Evidence That Lipoproteins Are Carriers of Bioactive Factors

Agapios Sachinidis, Ralf Kettenhofen, Stefan Seewald, Ioanna Gouni-Berthold, Udo Schmitz, Claudia Seul, Yon Ko, Hans Vetter

Abstract—We recently demonstrated that the mitogenic effect of LDL (100 μg/mL) as well as its early intracellular signaling pathway are mediated by a pertussis-toxin (PTX)-sensitive G protein-coupled receptor that is independent from its classical receptor and involves activation of extracellular response kinases (ERK1/2) (also known as p44MAPK/p42MAPK). In the present study we examined whether LDL-adherent factors may be responsible for some of the effects of LDL. The term “signaling activity” is used to characterize fractions that cause an increase in intracellular free Ca$^{2+}$ concentration or stimulate ERK1/2 and c-fos mRNA expression. LDL, HDL, and VLDL stimulate ERK1/2 with the following order of potency: LDL $>$ HDL $>$ VLDL. After delipidation of LDL with chloroform/methanol/water mixtures a PTX-sensitive signaling activity was found in one fraction arbitrarily called LDL-F. After further analysis of LDL-F compounds by high pressure liquid chromatography, a PTX-sensitive signaling activity was detected only in the fraction with a retention time of 33 minutes (arbitrarily called LDL-F33). Similarly, after separation of sphingosine-1-phosphate (SPP) and sphingosylphosphorylcholine (SPC) by high pressure liquid chromatography, a PTX-sensitive signaling activity was found in the fractions 33 and 33 to 35, respectively. These findings demonstrate that the effects of LDL-F33 are mimicked by similar fractions collected from SPP/SPC, hence suggesting that these LDL-adherent molecules are possibly closely related to SPP/SPC. A PTX-sensitive signaling activity was also detected in HDL and HDL-F33. Therefore, LDL and other lipoproteins may function as carriers for bioactive phospholipids thereby contributing to the development of coronary artery disease. Our findings support a new research concept that may contribute in elucidating cellular mechanisms promoting coronary artery disease.

Key Words: lipoproteins ■ vascular smooth muscle cells ■ MAP kinases ■ pertussis toxin

In addition to deposition of cholesterol, prominent features of the atherosclerotic lesions include the proliferation of vascular smooth muscle cells (VSMC), cholesteryl ester-loaded macrophage foam cells, extracellularly trapped lipoproteins, and aggregated lipoproteins. The majority of cholesterol is transported into the cells by a receptor-mediated endocytosis through binding to its classic LDL receptor via apo B100. There is evidence that LDL stimulates the phosphoinositide catabolism, elevates (p44 MAPK/p42 MAPK) isoforms as well as elevates [Ca$^{2+}$]i, stimulates the Na'/H' exchange, and promotes the expression of c-fos and egr-1 in VSMC. Several laboratories reported that LDL itself and in combination with classical growth factors exerts mitogenic effects on VSMC, endothelial cells, and fibroblasts. Recently we demonstrated that LDL activates the 44 kDa and the 42 kDa mitogen-activated protein (MAP) kinase (p44MAPK/p42MAPK) isoforms as well as elevates [Ca$^{2+}$], via a pertussis-toxin (PTX)-sensitive guanosine triphosphate binding protein (G protein)-coupled receptor that is independent from its classical receptor. Therefore, we hypothesized that LDL-adherent factors may be responsible for the early cellular events elicited by LDL. Possible candidates for bioactive LDL-adherent factors are phospholipids such as lysophosphatidic acid (LPA), sphingosine-1-phosphate (SPP), sphingosylphosphorylcholine (SPC), and platelet activating factor (PAF). For this purpose, lipids were extracted from lipoproteins and their efficacy to stimulate early intracellular events such as an increase of [Ca$^{2+}$], a stimulation of p44MAPK/p42MAPK, and c-fos mRNA expression was tested. Like the intracellular signaling pathway of LDL, one characteristic feature of these bioactive lipids is that their intracellular signaling pathway is mediated by a PTX-sensitive G protein. Furthermore, subsequent separation of lipid fractions was accomplished by HPLC, and the effects of the resulting fractions were examined regarding the p44MAPK/p42MAPK activation and [Ca$^{2+}$]. The term “signaling activity” was used to characterize fractions that were able to stimulate an increase in [Ca$^{2+}$], phosphorylation of p44MAPK/p42MAPK, or c-fos mRNA expression. Similar experiments were performed with HDL and VLDL.

