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Mitogenic effect of oxidized low-density lipoprotein on vascular smooth muscle cells mediated by activation of Ras/Raf/MEK/MAPK pathway

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1 It has been demonstrated that oxidized low-density lipoprotein (OX-LDL) is a risk factor in atherosclerosis by stimulating vascular smooth muscle cell (VSMC) proliferation. However, the mechanisms of OX-LDL-induced cell proliferation are not completely understood. Therefore, we investigated the effect of OX-LDL on cell proliferation associated with mitogen-activated protein kinase (MAPK) activation in rat cultured VSMCs.

2 Both native-LDL (N-LDL) and OX-LDL induced a time- and concentration-dependent incorporation of [^{3}H]-thymidine in VSMCs.

3 OX-LDL induced time- and concentration-dependent phosphorylation of p42/p44 MAPK. Pretreatment of these cells with pertussis toxin or U73122 attenuated the OX-LDL-induced responses.

4 Pretreatment with PMA for 24 h, preincubation with a PKC inhibitor staurosporine or the tyrosine kinase inhibitors, genistein and herbimycin A for 1 h, substantially reduced [^{3}H]-thymidine incorporation and p42/p44 MAPK phosphorylation induced by OX-LDL.

5 Removal of Ca^{2+} by BAPTA/AM or depletion of the internal Ca^{2+} pool by thapsigargin significantly inhibited OX-LDL-induced [^{3}H]-thymidine incorporation and p42/p44 MAPK phosphorylation.

6 OX-LDL-induced [^{3}H]-thymidine incorporation and p42/p44 MAPK phosphorylation was inhibited by PD98059 (an inhibitor of MEK1/2) and SB203580 (an inhibitor of p38 MAPK) in a concentration-dependent manner.

7 Overexpression of dominant negative mutants of Ras (H-Ras-15A) and Raf (Raf-N4) significantly suppressed MEK1/2 and p42/p44 MAPK activation induced by OX-LDL and PDGF-BB, indicating that Ras and Raf may be required for activation of these kinases.

8 These results suggest that the mitogenic effect of OX-LDL is mediated through a PTX-sensitive G protein-coupled receptor that involves the activation of the Ras/Raf/MEK/MAPK pathway similar to that of PDGF-BB in rat cultured VSMCs.

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Abbreviations: BCA, bicinchoninic acid; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; EC_{50}, concentration required for half-maximal stimulation; ECL, enhanced chemiluminescence; LDL, low-density lipoprotein; MAPK, mitogen-activated protein kinase; MEK1/2, MAPK kinase; N-LDL, native low-density lipoprotein; OX-LDL, oxidized low-density lipoprotein; PDGF-BB, PDGF B-chain homodimer; PI-PLC, phosphoinositide-phospholipase C; PKC, protein kinase C; PLA_2, phospholipase A_2; PLD, phospholipase D; PTX, pertussis toxin; VSMCs, vascular smooth muscle cells

Introduction

Several lines of evidence have demonstrated that oxidative modification of low-density lipoprotein (LDL) is important for many of the atherogenic effects of LDL (Steinberg *et al*., 1989). In fact, oxidized LDL (OX-LDL) exerts many effects *in vitro* that could contribute to the progression of atherosclerosis if the oxidative process is allowed to continue and OX-LDL accumulates to higher levels *in vivo*. For example, OX-LDL is implicated in the recruitment of monocytes and foam cell formation (Steinberg *et al*., 1989), the chemotaxis of smooth muscle cells (Autio *et al*., 1990) which accumulate in the subendothelial area (Stary, 1990) and the cytotoxicity toward cultured cells (Auge *et al*., 1995). Both native-LDL (N-LDL) and OX-LDL have also been shown to stimulate the proliferation of VSMCs in several species as revealed by [^{3}H]-thymidine incorporation into DNA (Heery *et al*., 1995; Auge *et al*., 1995; Sachinidis *et al*., 1997). The

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mitogenic effect of OX-LDL may be indirectly mediated through synthesis of platelet-derived growth factor (PDGF) in macrophages and VSMCs (Stiko-Rahm *et al*., 1992) and from other cells in the vessel walls (Boulanger *et al*., 1992). Therefore, the proliferation of VSMCs caused by OX-LDL in the intima of arterial wall is considered to be a critical event in the development of atherosclerotic plaque.

It is conceivable that phosphorylation of cellular proteins by protein kinases plays an important role in regulation of cellular functions including cell growth, migration, differentiation and the response to extracellular stimuli. Among these kinases, two distinct intracellular pathways activated by the various second messengers that lead to cell proliferation have been established. One is activated by the receptor that contains the intrinsic protein tyrosine kinase. This type of receptor is activated by classic growth factors such as PDGF and EGF (Pouyssegur & Seuwen, 1992). It has been demonstrated that the classic growth factor PDGF-BB propagates its mitogenic signal *via* autophosphorylation of a PDGF-β receptor on tyrosine residues, resulting in tyrosine phosphorylation of different substrate proteins (Kaplan *et al*., 1990). It has recently been recognized that further transmission of growth signals from the receptor to the nucleus is mediated by sequential activation of protein kinases. The activation of MAPKs, in particular the p42 and p44 MAPK isoforms, seems to be a key component in growth signal transduction through tyrosine kinase receptors such as the PDGF-β receptor (Blenis, 1993).

The other pathway is activated by G protein-coupled receptors (Lange-Carter *et al*., 1993; Post & Brown, 1996). Several studies reveal that cellular growth mediated by the intracellular signal pathway for the G protein-coupled receptor also involves MAPKs (Post & Brown, 1996; Pouyssegur & Seuwen, 1992). These MAPKs are activated during proliferation and cell cycle transition triggered by various stimuli (Blenis, 1993). Thus, MAPKs are important integrators of G protein-coupled receptor- and tyrosine kinase receptor-mediated signals for cell growth. Moreover, many growth factor receptors have been shown to activate a signal transduction pathway that includes MAPK kinase (MEK) and MAPK (Marshall, 1995; Lange-Carter *et al*., 1993). The requirement for the activation of the Ras, Raf, MEK, and MAPK, associated with cell proliferation, had been demonstrated for receptor tyrosine kinases such as PDGF in several cell types (Blenis, 1993; Marrero *et al*., 1997; Weber *et al*., 1997). Utilizing selective expression of dominant negative mutants, Ras, Raf, or MEK has further been shown to play a key step in MAPK activation (Stacey *et al*., 1991; Schaap *et al*., 1993; Abdellatif *et al*., 1998). However, whether the activation of MAPK and cell proliferation by OX-LDL, is similar mechanically to that of PDGF-BB in VSMCs, has not been completely elucidated.

Although OX-LDL had been known to stimulate DNA synthesis, the biochemical mechanisms for OX-LDL-induced VSMC proliferation and activation of MAPKs, were not completely understood. Therefore, the purpose of this study was to define the mechanisms underlying the mitogenic effects of OX-LDL associated with the activation of Ras/Raf/MEK/MAPK in rat cultured VSMCs.

