Remnant Lipoprotein–Induced Smooth Muscle Cell Proliferation Involves Epidermal Growth Factor Receptor Transactivation

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Background—Remnant lipoproteins (RLPs) have been shown to play a causative role during atherosclerosis. Furthermore, it is known that vascular smooth muscle cell (SMC) proliferation is crucial for the development of atherosclerosis and restenosis after percutaneous coronary intervention. We examined the direct effect of RLPs on the proliferation and signal transduction of SMCs.

Methods and Results—Incubation in the presence of RLPs (20 mg cholesterol per dL) for 48 hours induced rat aortic SMC proliferation (2.3-fold over medium alone). RLPs also induced the phosphorylation of epidermal growth factor (EGF) receptor in SMCs, which was followed by the activation of mitogen-activated protein kinases. Moreover, the activation of protein kinase C (PKC) as well as the shedding of membrane-bound soluble heparin-binding EGF-like growth factor (HB-EGF) was observed after RLP treatment of SMCs, whereas PKC inhibitors and metalloprotease inhibitors inhibited RLP-induced EGF receptor transactivation and HB-EGF shedding in SMCs. Furthermore, anti-HB-EGF neutralizing antibody inhibited RLP-induced EGF receptor transactivation. Phosphorylation of EGF receptor and HB-EGF shedding were also observed in the aortas of apolipoprotein E–knockout mice but not in those of C57BL6 mice.

Conclusions—These results suggest that RLPs transactivate EGF receptor via PKC and HB-EGF shedding from SMCs, resulting in SMC proliferation. (Circulation. 2003;108:2679-2688.)

Key Words: lipoproteins ■ muscle, smooth ■ signal transduction ■ atherosclerosis

Recent studies have demonstrated that serum remnant lipoproteins (RLPs) are atherogenic and may be a risk factor for ischemic heart disease, independent of LDL and HDL.1 Our previous results have also suggested a causative role of RLPs in atherosclerosis.2,3

The proliferation of smooth muscle cells (SMCs) plays a critical role in intimal thickening of arteries associated with atherosclerosis or restenosis after percutaneous coronary intervention (PCI).4 Several lipoproteins have been reported to induce the migration and proliferation of SMCs via activation of mitogen-activated protein kinase (MAPK), in which G protein–coupled receptor (GPCR)–dependent protein kinase C (PKC) activation was shown to be involved.5,6

It is known that RLPs enter vessel walls, where they are easily taken up by macrophages via LDL receptors,7 resulting in foam cell formation. However, the direct effects of RLPs on SMCs in media have not been fully elucidated, although we recently showed that RLPs directly stimulate porcine coronary artery SMC proliferation, independent of oxidative stress.8 In line with those results, we examined the effects of RLPs on MAPK pathway and epidermal growth factor (EGF) receptor transactivation, along with the involvement of G protein–coupled receptor in rat aortic SMCs. Herein, we report for the first time that atherogenic RLPs from patients with hypertriglyceridemia stimulated rat SMC proliferation via heparin-binding EGF-like growth factor (HB-EGF) shedding and EGF receptor transactivation.