Materials and Methods

Materials

Dulbecco’s modified Eagle’s medium (DMEM), Ham’s F-10 and PBS were obtained from Gibco BRL. Fetal calf serum was obtained from Boehringer Mannheim. Platelet derived growth factor-BB was a gift from Prof Dr Jürgen Hoppe, Physiological Chemistry, Univer-

Received November 11, 1999; revision accepted February 25, 1999.
From the Medizinische Universitäts-Poliklinik, Wilhelmstr. 35-37, Bonn, Germany. Correspondence to Prof Dr A. Sachinidis, Medizinische Universitäts-Poliklinik, Wilhelmstr. 35-37, 53111 Bonn, Germany. E-mail sachinidis@uni-bonn.de
© 1999 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org

2412
sity of Würzburg, Germany. PhosphoPlus™ Antibody Kit was obtained from New England BioLabs, Inc. Thin layer chromatography (TLC) silica gel 60-glass plates were from Merck. SPP, PAF, and fura 2/pentaacetoxymethyl ester (fura 2/AM) were obtained from Calbiochem. LPA was from Sigma. Nucleosil 100 to 3 (250×4 mm) was from Machery and Nagel.

Isolation and Culture of Vascular Smooth Muscle Cells

Rat aortic VSMC were isolated from thoracic aorta from Wistar-Kyoto rats (6 to 8 weeks old, Charles River Wiga GmbH) by enzymatic dispersion using a slight modification of the method of Chamley.17 as described previously.18 Cells were cultured in DMEM supplemented with 10% fetal calf serum, nonessential amino acids, penicillin 100 IU/mL, and streptomycin 100 µg/mL at 37°C in the Steri-Cult incubator (Forma Scientific) in a humidified atmosphere of 95% air and 5% CO₂. The purity of VSMC cultures was confirmed by immunocytochemical localization of smooth muscle specific α-smooth muscle actin.

Lipoprotein Isolation

LDL (d=1.019 to 1.063 g/mL), HDL, and VLDL were isolated from the plasma of 4 normcholesterolemic subjects (serum cholesterol<6.2 mmol/L) by potassium bromide density-gradient ultracentrifugation according to Redgrave et al.19 The LDL, HDL, and VLDL fractions were dialysed against 0.15 mol/L NaCl containing 1 mmol/L EDTA, concentrated to 5 mg/mL with the ultrafilters Amicon, and stored under nitrogen atmosphere. No oxidation of LDL was observed at least 4 weeks after LDL preparation as assessed by measurement of malondialdehyde by the thiorbitaric acid method.12 Quantification of lipoproteins was performed by determination of the protein-component according to the method of Bradford.20 The purity of LDL, HDL, and VLDL was examined as described previously.6

Extraction of Lipid and Protein Components From Lipoproteins

Analysis and isolation was performed as described previously.21 Briefly, 0.4 mL of methanol was added to 0.1 mL of the LDL sample. After vortexing, 0.1 mL chloroform was added and samples were vortexed again. For phase separation, 0.3 mL of water was added, and the samples were vortexed vigorously and centrifuged for 1 minute at 9000g. Three phases were observed. (1) The upper phase that was arbitrarily called the LDL-F fraction was removed and dried under nitrogen stream. (2) The small interphase contained precipitated proteins, (3) the lower the chloroform phase. The interphase and the lower chloroform phase were mixed with 0.3 mL methanol and centrifuged for 2 minutes at 9000g to pellet the protein. The supernatant that was arbitrarily called the LDL-P fraction was removed and dried under nitrogen stream. Precipitated protein components were arbitrarily called LDL-Pr.

Thin Layer Chromatography of LDL-F and LDL-P Fraction

Components of the LDL-F fraction were dissolved in water whereas components of the LDL-P fraction were dissolved in methanol:chloroform (1:1). Aliquots of those 2 fractions were applied on silica gel TLC plates (20 cm×20 cm) precoated with concentrating zone. Separation of the components was performed using a first solvent system petroil ether/acetone (3:1, vol:vol) followed by a solvent system of chloroform:methanol:water (60:40:8, vol:vol:vol). Detection of the lipid components was performed by iodine vapor. Staining of substances containing a NH₂ group was performed using phospho-specific antibodies. Staining of substances containing a NH₂ group was performed using phospho-specific antibodies.