Methods

Preparation of LDL and OX-LDL

Human LDL (d $1.019-1.063$ g ml^{-1}) was prepared by sequential ultracentrifugation (4°C) of plasma from healthy blood (Olofsson *et al*., 1980). The LDL preparation was filtered through 0.22 μm filters and stored at 4°C.

After extensive dialysis against PBS for 24 h, oxidation of LDL was performed by dialyzing against 5 μM $CuSO_4$ in PBS for 10–12 h at 37°C as described by Steinbrecher *et al*. (1984). The OX-LDL was dialyzed against PBS containing 0.1 mM EDTA for 3–4 days. The extent of LDL oxidation was monitored by measuring thiobarbituric acid-reactive substance (TBARS), lipid peroxides, and conjugated dienes using the method described by Morel *et al*. (1984). The OX-LDL preparation was filtered through 0.22 μm filters and stored at 4°C. The protein content of OX-LDL was determined by BCA reagents (Pierce, Rockford, IL, U.S.A.).

Vascular smooth muscle cell culture

VSMCs were isolated from Sprague-Dawley rat. Aortic strips were cut into small rings and placed in 6-well culture plates (Linas *et al*., 1988). These explants were grown in DMEM containing 10% (v v^{-1}) FBS, 2 mM glutamine, non-essential amino acid and antibiotics (100 μml^{-1} penicillin G, 100 μg ml^{-1} streptomycin, and 250 ng ml^{-1} fungizone) at 37°C in a humidified 5% CO_2 atmosphere. When the cultures reach confluence (7 days), cells were treated with 0.25% (w v^{-1}) trypsin/1 mM EDTA for 15 min at 37°C. The cells were counted and diluted with DMEM with 10% FBS to a final concentration of 2×10^4 cells ml^{-1}. The cells were plated onto (0.5 ml per well) 24-well and (10 ml per dish) 100-mm culture dishes for [^{3}H]-thymidine incorporation and MAPK phosphorylation, respectively. The medium was changed after 24 h and then every 3 days. VSMCs were identified by their characteristic 'hill and valley' growth pattern, the absence of factor VIII antigen and the presence of SMC-specific α-actin.

[^{3}H]-thymidine incorporation

Cells were growth-arrested by incubating the cells in serum-free DMEM for 24 h. The cells were stimulated with OX-LDL at concentrations ranging from 12.5 to 200 μg ml^{-1}. After 8 h of stimulation, cells were labelled with 1 μCi ml^{-1} of [^{3}H]-thymidine for another 16 h in the presence of OX-LDL. When inhibitors were used, they were applied 1 h prior to the addition of OX-LDL. The experiments were terminated by washing the cells with cold PBS, precipitation of acid-insoluble materials with 10% (w v^{-1}) TCA and extraction of the DNA with 0.1 N NaOH. The prepicipitants were filtered through Whatman GF/B filters and washed three times with cold PBS using a cell harvester. The radioactivity was counted using a scintillation counter (Beckman LS5000TA, Fullerton, CA, U.S.A.).

Plasmids and transfection

The plasmids encoding H-Ras-15A and Raf-N4 (dominant negative mutants of Ras and Raf-1), cloned into pZIP-NeoSV and pCGN, respectively, were kindly provided by Dr

Channing J. Der (Department of Pharmacology, University of North Carolina at Chapel Hill). All plasmids were prepared by using QIAGEN plasmid DNA preparation kits.

VSMCs were plated at $3-10^5$ cells ml^{-1} (2 ml per well) in 6-well culture plates for 24 h, reaching about 80% confluence. Cells were washed once with PBS and once with serum-free DMEM, and 0.8 ml of serum-free OPTI-MEM I medium was added to each well. The DNA PLUS-Lipofectamine reagent complex was prepared according to instructions of the manufacturer (GIBCO–BRL). The amount of transfected plasmid was kept constant (2 μg of H-Ras-15A, Raf-N4, pZIP-NeoSV, and pCGN, for each well). The DNA PLUS-Lipofectamine reagent complex (0.2 ml) was added to each well, incubated at 37°C for 5 h, and then 1 ml of OPTI-MEM I medium containing 20% FBS added and further incubated for 19 h. After 24 h of transfection, the cells were washed twice with PBS and maintained in DMEM containing 10% FBS for 48 h. Cells were then washed once with PBS and incubated with serum-free DMEM for 24 h before treatment with either OX-LDL or PDGF-BB.

Preparation of cell extracts and Western blot analysis of MAPK isoforms

Cells were plated in 100-mm dishes and rendered quiescent at confluence by incubation in fresh DMEM for 24 h. Growth-arrested VSMCs were incubated with or without OX-LDL at concentrations ranging from 12.5 to 200 μg ml^{-1} at 37°C for various times. When inhibitors were used, they were applied 1 h prior to the addition of OX-LDL. After incubation, the cells were then rapidly washed with ice-cold PBS, scraped and collected by centrifugation at $1000\times g$ for 10 min. The collected cells were lysed with ice-cold lysis buffer containing (mM): Tris-HCl 25, pH 7.4, NaCl 25, NaF 25, sodium pyrophosphate 25, sodium vanadate 1, EDTA 2.5, EGTA 2.5, Triton X-100 0.05% (w v^{-1}), SDS 0.5% (w v^{-1}), deoxycholate 0.5% (w v^{-1}), NP-40 0.5% (w v^{-1}), leupeptin 5 μg ml^{-1}, aprotinin 5 μg ml^{-1}, and PMSF 1. The lysates were centrifuged at $45{,}000\times g$ for 1 h at 4°C to yield the whole cell extract in the supernatants. Concentration of protein was determined by the BCA reagents according to the manufacturer's manual. Samples (100 μg protein) were denatured and subjected to SDS–PAGE using a 10% (w v^{-1}) running gel. Protein was transferred to nitrocellulose membrane and the membrane was incubated successively at room temperature with 5% (w v^{-1}) BSA in TTBS for 1 h. The phosphorylation of MEK1/2 and p42/p44 MAPK were identified and quantified by Western blot analysis using anti-phospho-MEK1/2 and anti-phospho-MAPK polyclonal antibody kits according to the manufacturer's manual. Briefly, membranes were then incubated overnight at 4°C with the anti-phospho-MEK1/2 or anti-phospho-p42/p44 MAPK polyclonal antibody used at a dilution of 1 : 1000 in TTBS. Membranes were washed with TTBS four times for 5 min each, incubated with a 1 : 1500 dilution of anti-rabbit horseradish peroxidase antibody for 1 h. During the end of incubation, the membrane was washed extensively with TTBS. The immunoreactive bands detected by ECL reagents were developed by Hyperfilm-ECL (Amersham International).

