{2+}]_i$ response to LDL was found to be weakened in the presence of EDTA (with loss of the second, sustained, phase of Ca^{2+} entry and therefore a reduced maximum increase) (Fig. 7B). Heparin, which is known to block the interaction of LDL with the apo B/E receptor (29), inhibited ^{125}I-LDL binding in fibroblasts but had no effect on the binding of LDL (Fig. 2) or the activation of PI turnover (Fig. 8) in VSMC. Lysine and arginine residues in apo B are crucial for interaction of LDL with the apo B/E receptor (4). Chemical modification of lysine residues (by acetylation or carbamylation) or arginine residues (by treatment with 1,2-cylcohexanedione) prevented binding of LDL to high affinity sites in fibroblasts (Figs. 3 and 4) but had only a minor effect on ^{125}I-LDL binding and the activation by LDL of PI catabolism (Fig. 9). Modification of lysine residues by malondialdehyde treatment (32) also did not impede the

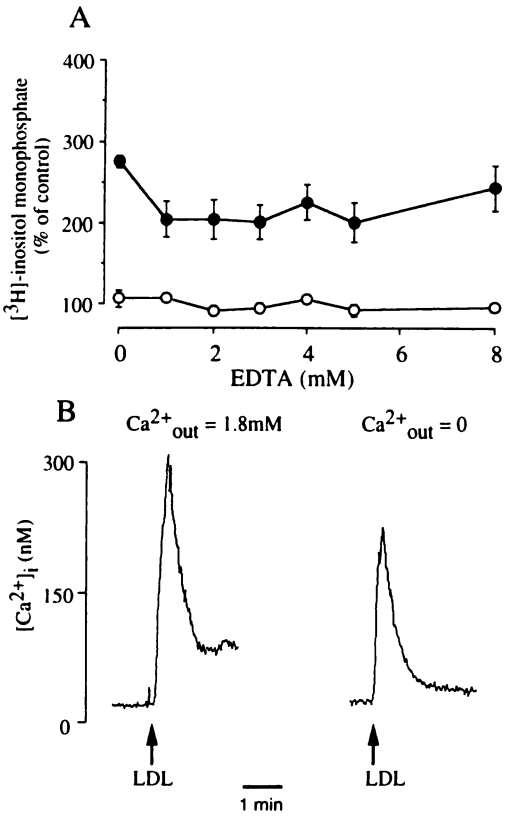

Fig. 7. Effects of EDTA on LDL-induced PI catabolism in VSMC. A, *myo*-[2-^{3}H]Inositol-prelabeled VSMC were incubated without (○) or with (●) LDL (100 μg/ml, for 6 min, in the presence of 15 mM LiCl), in the presence of the indicated concentrations of EDTA. Changes in [^{3}H]IP content are expressed relative to the level of [^{3}H]IP in samples not exposed to either EDTA or LDL. Data (mean values of triplicate determinations from a single experiment) are representative of four independent experiments. B, Monolayers of fura-2-loaded VSMC were incubated at 37° with 100 μg/ml LDL, either in Ca^{2+}-containing (1 mM $CaCl_2$) buffer or in Ca^{2+}-free (containing 1 mM EGTA) buffer. A typical fluorescence signal tracing of $[Ca^{2+}]_i$ is presented. The experiment was performed on at least four separate occasions, and cumulative values (mean ± standard deviation) for LDL-stimulated increases in $[Ca^{2+}]_i$ (at peak levels) were 259 ± 60 nM (in Ca^{2+}-containing buffer) and 156 ± 45 nM (in Ca^{2+}-free buffer).

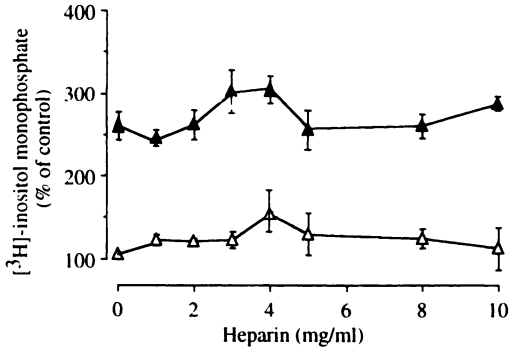

Fig. 8. Effect of heparin on accumulation of [^{3}H]IP in LDL-treated VSMC. *myo*-[2-^{3}H]Inositol-prelabeled VSMC were incubated without (▲) or with (△) LDL (100 μg/ml, for 6 min, in the presence of 15 mM LiCl), in the presence of the indicated concentrations of heparin. Changes in [^{3}H]IP content are expressed relative to the level of [^{3}H]IP in samples not exposed to either heparin or LDL. Data (mean values of triplicate determinations from a single experiment) are representative of three independent experiments. Heparin by itself did not influence the level of [^{3}H]IP.

ability of LDL to stimulate PI catabolism in VSMC (data not shown). Oxidatively modified (Cu^{2+}-treated) LDL, which also does not interact with the apo B/E receptor (1–3), is also an efficient stimulator of intracellular signaling in quiescent VSMC (33).