Separation of LDL-F Fraction or Commercial Bioactive Lipids by HPLC

Separation was performed in a Nucleosil 100-3 (250×4 mm) column using the HPLC system from Beckman with the Gold software system (Beckman).22 Different compounds were eluted with a mobile phase consisting of chloroform (arbitrarily called A) and chloroform:methanol:n-heptane:water:trifluoroacetic acid (100:40:50:15:5, vol:vol:vol:vol:vol) (arbitrarily called B) in a gradient mode by a flow of 1 mL/min. After an initial 1 minute with chloroform alone, the gradient was programmed so that the A-B changed to 100% B over a period of 25 minutes and held at B for 34 minutes. Finally, the HPLC pumps were programmed to 100% A for 10 minutes. After separations, the fractions were dried after evaporation of the organic solvents by nitrogen and the remaining traces of substances were dissolved in 100 µL water. Aliquots were tested for their ability to stimulate an increase in [Ca²⁺], or to stimulate p44/p42 phosphorylation.

Measurement of [Ca²⁺],

VSMC were cultured on round glass microscope slides (diameter, 12 mm) under normal tissue culture conditions until confluence. The medium was then replaced by serum-free medium consisting of a mixture of DMEM and Ham’s F-10 medium (1:1). After 24 hours cultivation in serum-free medium, VSMC were loaded with 2 µmol/L fura-2/pentaacetoxymethyl, and Ca²+-fura-2 fluorescence was measured at 37°C in a Perkin-Elmer LS50 fluorescence spectrofluorometer at excitation wavelengths of 340 and 380 nm and at an emission wavelength of 505 nm in HEPES buffer.23 Maximum (Rmax) and minimum (Rmin) fluorescence was determined by adding digitonin (30 µmol/L) followed by the addition of 1% Triton X-100 (vol:vol) and [Bis(aminooethyl)-]glycine [Sigma], N' nitro-acetic acid; 3,6-Dioxaacertamethylene[triglyceric acid] (EGTA) at a final concentration of 100 mM. Tris-base/25 mM EGTA. Fluorescence was corrected for cellular autofluorescence. Fluorescence signals were calibrated according to Grynkiewicz et al23 using the following equation: [Ca²⁺]=K₅×(R-Rmin)/(Rmax-Rmin)×(Sf/Sb). K₅ is the fura-2/Ca²⁺ complex at 37°C is assumed to be 224 mM/L. Sf is the 380 nm-excited fluorescence in the absence of Ca²⁺ (EGTA added) and Sb is the 380 nm-excited fluorescence in the presence of saturating Ca²⁺ (1 mM/L Ca²⁺).

Gel Electrophoresis and Immunostaining

Confluent VSMC in 3 cm diameter dishes were preincubated in 1 mL serum-free medium consisting of a mixture of DMEM and Ham’s F-10 medium (1:1) for 24 hours before stimulation. VSMC were then lysed with the SDS sample buffer containing 62.5 mM/L Tris-HCl, pH 6.8, 2% SDS (wt:vol), 10% glycerol, 50 mmoL dithiorthiole. Thirty µg of protein were analyzed by SDS-PAGE in a 10% polyacrylamide gel using the Mini Gel Protein system (Bio-Rad). After transfer of proteins to a polyvinylidenedifluoride membrane, blocking and antibody incubations were performed according to instruction manual of the PhosphoPlus MAPK Antibody Kit (New England BioLabs) using a phospho-specific mapk rabbit polyclonal IgG primary antibody and the alkaline phosphatase-conjugated anti-rabbit secondary antibody. The primary antibody recognized p42/p44 (Biosciences) as well as phospho-specific antibodies. The primary antibody recognized p42/p44 (Biosciences) as well as phospho-specific antibodies. The primary antibody recognized p42/p44 (Biosciences) as well as phospho-specific antibodies. The primary antibody recognized p42/p44 (Biosciences) as well as phospho-specific antibodies.

RNA Extraction and Analysis

Confluent VSMC in 75 cm² culture flasks were preincubated in 5 mL serum-free medium before stimulation with LDL or LDL-F for 30-minute periods. VSMC were then lysed with 1 mL TRI Reagent (Sigma) and total RNA was extracted according to manufacturer’s protocol. Northern blotting was performed after separation of 10 µg total RNA by electrophoresis as previously described.19 C-fos and β-actin mRNA were detected with a [1P]deoxyctydyme triphosphate ([3P]-dCTP)-labeled 1.0 kb v-fos cDNA and a [3P]-dCTP-labeled 77 kb σDNA probe (Dianova/Oncor), respectively. The size in kilobases (kb) of the detected mRNA was calculated by the 18S (1.8 kb) and 28S (4.6 kb) ribosomal RNA migration from the gel wells.
Determination of DNA Synthesis
The effect of LDL on [3H]thymidine incorporation into cell DNA was assessed as previously described. VSMC were seeded in 24-well culture plates and grown to confluence. Then the medium was replaced by serum-free medium consisting of a mixture of DMEM and Ham’s F-10 medium (1:1). After another 24 hours cultivation in serum-free medium, lipoproteins at a concentration of 100 μg/mL were added to the cells. Then cultures were exposed to the stimulating agents for 20 hours before 3 μCi/mL [3H]thymidine were added to the serum-free medium. Four hours later, experiments were terminated as described previously. Acid-insoluble [3H]thymidine was determined by using a liquid scintillation counter, model Beckman LS 3801, Duesseldorf, Germany, and determination of protein was performed using the Bio-Rad protein assay according to the method of Bradford.