Analysis of data

Concentration-effect curves were fitted and EC_{50} values were estimated by GraphPad Prizm Program (GraphPad, San Diego, CA, U.S.A.). Data are expressed as the mean ± s.e.mean. ANOVA was used to make comparisons with Bonferroni's test at a 0.05 level of significance. Error bars were omitted when they fell within the dimensions of symbol.

Materials

Dulbecco's modified Eagle's medium (DMEM), OPTI-MEM I medium, Lipofectamine Plus reagent, and foetal bovine serum (FBS) were purchased from Gibco–BRL (Gaithersburg, MD, U.S.A.). [^{3}H]-methyl thymidine, Hybond C membrane, and ECL Western blotting detection system were from Amersham (Buckinghamshire, U.K.). PhosphoPlus p42/p44 MAPK and phosphoPlus MEK1/2 antibody kits were from New England Biolabs (Beverly, MA, U.S.A.). p42MAPK antibody was from Santa Cruz (Santa Cruz. CA, U.S.A.). Genistein, herbimycin A, staurosporine, BAPTA/AM, PD98059 and SB203580 were from Calbiochem (San Diego, CA, U.S.A.). BCA protein assay kit was from Pierce (Rockford, IL, U.S.A.). Enzymes and other chemicals were from Sigma (St. Louis, MO, U.S.A.).

Results

DNA synthesis

Figure 1A shows the influence of different incubation periods with N-LDL or OX-LDL on DNA synthesis in VSMCs. N-LDL stimulated a rapid [^{3}H]-thymidine incorporation, a maximal incorporation was obtained after 16 h incubation which then declined to the basal level. In contrast, OX-LDL induced a slow [^{3}H]-thymidine incorporation, a maximal incorporation was obtained after incubation with OX-LDL for 12 h, which was sustained for up to 24 h during the period of incubation. These results suggest that N-LDL and OX-LDL induce [^{3}H]-thymidine incorporation in a time-dependent manner. Furthermore, the potency of N-LDL and OX-LDL to stimulate DNA synthesis was evaluated in VSMCs. Incubation of VSMCs rendered quiescent by 24 h serum deprivation with increasing concentrations of N-LDL or OX-LDL induced a marked elevation in DNA synthesis (Figure 1B). Both N-LDL and OX-LDL displayed similar potency to stimulate [^{3}H]-thymidine incorporation in VSMCs. The concentrations of N-LDL and OX-LDL that produced a half-maximal increase (EC_{50}) in [^{3}H]-thymidine incorporation were about 25 μg ml^{-1}, $n=3$. A maximal response was seen with 100 μg ml^{-1} of N-LDL and OX-LDL.

OX-LDL-stimulated p42/p44 MAPK activation

Several lines of evidence have shown that activation of MAPKs is linked to cell proliferation during exposure to various stimuli. Therefore, we determined whether OX-LDL stimulated cell proliferation associated with activation of MAPKs in VSMCs. Tyrosine phosphorylation of p42/p44 MAPK was monitored by Western blot with polyclonal antisera reactive for the tyrosine phosphorylated state of

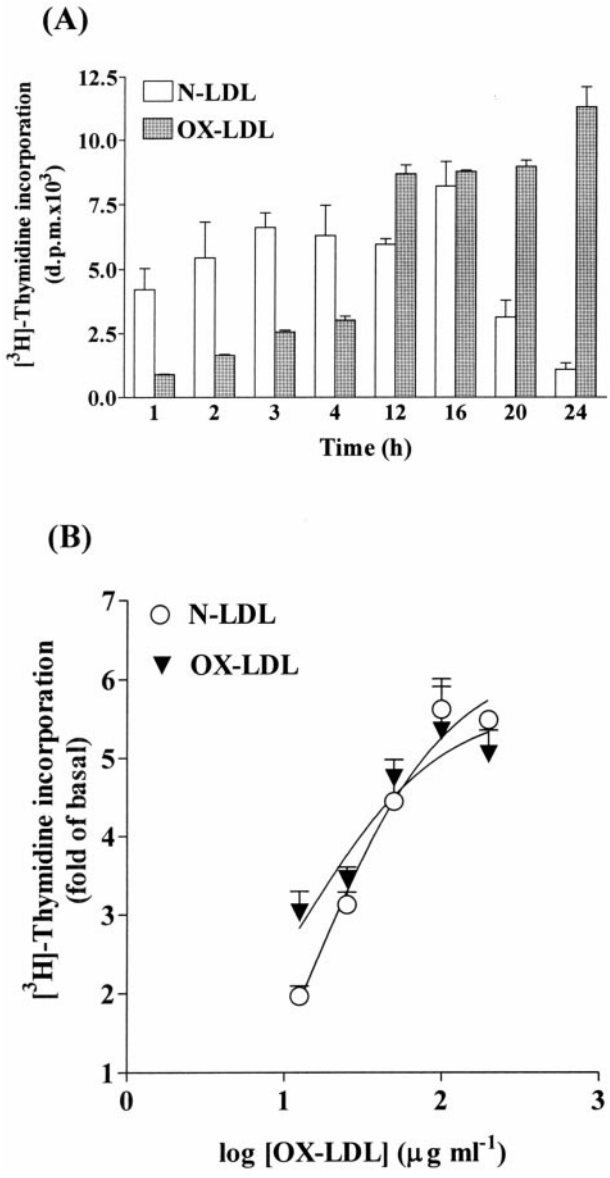

Figure 1 [^{3}H]-thymidine incorporation induced by N-LDL or OX-LDL in VSMCs. (A) Time course, after 24 h in serum-free medium, the cells were stimulated with vehicle, N-LDL or OX-LDL at a concentration of 100 μg ml^{-1}. The cells were labelled with 1 μCi ml^{-1} [^{3}H]-thymidine for the times indicated in the continuous presence of N-LDL or OX-LDL. (B) Concentration-dependence, the cells were stimulated with various concentrations of N-LDL or OX-LDL. After stimulation for 8 h, VSMCs were labelled with 1 μCi ml^{-1} [^{3}H]-thymidine for another 16 h in the continued presence of N-LDL or OX-LDL. The incorporation of [^{3}H]-thymidine was determined as described in Methods. Data are expressed as the mean$\pm$s.e.mean of three separate experiments determined in triplicate.