Methods

Cell Culture Reagents and Animals

SMCs were prepared from rat thoracic aortas and grown in DMEM (Sigma) supplemented with 10% FCS, 2 mmol/L L-glutamine, 100 μg/mL penicillin, and 100 U/mL streptomycin. Apolipoprotein E (apoE)–knockout male mice and C57BL6 male mice, each weighing between 29 and 32 g, were obtained from CLEA Japan (Tokyo, Japan) and Jackson Laboratory (Bar Harbor, Maine), respectively. All received water ad libitum and were fed a normal standard chow diet for 20 weeks, after which they were killed by heart puncture under diethyl-ether anesthesia. At 20 weeks of age, the apoE-knockout mice had already developed atherosclerotic lesions in the aorta. The aortas were carefully removed intact from the aortic arch to the iliac bifurcation and homogenized immediately for protein...
extraction. All animal procedures were approved by the Institutional Animal Care Committee of Tokyo Medical and Dental University (approval No. 0010209) and conducted according to Guidelines for Animal Experimentation at Tokyo Medical and Dental University. The antibodies used in the present study were as follows: rabbit anti-EGF receptor polyclonal antibody, rabbit anti-phospho-EGF receptor polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Calif), rabbit anti-MEK1/2 polyclonal antibody, rabbit anti-phospho-MEK1/2 polyclonal antibody, rabbit anti-ERK1/2 polyclonal antibody, rabbit anti-phospho-ERK1/2 polyclonal antibody, mouse anti-PKCα, β, δ, ε, γ, λ, η, ι, and θ monoclonal antibodies (New England Biolabs, Beverly, Mass), and anti-phosphotyrosine monoclonal antibody (pY20) (Cell Signaling, Beverly, Mass). A neutralizing goat anti-HB-EGF polyclonal IgG was obtained from Oncogene, and its specific inhibition was confirmed with rat SMCs stimulated by rat recombinant HB-EGF (kindly provided by Dr Shigeki Higashiyama, Ehime University, Matsuyama, Japan).

**Lipoprotein Preparation**

EDTA plasma was obtained from 20 patients with hypertriglyceridemia who showed elevated fasting serum RLP concentrations (>7.5 mg cholesterol per dL) 4 hours after eating breakfast. They had no cardiovascular diseases or diabetes and had not received cardiovascular medicine or antioxidants. RLPs were isolated from plasma samples using an RLP-C Kit (Japan Immunoresearch Laboratories), as described previously. The prepared RLPs were found to mainly consist of VLDL remnants and a few CM remnants.

Lipoproteins and lipids were dialyzed overnight against 10 mM EDTA (pH 7.4) and then sterilized using a 0.22-μm filter unit (Millipore). RLP lipid fractions were extracted using chloroform/methanol and then dried under N₂ gas. Trypsinized RLPs (devoid of immunochemically detectable apoE) were prepared as described previously. Endotoxin levels in the lipoprotein and lipid preparations, measured using a Limulus test assay kit (Wako), were less than 0.03 EU/mL.

**Cell Proliferation Assay**

Subcultured SMCs (passages 2 through 7) were seeded into 96-well microplates (3×10⁵ cells/well) or 35-mm dishes (2×10⁴ cells/dish). The culture medium was changed to DMEM supplemented with 1.0% FCS at 24 hours after seeding, and SMCs were made quiescent by incubating for 3 days, after which they were incubated with RLPs at the indicated concentrations or medium alone for the indicated hours. At the end of incubation, the incorporation of 5-bromo-2′-deoxyuridine (BrdU) into SMCs was examined using a microplate reader (CytoFlour II, Perceptive Biosystem). In parallel with the BrdU incorporation assay, SMCs were also stained with 4′,6-Diamidino-2-phenylindole, dihydrochloride (DAPI) (200 ng/mL) and viable cells were counted at the required time point using a hemacytometer.

**Immunoprecipitation and Immunoblotting**

A cell lysate from homogenized aorta samples was immunoprecipitated with anti-EGF receptor antibody. Fifty microliters of anti-rabbit IgG affinity gel (ICN Biomedicals) was then added for an additional 2 hours. The cell lysate was then added to the supernatant and incubated for an additional 2 hours. The immunoprecipitates were then washed and subjected to SDS-PAGE and Western blotting using the appropriate antibodies.