Statistical Analysis
Data are presented as means±SE. Statistical analysis was performed by the one factor ANOVA test with Scheffe’s procedure.

Results

Analysis of Lipoproteins
The following lipids have been identified in LDL and LDL-P fractions (Figure 1A) after detection with iodine vapor: sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, cholesterol, triglycerides, and cholesterol ester. After evaporation of iodine, the plate was stained with ninhydrin for detection of NH2-group. The main component detected in LDL and LDL-P fraction by ninhydrin was phosphatidylethanolamine (Figure 1B). Furthermore, a component with an Rf value of 0.045 and small amounts of a component with an Rf value of 0.12 were detected by ninhydrin in LDL and LDL-P. The Rf value of commercially obtained SPP and SPC (both possess a NH2-group) was 0.29 and 0.04, respectively. The main apolipoproteins found in LDL is apo B100 with an apparent molecular weight of >200 kDa. The protein with an apparent molecular weight of 65 kDa (band between 84 and 42 kDa) has been identified as albumin. The small amount of protein with an apparent molecular weight of 28 kDa has been identified as ApoA-I. No significant amounts of apo B100 or albumin could be detected in the LDL-F and LDL-P fractions.

Effect of Lipoproteins on the Phosphorylation of MAP Kinase Isoforms in VSMC
When VSMC were stimulated with 100 μg/mL LDL (Figure 2A), HDL (Figure 2B), and VLDL (Figure 2B), maximal stimulation of the p44^{erek} and p42^{erek} occurred at 5 minutes. No stimulation of either isoforms could be observed after 30 minutes. Stimulation with PDGF-BB for 5 minutes resulted in a marked increase of the phosphorylated isoforms. As demonstrated in Figures 2C and 2D, the LDL-, HDL-, and VLDL-induced phosphorylation of both isoforms was dose-dependent, showing a maximal effect with a concentration of 100 μg/mL. Figure 2E shows the stimulation of p44^{erek}/p42^{erek} phosphorylation by the lipoproteins detected on the same blot. Statistical analysis of the band densities by laser densitometry obtained by separate experiments revealed that maximal stimulation by LDL, HDL, and VLDL at 5 minutes was 88.7±6.8% (n=12), 56.6±7.1% (n=5), and 22.8±3.7% (n=5) of the maximal effect of PDGF-BB at 5 minutes, respectively (P<0.05 for LDL effect versus HDL or VLDL effect, P<0.05 for HDL effect versus VLDL effect). As demonstrated in Figure 2F, stimulation of PTX-pretreated VSMC with 100 μg/mL LDL, 100 μg/mL HDL, and 5 μg/mL SPP for 5 minutes resulted in an almost inhibition of the phosphorylation of the MAP kinase isoforms. Finally, the effect of HDL on the MAP kinases activation in PD98059-treated VSMC was examined. As shown in Figure 2G, treatment of VSMC with the specific MAP kinase kinase (MEK) inhibitor PD98059 caused a complete inhibition of the HDL-induced phosphorylation of both MAP kinase isoforms. Treatment of the VSMC with PD98059 induced a 50% inhibition of the PDGF-BB-induced phosphorylation of p44^{erek}/p42^{erek}. It is conceivable that a concentration of PD98059 higher than 20 μmol/L might be necessary for complete abolishment of the PDGF-BB effect. As shown in Figure 2H, SPC caused a maximal phosphorylation of...
lated (catalytically activated) p42mapk and p44mapk.