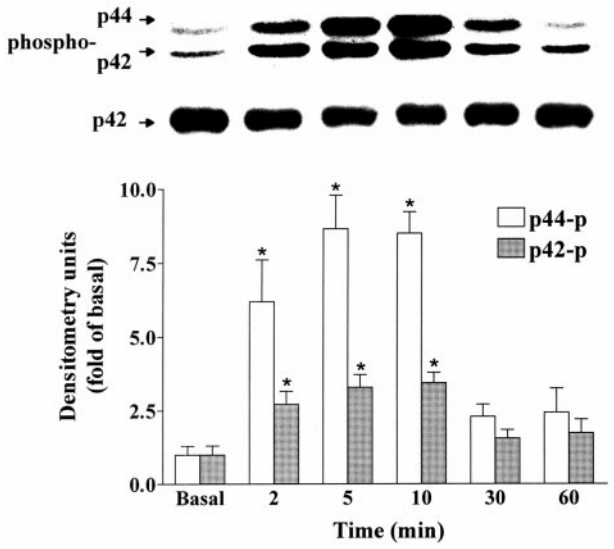

Figure 2 Time course of OX-LDL-stimulated p42/p44 MAPK phosphorylation in VSMCs. The cells were grown to confluency, made quiescent by serum-deprivation for 24 h and incubated with 100 μg ml^{-1} OX-LDL for 2–60 min. The cell lysates were subjected to 10% SDS–PAGE and transferred to nitrocellulose membrane. Western blot analysis was performed using an antiserum reactive with anti-phospho-p42/p44 MAPK polyclonal antibody for activated p42/p44 MAPK and anti-p42 MAPK antibody for the total p42 MAPK as an indicator of protein loading in each well. Bands were visualized by an ECL method. Data are expressed as the mean$\pm$s.e.mean of three separate experiments. *$P<0.01$, compared with the respective basal level.

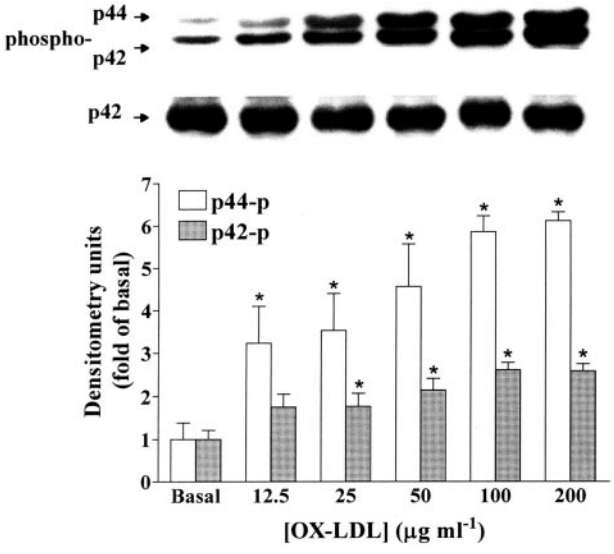

Figure 3 Concentration-dependence of OX-LDL-stimulated p42/p44 MAPK phosphorylation in VSMCs. The cells were grown to confluency, made quiescent by serum-deprivation for 24 h and incubated with various concentrations of OX-LDL for 5 min. The cell lysates were subjected to 10% SDS–PAGE and transferred to nitrocellulose membrane. Bands were visualized by an ECL method as described in Figure 2. Data are expressed as the mean$\pm$s.e.mean of three separate experiments. *$P<0.01$, compared with the respective basal level.

these two forms of MAPK. As shown in Figure 2, OX-LDL (100 μg ml^{-1}) stimulated a marked increase in the levels of p42 and p44 kDa phosphorylation within 2 min ($P<0.01$, $n=3$). Densitometric analysis of the blot revealed that at 5 min, OX-LDL induced a 3.3 ± 0.4 and 8.7 ± 1.1 fold increase in both p42 and p44 MAPK isoforms, respectively. After 30–60 min, phosphorylation rapidly declined to near the basal level. Furthermore, as demonstrated in Figure 3, the OX-LDL-induced phosphorylation of p42 and p44 MAPK isoforms and a maximal effect was achieved with 100 μg ml^{-1} OX-LDL. These results indicated that the effect of OX-LDL was not dose-related as compared between cells treated with

different concentrations of OX-LDL. After blotting with anti-phospho-p42/p44 MAPK antibody, the membranes were tripped and the amount of total p42 MAPK was determined with the use of an antibody that recognized total p42 MAPK as an indicative of protein loading in each well. There was no significant difference in the total amount of p42 MAPK among these samples (Figures 2 and 3). In addition, N-LDL also induced a time- and concentration-dependent phosphorylation of p42/p44 MAPK similar to that of OX-LDL in these cells (data not shown), which is consistent with other results obtained in the same cell type (Sachinidis *et al*., 1997).

Effects of pertussis toxin and U73122 on OX-LDL-induced DNA synthesis and MAPK phosphorylation

To investigate whether the effect of OX-LDL on DNA synthesis and MAPK activation was mediated by activation of a receptor coupled to a PTX-sensitive G protein and activation of phosphinositide phospholipase C (PI-PLC), the cells were pretreated with 100 ng ml^{-1} PTX for 24 h or 10 μM U73122 (an inhibitor of PI-PLC) for 1 h and then stimulated with 100 μg ml^{-1} OX-LDL. As shown in Figure 4, pretreatment of VSMCs with PTX or U73122 reduced [^{3}H]-thymidine incorporation ($P<0.01$, $n=3$) and p42/p44 MAPK activation ($P<0.01$, $n=3$), as compared with the cells exposed to OX-LDL alone, indicating that the effect of OX-LDL was mediated through a PTX-sensitive G protein and activation of PI-PLC. It should be noted that treatment with U73122 significantly increased the basal level of [^{3}H]-thymidine incorporation as compared with non-treated cells. The precise mechanism of this action of U73122 is not known.

Effects of PKC inhibitors on OX-LDL-induced DNA synthesis and MAPK phosphorylation

To examine the role of PKC in OX-LDL-mediated p42/p44 MAPK activation associated with cell proliferation, VSMCs were preincubated with a PKC inhibitor and by down-regulation of PKC with prolonged exposure to PMA for 24 h. As shown in Figure 5A, pretreatment of VSMCs with 1 μM staurosporine significantly reduced [^{3}H]-thymidine incorporation ($P<0.01$, $n=3$) in response to OX-LDL. In addition, OX-LDL-stimulated p42/p44 MAPK phosphorylation was inhibited by staurosporine ($P<0.05$, $n=3$) in VSMCs as revealed by Western blot analysis (Figure 5B). Moreover, long-term treatment of VSMCs with 1 μM PMA for 24 h significantly attenuated the [^{3}H]-thymidine incorporation ($P<0.01$, $n=3$) and p42/p44 MAPKs activation ($P<0.05$, $n=3$) in response to OX-LDL (Figure 5). These results suggest that OX-LDL-stimulated [^{3}H]-thymidine incorporation and p42/p44 MAPK phosphorylation was, at least in part, mediated through the activation of PKC in VSMCs.