**Figure 1. Effects of RLPs on SMC DNA synthesis and proliferation.** A and C, SMCs were incubated in the presence of increasing concentrations of RLPs for up to 24 (A) or 48 (C) hours, and then a BrdU incorporation assay was carried out (n=6) (A) or the number of SMCs was counted using a hemacytometer (n=6) (C). *P<0.05, **P<0.01 vs 0 mg cholesterol per dL. B and D, SMCs were incubated in the absence (−) or presence (+) of RLPs (20 mg cholesterol per dL) for the indicated hours, and then a BrdU incorporation assay was carried out (n=6) (B) or the number of SMCs was counted using a hemacytometer (n=6) (D). *P<0.05, **P<0.01 vs 0 hours.
60 minutes, after which the immune complexes were collected and resuspended in SDS-PAGE sample buffer. SMCs were also incubated with RLPs (20 mg cholesterol per dL) for the indicated minutes, after which the membrane fractions and total cell lysates of SMCs were collected, as described previously. An equal amount of protein (10 μg) from each condition was subjected to SDS-PAGE. Immunoblots were developed with ECL plus (Amersham Pharmacia Biotech).

**SMC Transfection**

Quiescent SMCs were transfected with dominant-negative (DN) Raf-1 cDNA in pUSEamp (Upstate Biotechnology) or in pUSEamp alone using a calcium phosphate method. To access the transfection efficiency, pCXEGFP was cotransfected and green fluorescent protein–positive SMCs were detected using fluorescent microscopy (IX70) (Olympus). The estimated transfection efficiency based on green fluorescent protein staining was from 26% to 29%. Transfected SMCs at 24 hours after transfection were used for cell proliferation and Western blotting assays.

**Statistical Analysis**

Results are presented as mean±SEM. Data were analyzed using ANOVA, with P<0.05 considered significant.
Results

RLPs Induce SMC Proliferation

First, we examined the effects of RLPs on rat aortic SMC proliferation. When SMCs were incubated in the presence of RLPs (20 mg cholesterol per dL) for 24 hours, BrdU incorporation into SMCs was significantly increased at pathophysiological concentrations (>7.5 mg cholesterol per dL) in a dose-dependent manner (Figure 1A), and RLP-induced BrdU incorporation reached a peak after 24 hours of incubation (Figure 1B). The total number of SMCs simultaneously counted under the same conditions increased in a dose- and time-dependent manner (Figures 1C and 1D). In the following experiments, we chose to incubate SMCs with RLPs at 20 mg cholesterol per dL for 24 hours, unless otherwise indicated.

Involvement of MAPK Signal Transduction in RLP-Induced SMC Proliferation

Next, activation of MAPK pathway in RLP-treated SMCs was examined. Phosphorylated ERK1/2 increased after treatment with RLPs (Figure 2A). RLPs induced a sequential activation of Raf-1-MEK1/2-ERK1/2 (data not shown). Pretreatment with PD98059 (Calbiochem), a specific MEK1 inhibitor, significantly inhibited RLP-induced SMC BrdU incorporation (Figure 2B). Furthermore, the transfection of DN Raf-1 reduced RLP-induced MEK1/2 activation and SMC BrdU incorporation (Figures 2C and 2D).

RLP-Induced EGF Receptor Transactivation in SMCs

We then examined activation of the EGF receptor in RLP-treated SMCs. As shown in Figure 3A, the receptor was
Figure 4. Involvement of G protein–coupled receptor and PKC in RLP-induced SMC proliferation. A and B, SMCs were preincubated in the absence (−) or presence (+) of pertussis toxin (PTX) (100 ng/mL) for 24 hours and then incubated in the absence (−) or presence (+) of RLPs (20 mg cholesterol per dL) for 24 hours (A) or the indicated minutes (B) before a BrdU incorporation assay (n=4) (A) or Western blotting (blots are representative of 3 separate experiments) (B). *P<0.05 vs RLP (−)/PTX (−), #P<0.05 vs RLP (+)/PTX (−).