Specific MAP kinase antibody which recognizes the phosphorylation at Tyr 204 in VSMC. VSMC were stimulated with 100 µg/mL LDL, HDL, and VLDL for 5 minutes. E, VSMC were stimulated with different concentrations of LDL (C) and VLDL (D) for 5 minutes. F, Confluent VSMC were preincubated in serum-free medium in the presence and absence of 100 nmol/L PTX for 24 hours before stimulation with LDL, HDL, and SPP for 5 minutes. G, VSMC were preincubated 2 hours with 20 µmol/L PD98059. Cells were then stimulated with 100 µg/mL HDL for 5 minutes. H, Confluent VSMC were preincubated in serum-free medium in the presence and absence of 100 nmol/L PTX for 24 hours before stimulation with 5 µg/mL SPC for different time periods. MAP kinase was detected by a specific MAP kinase antibody which recognizes the phosphorylated (catalytically activated) p42mapk and p44mapk at 15 minutes. Maximal phosphorylation was attenuated by 70% in PTX-treated cells.

Effect of Lipoproteins on the DNA Synthesis in VSMC

LDL, HDL, and VLDL at a concentration of 100 µg/mL caused an increase in [3H]thymidine incorporation from 100±8 (unstimulated cells) to 355±30%, 302±21%, and 223±12%, respectively (Figure 3).

Effect of Lipoprotein Compounds on [Ca²⁺] in VSMC

LDL (100 µg/mL) induced a maximal elevation in [Ca²⁺], from 40 to 140 nmol/L with a peak occurring at 10 seconds (Figure 4A). Although stimulation of VSMC with the corresponding LDL-F resulted in an elevation in [Ca²⁺], from 30 to 95 nmol/L at 10 seconds (Figure 4B), the corresponding LDL-P fraction had a negligible effect on [Ca²⁺], (Figure 4C). The effect of the LDL and LDL-F was evaluated by calculating the maximal effect of LDL and LDL-F within the first 10 seconds. LDL and LDL-F caused at 10 seconds an increase in [Ca²⁺], from 35±3 nmol/L (basal value, n=12) to 123±14 nmol/L (n=4) (P<0.05 for LDL effect versus basal value) and 115±9 nmol/L (n=4) (P<0.05 for LDL-F versus basal value). Because apo B₁₀₀, the main compound of LDL, was not soluble in water, a suspension of the corresponding LDL-protein (designated as LDL-Pr) was centrifuged and the supernatant used for testing its signaling activity. Assuming that the LDL-protein fraction might contain water soluble bioactive peptide(s) or proteins, we should expect an increase in [Ca²⁺]. As shown in Figure 4D, LDL-Pr had no effects on [Ca²⁺]. Like LDL-F (E), the corresponding VLDL-F fractions (F) and HDL-F fraction (G) from 100 µg/mL lipoprotein also stimulated an increase in [Ca²⁺], (please note that the lipoproteins in Figures 4E to 4G were isolated from a different donor than in Figures 4A to 4D).

Effect of LDL and LDL-F on p44mapk/p42mapk Phosphorylation and c-fos mRNA Expression

Stimulation of VSMC with 100 µg/mL LDL and the corresponding LDL-F for 5 minutes resulted in a stimulation of p44mapk/p42mapk (Figure 5A). The effect of LDL-F at 5 minutes was 54±4% (n=3) of the maximal effect of PDGF-BB at 5 minutes. Stimulation of VSMC with LDL and LDL-F for 30 minutes resulted in an expression of c-fos mRNA (Figure 5B). Laser densitometry of the band densities revealed that the effect of LDL and LDL-F on c-fos mRNA expression was 72±8% (n=3) and 56±5% (n=3) of the maximal effect of PDGF-BB at 30 minutes, respectively.

Effect of HPLC-Fractions on [Ca²⁺] After Separation of LDL-F by HPLC

Figure 6 (LDL-F) shows the HPLC profile of LDL-F at 280 nm. Fractions 10 to 45 (fraction volume 1 mL/min) were dried by nitrogen and then traces observed at the bottom of the glass tubes were dissolved in water. Aliquots were then tested regarding their ability to increase [Ca²⁺], in VSMC.
shown in Figure 6, signaling activity was detected only in fraction 33. LDL-F33 caused an increase in \([\text{Ca}^{2+}]_i\) at 10 seconds from 35±3 (basal value, n=12) to 116±9 nmol/L (n=4) (P<0.05 for LDL-F33 effect versus basal value).

To examine whether the signaling activity found in LDL-F33 may be exerted by adherent biolipids such as LPA, PAF, SPP, and SPC, separation of available commercial lipids by HPLC was performed, and fractions were tested for their ability to increase \([\text{Ca}^{2+}]_i\). As demonstrated after separation of LPA by HPLC, signaling activity was found only in fractions 29 to 30 (Figure 6, LPA). After separation of PAF, signaling activity was found only in fractions 38 to 40 (Figure 6, PAF). Remarkably, after separation of SPP (Figure 6, SPP) or SPC (Figure 6, SPC), signaling activity was found in fraction 33 or fractions 33 to 35, respectively.