Discussion

The data in this paper demonstrate that the apo B/E receptor (detected as the high affinity site on fibroblasts) and an atypical, low affinity, LDL receptor (representing the bulk of specific ^{125}I-LDL binding in quiescent VSMC) differ in several respects. (a) The affinity of LDL binding in VSMC is 25–50 times lower than that in fibroblasts (Fig. 2). (b) The kinetics of LDL association and dissociation in VSMC are much faster than those in fibroblasts (Fig. 1). (c) In contrast to apo B/E receptor-mediated binding of LDL, neither heparin nor chemical modification of lysine residues inhibits LDL binding in VSMC, indicating that recognition of LDL by low affinity binding sites does not require intact, positively charged, amino acid residues (Figs. 2 and 3). (d) Whereas LDL binding to the apo B/E receptor is obligatorily dependent on the presence of divalent cations, binding of LDL to VSMC is insensitive to chelation (with EDTA) of divalent cations (Fig. 4). (e) Apo E-free HDL_3 more effectively displaces labeled LDL in VSMC than in fibroblasts (Fig. 3). (f) The ratio of bound/internalized LDL to degraded LDL differs markedly between fibroblasts and VSMC (Fig. 6). The presence of the atypical, low affinity, binding sites in VSMC are unlikely to represent an artifact of VSMC isolation or culture, because binding of ^{125}I-LDL to membranes isolated from the medial layer of human aortic tissue (without proteolytic enzymes) exhibits comparable affinity and kinetic characteristics (data not shown).

Previous observations of intracellular Ca^{2+}-mobilizing effects of LDL in fibroblasts and platelets of patients with hereditary defects in apo B/E receptor expression (14, 15), as well as the ability of oxidized LDL to stimulate PI catabolism in VSMC (33), have given rise to the hypothesis that lipoproteins stimulate intracellular signaling via a mechanism that is independent of the classical LDL receptor. Both LDL and HDL elicit a rapid (within seconds to minutes) and transient PI turnover

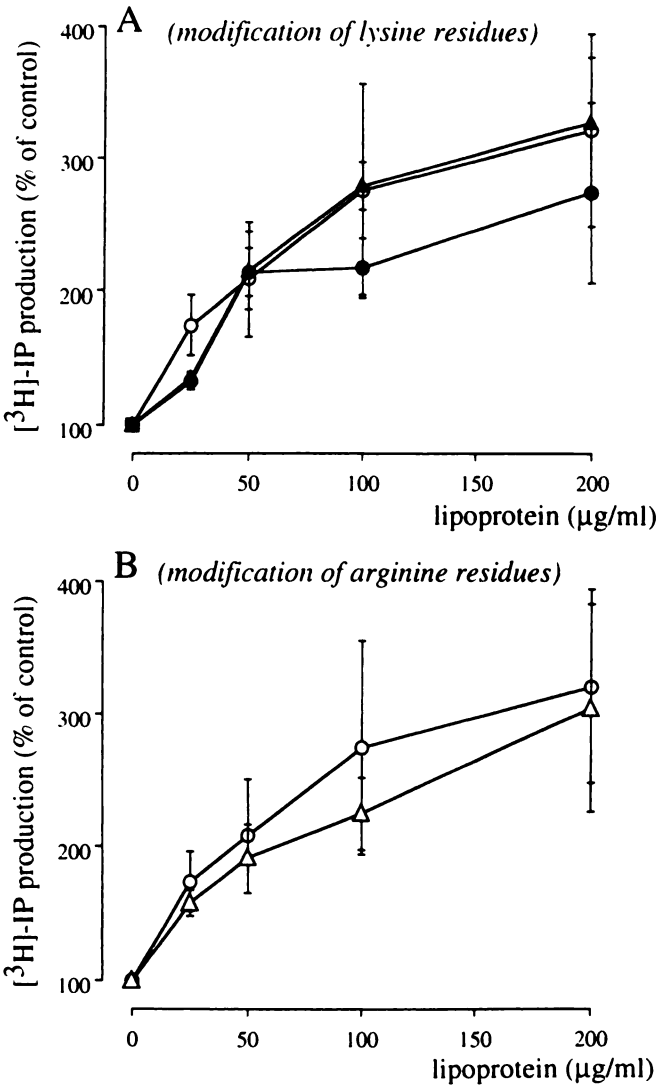

Fig. 9. Effect of chemical modification of apo B on the ability of LDL to stimulate PI catabolism in VSMC. *myo*-[2-^{3}H]Inositol-prelabeled VSMC were stimulated (for 5 min, in the presence of 15 mM LiCl) with the indicated concentrations of native LDL (○) or LDL preparations chemically modified on apo B either at lysine residues (●, Ac-LDL; ▲, Carb-LDL) (A) or at arginine residues (△, 1,2-cyclohexanedione-LDL) (B). Data given for [^{3}H]IP production are mean values of triplicate determinations in a single experiment. Similar results were obtained in two other independent experiments.