C and D, SMCs were preincubated in the absence (−) or presence (+) of Go6976 (Go) (2.5 μmol/L) or rottlerin (rot) (5 μmol/L) for 30 minutes and then incubated in the absence (−) or presence (+) of RLPs (20 mg cholesterol per dL) for 30 minutes (C) or 24 hours (D) before Western blotting (blots are representative of 3 separate experiments) (C) or a BrdU incorporation assay (n=4) (D). *P<0.01 vs RLP (−)/Go (−)/rot (−), #P<0.01 vs RLP (+)/Go (−)/rot (−).
Figure 5. Involvement of HB-EGF shedding in RLP-induced SMC proliferation. A, SMCs were preincubated in the absence (−) or presence (+) of Go6976 (Go) (2.5 μmol/L) or rottlerin (rot) (5 μmol/L) for 30 minutes and then incubated in the absence (−) or presence (+) of RLPs (20 mg cholesterol per dL) for 30 minutes before Western blotting (blots are representative of 3 separate experiments). *P<0.01 vs RLP (−)/Go (−)/rot (−), #P<0.01 vs RLP (+)/Go (−)/rot (−). B and C, SMCs were preincubated in the absence (−) or presence (+) of TIMP-1 (0.5 μmol/L), TIMP-2 (0.5 μmol/L), MMP3 inhibitor (MMP3-I) (100 μmol/L), or MMP2/9 inhibitor (MMP2/9-I) (100 μmol/L) for 4 hours and then incubated in the absence (−) or presence (+) of RLPs (20 mg cholesterol per dL) for 30 minutes (B) or 24 hours (C) before Western blotting (blots are representative of 3 separate experiments) (B) or a BrdU incorporation assay (n=4) (C). *P<0.01 vs RLP (−)/TIMP-1 (−)/TIMP-2 (−)/MMP3-I (−)/MMP2/9-I (−), #P<0.01 vs RLP (+)/TIMP-1 (−)/TIMP-2 (−)/MMP3-I (−)/MMP2/9-I (−). D, SMCs were preincubated in the absence (−) or presence (+) of goat IgG (1.0 μg/mL) or anti-HB-EGF neutralizing antibody (anti-HB-EGF) (1.0 μg/mL) for 30 minutes before a BrdU incorporation assay (n=4). *P<0.01 vs RLP (−)/goat IgG (+)/anti-HB-EGF (−), #P<0.01 vs RLP (+)/goat IgG (+)/anti-HB-EGF (−).
phosphorylated as early as 15 minutes after incubation with RLPs, which preceded MAPK activation. Pretreatment with AG1478 (Calbiochem), a specific EGF receptor inhibitor, significantly inhibited RLP-induced SMC ERK activation and BrdU incorporation (Figures 3B and 3C).

**Involvement of GPCR and PKC in RLP-Induced EGF Receptor Transactivation and MAPK Activation**

The potential involvement of GPCR and PKC in RLP-induced SMC proliferation was then examined. Pertussis toxin (List Biological Laboratories), a G protein inhibitor, significantly reduced RLP-induced SMC BrdU incorporation (Figure 4A) and attenuated RLP-induced EGF receptor transactivation (data not shown). The membrane translocation of PKCα and PKCδ was increased after 15 minutes of incubation with RLPs and remained after 60 minutes (Figure 4B). Other PKC isoforms were not activated on RLP treatment (data not shown). Pretreatment of SMCs with pertussis toxin attenuated PKCα activation. In contrast, pertussis toxin treatment resulted in a partial inhibition of PKCδ activation (Figure 4B). Rottlerin (BIOMOL Research Laboratories), a specific PKCδ inhibitor, significantly reduced RLP-induced EGF receptor transactivation and SMC BrdU incorporation, as did Go6976 (Calbiochem), a specific PKCα inhibitor, though to a lesser extent (Figures 4C and 4D).