Figure 4. Effect of LDL, LDL-F, LDL-P, LDL-protein, HDL-F, and VLDL-F on \([\text{Ca}^{2+}]_i\) in VSMC. Confluent VSMC on slides were precultured for 24 hours in serum-free medium. A, LDL (100 µg/mL) and the corresponding LDL-F (B and E), LDL-P (C), LDL-Proteins (D), VLDL-F (F), and HDL-F (G) isolated from 200 µg of the respective lipoprotein were applied to fura-2-loaded VSMC in 2 mL HEPES buffer and changes in fluorescence were monitored. After subtraction of autofluorescence, changes in 340/380 nm excitation wavelength ratio at an emission wavelength of 505 nm were converted into corresponding levels of \([\text{Ca}^{2+}]_i\).
**Figure 5.** Effect of LDL and LDL-F on p44mapk/p42mapk phosphorylation at Tyr 204 and of c-fos mRNA expression in VSMC. Confluent VSMC were precultured for 24 hours in serum-free medium. A, VSMC were stimulated with 100 μg/mL LDL and LDL-F isolated from 100 μg/mL LDL or with 50 ng/mL PDGF-BB for 5 minutes. Cells were lysed and 20 μg of protein were analyzed by SDS-PAGE. MAP kinase was detected after blotting on PVDF membranes by a specific MAP kinase antibody that recognizes the catalytically activated p42mapk and p44mapk. B, Confluent cells in 75 cm² flasks were precultured in 5 mL serum-free medium for 24 hours. Then they were treated with 100 μg/mL LDL, LDL-F isolated from 100 μg/mL LDL, and 50 ng/mL PDGF-BB. Ten μg of total RNA was electrophoresed on formaldehyde-agarose gels, blotted onto Hybond N membranes by a specific MAP kinase antibody that recognizes the catalytically activated p42mapk and p44mapk. Arrows show the 28S (4.6 kb), the 18S ribosomal RNA (1.8 kb), the 2.2 kb c-fos mRNA, and the 2.0 kb β-actin mRNA.

**Effect of HPLC-Fractions on p44mapk/p42mapk Phosphorylation After Separation of LDL-F and LDL-P by HPLC**

As illustrated in Figure 7A, only fraction 33 was able to stimulate phosphorylation of MAP kinase. Laser densitometry of the band densities revealed that the effect of LDL-F33 on p44mapk/p42mapk phosphorylation was 51±11% (n=4) of the PDGF-BB effect at 5 minutes. Control experiments were performed by examining the effect of fractions obtained after separation of the LDL-F fraction by HPLC on p44mapk/p42mapk phosphorylation. Dried LDL-P sample was dissolved in chloroform:methanol (1:2, vol:vol) before injection in the column. As shown in Figure 7B, none of the fractions was able to stimulate phosphorylation of p44mapk/p42mapk. Also, after separation of the respective HDL-F, signaling activity was found only in fraction 33 (see Figure 7C). As shown in Figure 7C, treatment of VSMC with PTX resulted in an abrogation of the LDL, HDL, LDL-F33, HDL-F33, and LPA-induced phosphorylation of p44mapk/p42mapk.

**Effect of PTX on the LDL-F33 and SPC-Induced Elevation of [Ca²⁺]**

As demonstrated in Figure 8, stimulation of PTX-treated VSMC with LDL-F33 (b) resulted in an approximately 50% of the maximal induced increase of [Ca²⁺], at 10 seconds compared with unstimulated cells (a). Treatment of the PTX-treated cells with SPP resulted in an almost complete inhibition of the SPP-induced increase of [Ca²⁺]. In contrast, the effect of SPC on [Ca²⁺] in PTX-treated VSMC was inhibited by 60%.

**Discussion**

One interesting observation was that all 3 lipoproteins stimulate the phosphorylation of p44mapk/p42mapk and that the stimulation by LDL and HDL occurs with a similar time kinetic. Maximal stimulation by LDL and HDL occurred with 20 μg/mL, whereas a stimulation by VLDL occurred with 100 μg/mL. Lipoproteins stimulate p44mapk/p42mapk phosphorylation with the following order of potency: LDL > HDL > VLDL. In concordance with our findings, more recently it has been demonstrated that HDL activates MAP kinase via multiple signal transduction pathways in human fibroblasts.