Effect of Ca^{2+} on OX-LDL-induced DNA synthesis and MAPK phosphorylation

To elucidate whether Ca^{2+} is required for the activation of p42/p44 MAPK and [^{3}H]-thymidine incorporation in response to OX-LDL, VSMCs were preincubated with 30 μM

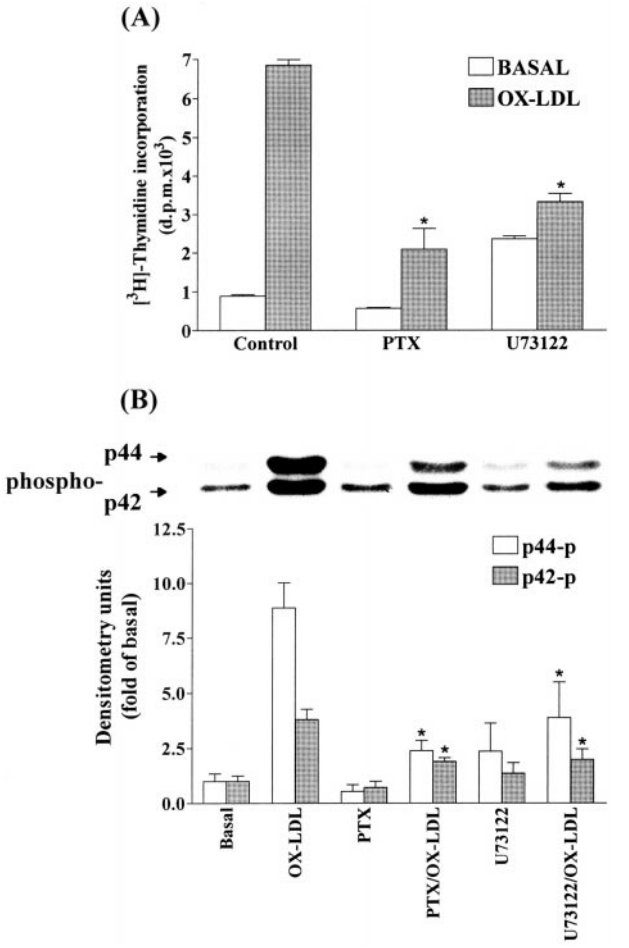

Figure 4 Effects of pertussis toxin and U73122 on DNA synthesis and p42/p44 MAPK phosphorylation induced by OX-LDL in VSMCs. (A) The cells were grown to confluency, made quiescent by serum-deprivation and preincubated with pertussis toxin (PTX, 100 ng ml^{-1}) for 24 h or U73122 (10 μM) for 1 h, and then stimulated with vehicle or 100 μg ml^{-1} OX-LDL. After 8 h incubation, cells were labelled with 1 μCi ml^{-1} [^{3}H]-thymidine for another 16 h in the continuous presence of OX-LDL. The incorporation of [^{3}H]-thymidine was determined as described in Methods. Data are expressed as the mean$\pm$s.e.mean of three separate experiments determined in triplicate. *$P<0.01$, compared with the cells exposed to OX-LDL. (B) For MAPK experiment, after incubation with PTX for 24 h or U73122 for 1 h, the cells were stimulated with 100 μg ml^{-1} OX-LDL for 5 min. The cell lysates were subjected to 10% SDS–PAGE and transferred to nitrocellulose membrane. Bands were visualized by an ECL method as described in Figure 2. Data are expressed as the mean$\pm$s.e.mean of three separate experiments. *$P<0.01$, compared with the cells exposed to OX-LDL.

BAPTA/AM (a potent intracellular Ca^{2+} chelator, Yang *et al*., 2000) and 5 mM EGTA. As shown in Figure 6, pretreatment of these cells with BAPTA significantly reduced both p42/p44 MAPK phosphorylation ($P<0.01$, $n=3$) and [^{3}H]-thymidine incorporation ($P<0.01$, $n=3$) induced by OX-LDL, suggesting that Ca^{2+} may be required for these responses. To further differentiate the mitogenic effect of OX-LDL dependent on the release of intracellular Ca^{2+} and influx of extracellular Ca^{2+}, the effect of OX-LDL on [^{3}H]-thymidine incorporation was observed in VSMCs preincubated with Ca^{2+} channel blocker (diltiazem), sacroplasmic reticulum Ca^{2+} transport blocker (ryanodine), and endoplas-

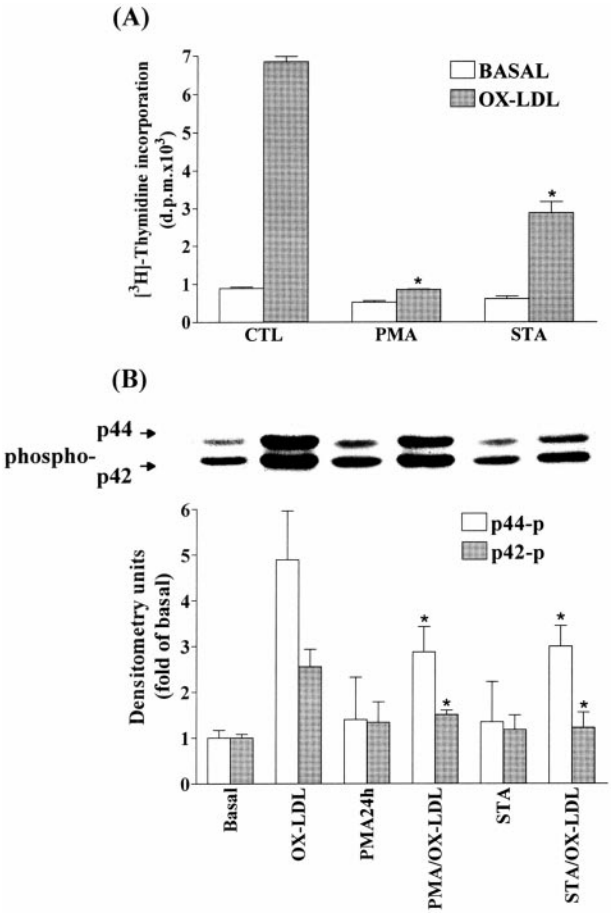

Figure 5 Involvement of PKC in DNA synthesis and p42/p44 MAPK phosphorylation induced by OX-LDL in VSMCs. (A) The cells were grown to confluence, made quiescent by serum-deprivation for 24 h. The cells were preincubated with 1 μM staurosporine (STA) for 1 h, or 1 μM PMA for 24 h before stimulated with vehicle or 100 μg ml^{-1} OX-LDL. The incorporation of [^{3}H]-thymidine was determined as described in Figure 4. Data are expressed as the mean$\pm$s.e.mean of three separate experiments determined in triplicate. *$P<0.01$, compared with the cells exposed to OX-LDL. (B) For MAPK experiment, after incubation with STA for 1 h, or PMA for 24 h, the cells were stimulated with 100 μg ml^{-1} OX-LDL for 5 min. The cell lysates were subjected to 10% SDS–PAGE and transferred to nitrocellulose membrane. Bands were visualized by an ECL method as described in Figure 2. Data are expressed as the mean$\pm$s.e.mean of three separate experiments. *$P<0.05$, compared with the cells exposed to OX-LDL.