**Involvement of HB-EGF Shedding in RLP-Induced EGF Receptor Transactivation**

We next examined whether HB-EGF shedding is involved in RLP-induced SMC proliferation. As shown in Figures 5A and 5B, treatment with RLPs increased the amount of soluble HB-EGF in SMC membranes. In contrast, the amount of pro HB-EGF in RLP-treated SMC membranes was decreased, suggesting that the cleavage of pro-HB-EGF occurred. Interestingly, RLP did not affect total amount of HB-EGF expression. In line with the BrdU incorporation assay results, pretreatment of SMCs with rottlerin inhibited HB-EGF shedding in RLP-treated SMCs, as did Go6976, although to a lesser extent (Figure 5A). We also examined the significance of metalloproteases in this process. Matrix metalloprotease3 (MMP3) inhibitor (Calbiochem), tissue inhibitor metalloprotease-1 (TIMP-1), and, to a lesser extent, TIMP-2 (Fuji Yakuhin) reduced HB-EGF shedding in RLP-treated SMCs (Figure 5B) and attenuated RLP-induced SMC BrdU incorporation (Figure 5C). In contrast, MMP2/9 inhibitor (Calbiochem) had little effect on HB-EGF shedding and BrdU incorporation. TIMP-1 and MMP3 inhibitor also inhibited EGF receptor activation (data not shown). RLP-induced pretreatment of SMCs with anti-HB-EGF neutralizing antibody significantly reduced RLP-induced SMC BrdU incorporation (Figure 5D), indicating that HB-EGF shedding is involved, at least in part, in RLP-induced EGF receptor transactivation.

**RLP-SMC Interaction and RLP-Induced SMC Proliferation**

Trypsinized RLPs and RLP lipid extracts induced moderate, but statistically significant, ERK activation and SMC proliferation (Figures 6A and 6B). ApoE (Sigma), a major apolipoprotein component of RLPs, did not have an effect on SMC proliferation. Moreover, pretreatment of SMCs with heparin and heparitinase, compounds known to remove cell-surface lipoprotein lipase (LPL) and heparan-sulfate proteoglycan (HSPG), respectively, reduced PKCα and PKCδ activation (Figure 6C). When SMCs were treated with heparin or heparitinase before RLP treatment, soluble HB-EGF was decreased compared with RLP treatment alone (Figure 6D), and heparin and heparitinase also reduced RLP-induced SMC BrdU incorporation (Figure 6E).

**EGF Receptor Transactivation and HB-EGF in Animal Models**

To confirm the relevance of the observed RLP-induced SMC proliferation in vivo, we attempted to determine whether activation of EGF receptor and HB-EGF shedding occurred in the aortas of apoE-knockout mouse. HB-EGF shedding was detected in the apoE-knockout mice aortas but not in those of wild-type mice (Figure 7A). Moreover, an increased tyrosine phosphorylation of the EGF receptor was observed only in apoE-knockout mice (Figure 7B).

**Discussion**

Our present results are the first to demonstrate that proliferation of vascular SMCs triggered by RLPs involves activation of PKCs and EGF receptor transactivation. ApoE-devoid RLP (trypsinized RLP and lipid extracts from RLPs) exerted stimulatory effects on SMC proliferation, which may support the importance of RLP lipid components, as has been reported with oxidized LDL.13 The inhibition of PKCα activation and SMC proliferation by pertussis toxin suggested that RLPs activate PKC via the GPCR signaling pathway. On the other hand, RLP-induced PKCδ activation was virtually unaffected by pertussis toxin, indicating a pertussis toxin–insensitive mechanism involved with RLP-induced SMC proliferation. In fact, both PKC and MAPK activation continued for longer than previously reported,8 which suggests the requirements of RLP uptake and lysosomal processing for this phenomenon to occur.

We also examined the potential interaction between RLPs and SMCs. RLPs not only bind directly to cell-surface LDL receptor families but also form a complex with LPL and HSPG on the cell surface via apoE.3,14 ApoE-devoid RLPs showed a limited effect on SMC proliferation, which may support the importance of apoE in RLP-SMC interaction. Heparin and heparitinase attenuated PKCδ and PKCα activation to a lesser extent and inhibited HB-EGF shedding, suggesting that the depletion of cell-surface LPL and HSPG decreases RLP-SMC interaction.