Lipid analysis of LDL, LDL-F, and LDL-P fraction revealed that cholesterol, cholesterol ester, triglycerides, phosphatidylethanolamine, phosphatidylcholine, and sphingomyelin were present in the LDL-F fraction but not in the LDL-F. Using ninhydrin, which stains NH₂-groups, we were able to detect one main component in LDL and LDL-F with an Rₗ value of 0.045 and a minor component in LDL-F with an Rₗ value of 0.12. Interestingly, the Rₗ value of the main component is similar to the Rₗ value of commercial SPC, which have an Rₗ value of 0.04. Analysis of protein compounds of LDL by SDS-PAGE demonstrated that LDL contained albumin, which is known to be a natural antioxidant preventing oxidation of LDL by the binding of Cu²⁺ in a nonspecific manner. It is well known that albumin is one contaminant of LDL observed in several preparation methods by ultracentrifugation (for review see reference 26). The LDL contained an intact apo B₁₀₀. An intact apo B₁₀₀ is also an indicator for native LDL because it has been repeatedly described that oxidation of LDL leads to degradation of these proteins to smaller peptides (for review see reference 27). These findings show that the LDL used in the present study was not oxidized. Oxidation of LDL was also routinely tested by the malondialdehyde method.

There is some evidence supporting our concept that the signaling pathway of LDL in different cell types including VSMC is mediated by lipoprotein-adherent factor(s) through a putative G₁ protein-coupled receptor. (1) An intact structure of lipoprotein particle is not a prerequisite for the signaling activity of lipoproteins because fractions obtained after delipidation (LDL-F, HDL-F, and VLDL-F) also stimulate intracellular events such as an increase in [Ca²⁺], and p44mapk/p42mapk phosphorylation. (2) After separation of LDL-F and HDL-F by HPLC, we found signaling activity only in fraction 33, and the effect of fraction LDL-F33 and HDL-33 on p44mapk/p42mapk phosphorylation was also PTX-sensitive similar to the effect of LDL and HDL. Furthermore, similar to the effect of LDL on [Ca²⁺], the effect of LDL-F33 on [Ca²⁺], in PTX-treated VSMC was partly PTX-sensitive. These results give direct evidence that most probably lipoprotein-adherent factor(s) are responsible for intracellular effects attributed to lipoproteins.

Because apo B₁₀₀ was not detected in LDL-F (see Figure 1C), involvement of apoB₁₀₀ in the LDL-induced signal transduction may be excluded. This conclusion may be supported by the observation that stimulation of human fibroblasts isolated from patients with familial hypercholesterolemia homozygote class I mutations, which are not able to produce the classical LDL receptor, also resulted in
stimulation of the p44mit and p42mit, DNA synthesis, and an increase in \([\text{Ca}^{2+}]\), and stimulation of the Na\(^+/\)H\(^-\)-exchanger. Bioactive phospholipids such as LPA and PAF have been excluded as candidate-bioactive lipoprotein-adherent factor(s) because after separation of commercial LPA or PAF by HPLC, signaling activity was found in fractions 29 to 30 or 38 to 40, respectively. In this context, Heery et al demonstrated that copper oxidatively modified LDL contains phospholipids with PAF-like activity that may be responsible for the growth promoting effects of LDL. Recently it has been demonstrated that native LDL stimulate MAP kinase, and the effect was potentiated by copper-oxidized LDL. Based on extraction experiments of oxidized LDL with organic solvent experiments, the authors suggest that the active moiety responsible for MAP kinase activation is a lipid moiety. However, it has been repeatedly described that copper-oxidized LDL is toxic to VSMC, endothelial cells, and fibroblasts (for review see reference 27). Furthermore, activation of the mitogen-activated protein MAP kinase occurs not only by growth factors but also stresses such as hypoxia, oxidative stress, osmotic imbalance, heat shock,
inhibition of protein synthesis, and irradiation resulting in cell death.\textsuperscript{30,31}

Therefore, we may propose that oxidized LDL may be one factor that causes injury of the endothelium and thereby may be implicated in the atherosclerotic process.