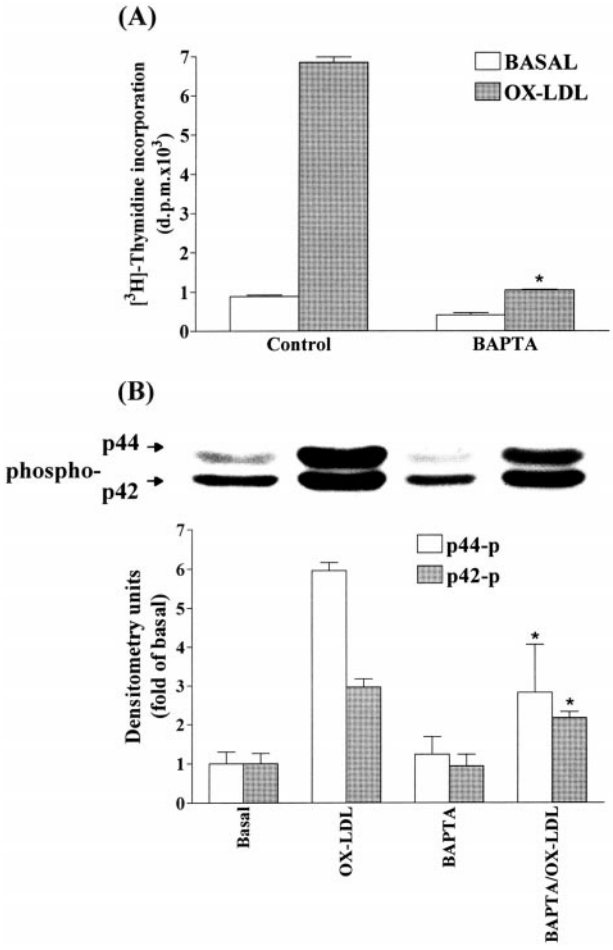

Figure 6 Effect of BAPTA plus EGTA on OX-LDL-induced [^{3}H]-thymidine incorporation and p42/p44 MAPKphosphorylation in VSMCs. (A) The cells were grown to confluence, made quiescent by serum-deprivation for 24 h. The cells were preincubated with 30 μM BAPTA and 5 mM EGTA for 1 h and then stimulated with vehicle or 100 μg ml^{-1} OX-LDL. The incorporation of [^{3}H]-thymidine was determined as described in Figure 4. Data are expressed as the mean$\pm$s.e.mean of three separate experiments determined in triplicate. *$P<0.01$, compared with the cells exposed to OX-LDL. (B) For MAPK experiment, after incubation with BAPTA and EGTA for 1 h, the cells were stimulated with 100 μg ml^{-1} OX-LDL for 5 min. The cell lysates were subjected to 10% SDS–PAGE and transferred to nitrocellulose membrane. Bands were visualized by an ECL method as described in Figure 2. Data are expressed as the mean$\pm$s.e.mean of three separate experiments. *$P<0.01$, compared with the cells exposed to OX-LDL.

mic reticulum Ca^{2+} ATPase inhibitor (thapsigargin). As shown in Figure 7, the OX-LDL-induced [^{3}H]-thymidine incorporation was almost completely inhibited by thapsigargin ($P<0.001$, $n=3$), but not by diltiazem and ryanodine. These results suggest that release of Ca^{2+} from internal stores may be sufficient for stimulation of cell proliferation in response to OX-LDL.

Effects of tyrosine kinase inhibitors on OX-LDL-induced DNA synthesis and MAPK phosphorylation

To determine whether the mitogenic effect of OX-LDL was mediated through the activation of tyrosine kinase, VSMCs were pretreated with 10 μM genistein or 10 μM herbimycin A for 1 h and then stimulated with 100 μg ml^{-1} OX-LDL. As shown in Figure 8, pretreatment of cells with genistein or herbimycin A completely inhibited OX-LDL-induced DNA synthesis ($P<0.01$, $n=3$). However, these two tyrosine kinase inhibitors had only a slight effect on the OX-LDL-stimulated p42/p44 MAPK phosphorylation in VSMCs.

Effects of MEK inhibitors on OX-LDL-induced DNA synthesis and MAPK phosphorylation

To ensure that the mitogenic effect of OX-LDL was mediated through the activation of MAPK pathway, the effect of OX-LDL on p42/p44 MAPK activation and DNA synthesis was examined after treatment of VSMCs with 10 μM PD98059 (a synthetic MEK1/2 inhibitor, Dudley *et al*., 1995) or 10 μM

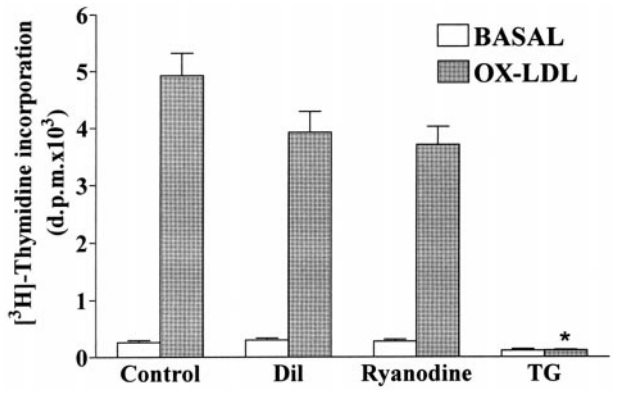

Figure 7 Effects of diltiazem, ryanodine, and thapsigargin on OX-LDL-induced [^{3}H]-thymidine incorporation in VSMCs. The cells were grown to confluence, made quiescent by serum-deprivation for 24 h. The cells were preincubated with 10 μM diltiazem (Dil), ryanodine or thapsigargin (TG) for 1 h and then stimulated with vehicle or 100 μg ml^{-1} OX-LDL. The incorporation of [^{3}H]-thymidine was determined as described in Figure 4. Data are expressed as the mean$\pm$s.e.mean of three separate experiments determined in triplicate. *$P<0.001$, compared with the cells exposed to OX-LDL.

SB203580 (a p38 MEK inhibitor, Cuenda *et al*., 1995) for 1 h. As shown in Figure 9A, treatment of VSMCs with these inhibitors caused a significant inhibition of the OX-LDL-induced [^{3}H]-thymidine incorporation ($P<0.01$, $n=3$). In addition, stimulation of VSMCs with OX-LDL increased p42/p44 MAPK phosphorylation (Figure 9B). This effect was inhibited in cells pretreated with increasing concentrations of PD98059 and SB203580. PD98059 was more potent than SB203580 to attenuate p42/p44 MAPK phosphorylation. At a concentration of 10 μM, PD98059, but not SB203580, caused a significant inhibition of p42/p44 MAPK phosphorylation in response to OX-LDL ($P<0.01$, $n=3$), confirming that MEK1/2 is required for MAPK activation in these cells. The phosphorylation of p42/p44 MAPK induced by OX-LDL was inhibited by SB203580 at higher concentration of 30 μM. Moreover, these two inhibitors (10 μM each) displayed an additive effect on this response when they were concurrently added to the cells. In addition, the maximal concentrations of these inhibitors used did not cause any toxic effect on VSMCs (data not shown). However, SB203580, at a concentration of 10 μM, caused a significant inhibition of [^{3}H]-thymidine incorporation, but no effect on phosphorylation of p42/p44 MAPK induced by OX-LDL, implying that an alternative pathway may be implicated in this mitogenic effect, perhaps the p38 MAPK pathway.