response in a number of cell types, including VSMC (12, 13), rendering it unlikely that these events are directly related to effects of LDL and HDL on lipid metabolism, because the two lipoproteins have much slower (hours) and opposing effects on cellular cholesterol content (1–3). The assumption that lipoproteins stimulate PI turnover via a specific signal transduction-coupled receptor is based on observations that lipoproteins, angiotensin II, and platelet-derived growth factor-BB generate qualitatively identical mixtures of inositol phosphate isomers (12) and that lipoprotein-induced PI signal transduction is coupled to a pertussis toxin-sensitive G protein and is inhibitable in VSMC pretreated with compounds that activate cAMP- and cGMP-dependent protein kinases and PKC (12, 13).

We have recently reported the existence of a low affinity

LDL binding site in quiescent VSMC (13). The present paper further characterizes this atypical binding site, and our data clearly demonstrate that the low affinity LDL receptor in VSMC is quite distinct from the apo B/E-recognizing receptor. The parallel insensitivity of LDL-stimulated PI catabolism and low affinity LDL binding to heparin, modification of lysine and arginine residues in LDL, and chelation of divalent cations allows us to propose that the atypical (low affinity) receptor mediates lipoprotein-stimulated signal transduction in VSMC. Atypical, low affinity, LDL binding sites have also been found in human platelets (34). LDL binding in platelets was characterized by a K_d of 5.1×10^{-7} M and forward and reverse rate constants of 1.47×10^4 M^{-1} sec^{-1} and 8×10^{-4} M^{-1} sec^{-1}, respectively. The binding was largely unaltered by divalent cations, chelating agents, HDL_3, heparin, or antibody (IgG-C7) against the apo B/E receptor. These characteristics of LDL binding in platelets resemble those described here for LDL binding in VSMC. Furthermore, atypical LDL binding sites are present in platelets of patients with familial hypercholesterolemia, and the Ca^{2+}-mobilizing action of LDL in these platelets and in platelets from normal donors is not different (15, 34). Such data further support the concept that atypical, low affinity, LDL binding sites mediate the effects of LDL on cell signaling.

The physiological role of LDL-induced PI catabolism, mobilization of intracellular Ca^{2+}, and activation of PKC has not yet been elucidated. However, because PKC and $[Ca^{2+}]_i$ are recognized to play pivotal roles in endocytosis, lipid metabolism, receptor expression, contraction, and growth, it is likely that the atypical receptor may mediate multiple cell functions. In human platelets, LDL-dependent cell signaling is associated with shape change, sensitization to adrenaline (35), and aggregation (5). In arterial rings, LDL-induced increases in $[Ca^{2+}]_i$ are associated with inhibition of relaxation and potentiation of vasoconstriction (11, 12). In cultured VSMC, LDL and HDL induce expression of early growth response genes (c-*fos* and c-*myc*) (36), suggesting a potential mitogenic role for lipoproteins. With respect to a role for LDL-stimulated signaling in growth regulation, it should be noted that activation of PI-specific phospholipase C can be mediated by G protein-coupled receptors or tyrosine kinase (growth factor-type) receptors. The sensitivity of LDL-stimulated PI catabolism to pertussis toxin (13) does not exclude the participation of growth factor-type receptors in LDL-promoted cell signaling. For the insulin-like growth factor receptor, which possesses intrinsic tyrosine kinase activity, direct interaction with and activation of the G_{i2} protein, reminiscent of those for G protein-coupled receptors, have been demonstrated (37). LDL- and HDL-induced PI catabolism and subsequent activation of PKC might also be involved in the regulation of cholesterol metabolism. PKC induces expression of mRNA encoding the apo B/E LDL receptor (38) and mediates HDL receptor-dependent efflux of intracellular cholesterol (17). The key roles of Ca^{2+} and PKC in regulating cell function, together with the existence of low affinity LDL binding sites that are apparently coupled to these regulatory systems, warrant future studies aimed at clearly defining the physiological/pathophysiological relevance of LDL-induced signaling in cells.

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