Furthermore, we demonstrated that the effect of SPP and SPC on stimulation of p44\textsuperscript{mpk}/p42\textsuperscript{mapk} phosphorylation and increase of \([\text{Ca}^{2+}]_{i}\) in VSMC is PTX-sensitive. However, the effect of SPP on \([\text{Ca}^{2+}]_{i}\) in VSMC was more PTX-sensitive compared with the effect of SPC. In this context, we showed that similar to LDL-F33, after separation of commercial SPP or SPC by HPLC, signaling activity was found in fraction 33 or fractions 33 and 34, respectively. These finding demonstrate that the effects of LDL-F33 are mimicked by similar fractions collected from SPP/SPC, suggesting that these LDL-adherent molecules are possibly closely related to SPP/SPC. From these results, we may deduce that lipoprotein-adherent factors with SPP/SPC-like activity are responsible for the stimulation of the early intracellular signaling pathway observed by native lipoproteins. Although further efforts are necessary to characterize the chemical structure of this(these) bioactive factor(s), we show for the first time that the signaling activity of the lipoproteins is derived from bioactive factors adsorbed on lipoproteins.

LDL is considered to be the main atherogenic class of lipoproteins, and elevated levels of LDL is one of the most important risk factors for atherosclerosis and cardiovascular morbidity.\textsuperscript{32} A close correlation between the concentration of LDL in human aortic intima and serum cholesterol level has been found.\textsuperscript{33} It has been proposed that most of circulating LDL is transported through vascular endothelium by transcytosis (classic LDL-receptor independent pathway) via plasmalemma vesicles that deliver LDL to other cells of the vascular wall.\textsuperscript{34} Moreover, it is assumed that in contrast to HDL, retention of LDL occurs through electrostatic interactions between apo B\textsubscript{100} and glycosaminoglycans, or hydrophobic interactions with elastin, both being connective tissue constituents of the vascular wall.\textsuperscript{35} Also, cardiovascular risk factors such as hypertension and hypercholesterolemia induce an elevation of the LDL transport from blood in the rat aortic intima.\textsuperscript{36} Furthermore, an increased transfer of LDL from blood to rat arterial vessels occurs after injection of animals with vasoactive substances such as serotonin, angiotensin II, and catecholamines.\textsuperscript{37} Thus it is likely that under such...
pathophysiological conditions, elevated lipoprotein-adherent factors with SPP/SPC-like activity may be transported by LDL to the intima and media of the vascular wall exerting their growth promoting effects. Therefore, we suggest that our in vitro findings significantly contribute to understanding mechanisms of coronary artery disease development.

On the other hand, there is an inverse correlation between an elevated plasma HDL and cardiovascular diseases. We demonstrated that although the effect of LDL on p44/42MAPK phosphorylation was more pronounced than that of HDL and VLDL, both HDL and VLDL are able to induce similar effects in VSMC including DNA synthesis. Our results are in concordance with the findings of other investigators showing that not only LDL but also HDL38,39 and VLDL38 stimulate DNA synthesis in VSMC. It is also described that HDL is able to stimulate an increase of c-fos mRNA in VSMC.40

We suggest that the atherogenic potential of SPP/SPC-like lipoprotein-adherent factors can be developed only after transportation by lipoproteins, especially by LDL. It is possible that in addition to the increased level of SPP/SPC-like factors in the LDL particle, an increased transcytosis through the endothelium or an increased retention of LDL particle may promote the development of coronary artery disease. In this context, Saxena et al41 demonstrated on cultured endothelial cells that lipoprotein lipase, apart from its enzymatic ability to hydrolyze plasma lipoprotein triglycerides, is capable of increasing the retention time of LDL and VLDL but not of HDL by interactions of lipoprotein lipase attached to matrix proteins with LDL or VLDL.41 In this context, it is possible that in addition to the increased level of SPP/SPC-like factors in LDL particle, the atherogenic potency of LDL or other lipoproteins may be dependent on the transcytosis ratio of lipoproteins through vascular endothelium and retention time in the vessel wall. Furthermore, it is conceivable that after an endothelium injury, all lipoproteins may be able to pass through endothelial barrier and promote atherosclerotic lesions through their growth promoting effects. Furthermore, oxidized LDL may cause injury of the endothelium, explaining its high atherogenic potency.

Acknowledgment

This work was supported by a grant of the Deutsche Forschungsgemeinschaft (Sa 568/4-1).

References


Evidence That Lipoproteins Are Carriers of Bioactive Factors
Agapios Sachinidis, Ralf Kettenhofen, Stefan Seewald, Ioanna Gouni-Berthold, Udo Schmitz,
Claudia Seul, Yon Ko and Hans Vetter

doi: 10.1161/01.ATV.19.10.2412
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272
Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://atvb.ahajournals.org/content/19/10/2412

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published
in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the
Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for
which permission is being requested is located, click Request Permissions in the middle column of the Web
page under Services. Further information about this process is available in the Permissions and Rights
Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online
at:
http://atvb.ahajournals.org//subscriptions/