Activation of MAPK requires Ras and Raf-1

Several lines of evidence have suggested that Ras plays an important role in a variety of cell functions mediated through sequential activation of Raf-1, MEK1/2, and MAPK (Blenis, 1993; Post & Brown, 1996). To elucidate whether the activation of Ras/Raf was required for MAPK phosphorylation induced by OX-LDL and PDGF-BB, VSMCs were transfected with or without a dominant negative Ras (H-Ras-15A) or Raf (Raf-N4) and then treated with OX-LDL or PDGF-BB. As shown in Figure 10, both OX-LDL and PDGF-BB induced phosphorylation of p42/p44 MAPK in VSMCs. In addition, transfection with H-Ras-15A or Raf-N4 abolished p42/p44 MAPK phosphorylation induced by these two agents. Moreover, both OX-LDL and PDGF also activated MEK1/2 that is an upstream component of p42/p44 MAPK pathway, these stimulatory effects were almost suppressed by transfection with H-Ras-15A or Raf-N4. There was no significant change in the phosphorylation of p42/p44 MAPK induced by OX-LDL and PDGF-BB when VSMCs were transfected with empty vectors pZIP-NeoSV and pCGN, respectively. Parallel blot ran as controls that used antibody directed against the total p42 MAPK did not show any change (Figure 10). In addition, transfection of VSMCs with H-Ras-15A or Raf-N4 attenuated OX-LDL-induced [^{3}H]-thymidine incorporation by 45–55% (data not shown). These results demonstrated that OX-LDL shared a common mechanism with PDGF, activated Ras/Raf/MEK/MAPK pathway in VSMCs.

Discussion

It has been shown that OX-LDL displays a range of biological activities including modulation of gene expression of growth factors, adhesion molecules, and tissue factors, alteration of the motility of monocytes and macrophages (Witztum & Steinberg, 1991) and is also mitogenic for VSMCs (Auge *et al*., 1995; Heery *et al*., 1995). Several lines of evidence indicate that N-LDL- and OX-LDL-mediated VSMC proliferation are important contributors to the development of atherosclerosis. The mitogenic effect of N-LDL associated with activation of p42/p44 MAPK has been well characterized in cultured rat VSMCs (Sachinidis *et al*., 1997). However, the signalling pathways involved in the mitogenic effects triggered by OX-LDL are poorly understood. The present study demonstrates that OX-LDL can induce VSMC proliferation mediated through the activation of the Ras/Raf/MEK/MAPK pathway which is consistent with results obtained in rabbit VSMCs (Chatterjee *et al*., 1997). OX-LDL exhibited mitogenic potency similar to that of N-LDL, which is consistent with other results obtained in the same cell type (Sachinidis *et al*., 1997). Moreover, both activation of p42/p44 MAPK and [^{3}H]-thymidine incorporation stimulated by OX-LDL were modulated by Ca^{2+}, PKC, PI-PLC, and tyrosine kinase in these cells.

Several lines of evidence revealed that activation of p42/p44 MAPK was associated with a PTX-sensitive G protein signalling in COS-7 cells (Crespo *et al*., 1994) and Swiss 3T3 cells (Inglese *et al*., 1995). In the present study, OX-LDL-stimulated [^{3}H]-thymidine incorporation and p42/p44 MAPK activation was blocked by PTX treatment in VSMCs. Pretreatment of these cells with PTX has been shown to inhibit intrinsic GTPase activity of G_i protein by ADP-ribosylation of specific residues. Complete abrogation of PTX-sensitive G_i protein has been revealed by [^{32}P]-ADP-ribosylation of cell membranes prepared from VSMCs treated with PTX (Sachinidis *et al*., 1995). We further determined whether the effect of OX-LDL was derived from its degraded products of fatty acid such as lysophosphatidic acid. Pretreatment of VSMCs with lysophosphatidic acid for 24 h to down-regulate its receptors did not change the stimulatory effect of OX-LDL on activation of p42/p44 MAPK and [^{3}H]-thymidine incorporation, indicating that this mitogenic effect

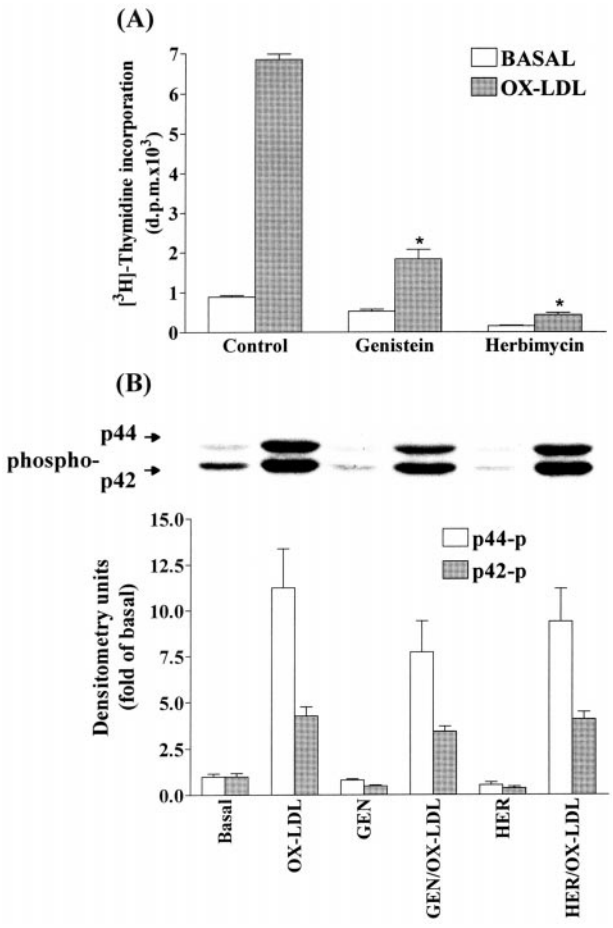

Figure 8 Involvement of tyrosine kinase in DNA synthesis and p42/p44 MAPK phosphorylation induced by OX-LDL in VSMCs. (A) The cells were grown to confluence, made quiescent by serum-deprivation for 24 h. The cells were preincubated with 10 μM genistein and 10 μM herbimycin A for 1 h, and then stimulated with vehicle or 100 μg ml^{-1} OX-LDL. The incorporation of [^{3}H]-thymidine was determined as described in Figure 4. Data are expressed as the mean$\pm$s.e.mean of three separate experiments determined in triplicate. *$P<0.01$, compared with the cells exposed to OX-LDL. (B) For MAPK experiment, after incubation with genistein (GEN) and herbimycin A (HER) for 1 h, the cells were stimulated with 100 μg ml^{-1} OX-LDL for 5 min. The cell lysates were subjected to 10% SDS–PAGE and transferred to nitrocellulose membrane. Bands were visualized by an ECL method as described in Figure 2. Similar results were obtained in three independent experiments.