It has been reported that PYK2 and Src family tyrosine kinases are involved in PKC-induced EGF receptor transactivation.15 However, PP1 and PP2, specific Src family tyrosine kinase inhibitors, had no effect on RLP-induced EGF receptor transactivation and RLPs did not affect PYK2 activity (data not shown), indicating that an alternative mechanism is involved in this process. Recently, the potential cleavage of membrane-anchored HB-EGF (pro-HB-EGF) or HB-EGF shedding by MMP/a disintegrin and metalloprotease (ADAM) families has been demonstrated in EGF...
Figure 6. RLP-SMC interaction and SMC proliferation. A and B, SMCs were incubated with native RLPs (20 mg cholesterol per dL), trypsinized (tryp)-RLPs (20 mg cholesterol per dL), RLP lipid (20 mg cholesterol per dL), apoE (10 μg/mL), or medium alone (control) for 120 minutes (A) or 24 hours (B) before Western blotting (blots are representative of 3 separate experiments) (A) or a BrdU incorporation assay (n=4) (B). *P<0.05 vs control, #P<0.05 vs native RLP.

C, D, and E, SMCs were preincubated in the absence (−) or presence (+) of heparin (10 μg/mL) or heparitinase (4 IU/mL) for 4 hours and then incubated with RLPs (20 mg cholesterol per dL) for 15 minutes (C), 30 minutes (D), or 24 hours (E) before Western blotting (blots are representative of 3 separate experiments) (C and D) or a BrdU incorporation assay (n=4) (E). *P<0.01 vs RLP (−)/heparin (−)/heparinase (−), #P<0.01 vs RLP (+)/heparin (−)/heparinase (−).
receptor transactivation in vitro and in vivo. Because most of the soluble HB-EGF binds to HSPG on the surface of SMCs, membrane-associated soluble HB-EGF reflects the amount of HB-EGF shedding. Thus, we examined whether RLPs induce HB-EGF shedding in SMCs and successfully found detectable levels of membrane-associated soluble HB-EGF produced by RLPs, which were lowered by PKC inhibitors, suggesting that RLPs induce HB-EGF shedding via PKCs. In the present study, several MMP inhibitors attenuated RLP-induced HB-EGF shedding and EGF receptor activation. However, identification of specific MMPs responsible for this process requires additional investigation. Moreover, a remarkable difference in the inhibition of HB-EGF shedding between TIMP-1 and TIMP-2 may point to a potential involvement of ADAM families, including ADAM10, in addition to MMPs. Furthermore, heparin has been shown to block the binding of soluble HB-EGF to EGF receptor. Thus, heparin and heparitinase may not only decrease RLP-SMC interaction but also interfere with the binding of soluble HB-EGF generated by RLPs to EGF receptor.

We were also able to detect EGF receptor activation and HB-EGF shedding in the aortas of apoE-knockout mice, a model of spontaneous atherosclerosis exhibiting hyperremnant lipoproteinaemia. Although careful examinations with these animals, including involvement of PKC and MMP/ADAM families, will be required in the future to elucidate the role of RLP in vivo, these results suggest that some of the observations regarding RLP-induced SMC proliferation may be operative in vivo as well.

In the present study, anti-HB-EGF antibody and EGF receptor inhibitor failed to completely inhibit RLP-induced SMC proliferation. A PKC-dependent and EGF receptor–independent signaling pathway may be involved in this process.

Taken together, we hypothesized that RLPs activate PKCs via a GPCR-dependent or -independent mechanism, followed by EGF receptor transactivation, which results in SMC proliferation. RLP-induced vascular SMC proliferation may be one of the direct causative roles of remnant lipoproteins in atherogenesis.

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References
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