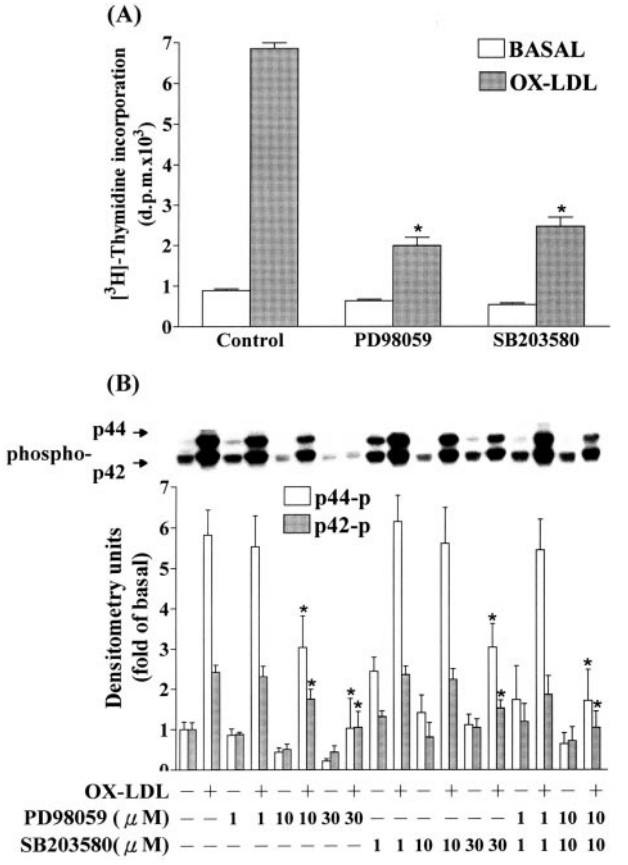

Figure 9 Effects of MAPK kinase inhibitors on OX-LDL-stimulated DNA synthesis and p42/p44 MAPK phosphorylation in VSMCs. (A) The cells were grown to confluence, made quiescent by serum-deprivation for 24 h. The cells were preincubated with 10 μM PD98059 and 10 μM SB203580 for 1 h, and then stimulated with vehicle or 100 μg ml^{-1} OX-LDL. The incorporation of [^{3}H]-thymidine was determined as described in Figure 4. Data are expressed as the mean$\pm$s.e.mean of three separate experiments determined in triplicate. *$P<0.01$, compared with the cells exposed to OX-LDL. (B) For MAPK experiment, after incubation with increasing concentrations of PD98059 and SB203580 for 1 h, the cells were stimulated with 100 μg ml^{-1} OX-LDL for 5 min. The cell lysates were subjected to 10% SDS–PAGE and transferred to nitrocellulose membrane. Bands were visualized by an ECL method as described in Figure 2. Data are expressed as the mean$\pm$s.e.mean of three separate experiments. *$P<0.01$, compared with the cells exposed to OX-LDL.

was not due to lysophosphatidic acid. These findings suggest that the mitogenic effect and activation of p42/p44 MAPK induced by OX-LDL were mediated through a receptor coupled to a PTX-sensitive G protein to stimulate signal transduction, possibly Ras/MAPK, that might play an important role in the initiation of DNA synthesis.

OX-LDL has been shown to activate PLC, PLA_2 and PLD to generate diacylglycerol (DAG) (Natarajan *et al*., 1995) and enhances PKC activity in VSMCs (Claus *et al*., 1996); this time course of PKC activation appears to be essential for late responses such as proliferation and differentiation (Nishizuka, 1992). In this study, we further investigated the regulatory mechanisms mediated by PI-PLC or PKC which were involved in OX-LDL-stimulated [^{3}H]-thymidine incorporation and activation of p42/p44 MAPK. These results demonstrated that long-term treatment with PMA or pretreatment with a PKC inhibitor staurosporine or PI-PLC inhibitor U73122 attenuated OX-LDL-stimulated [^{3}H]-thymidine incorporation and activation of p42/p44 MAPK, indicating that some components of the MAPK signal involve PI-PLC- and PKC-mediated activation of intermediate kinases. This is likely to be either Raf-1 which has been shown to be phosphorylated by PKC (Kolch *et al*., 1993) or possibly MEK1/2 which is also believed to be activated in a PKC-dependent manner (Lange-Carter *et al*., 1993). These results are consistent with studies which examine MAPK activation in response to other G-protein coupled agonists including angiotensin II and vasopressin (Molloy *et al*., 1993). Moreover, the effect of OX-LDL on MAPK phosphorylation may be mediated *via* indirect autocrine mechanisms including the release of VSMC-derived mitogenic neuroendocrine factors like endothelin-1 or angiotensin II

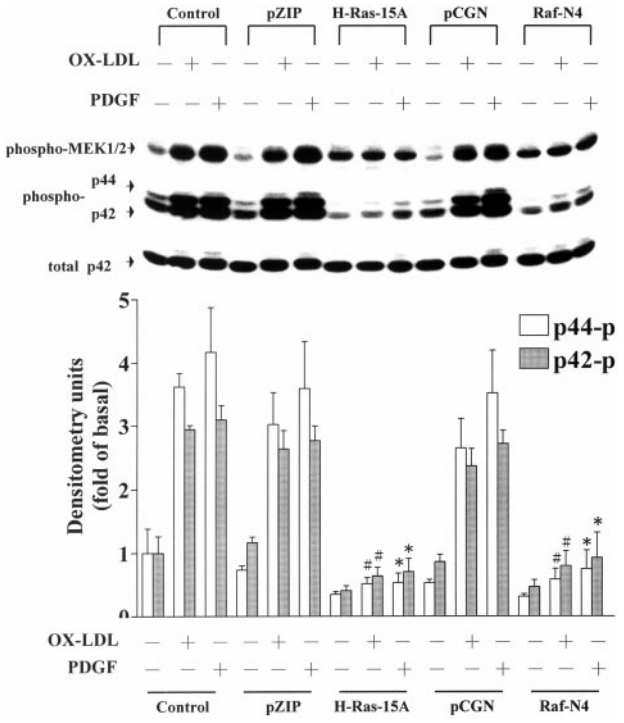

Figure 10 Requirement of Ras and Raf for OX-LDL- and PDGF-BB-induced activation of MEK1/2 and p42/p44 MAPK in VSMCs. Cells were transfected with plasmids encoding pZIP-NeoSV, H-Ras-15A, pCGN, or Raf-N4, and then stimulated with OX-LDL (100 μg ml^{-1}) or PDGF-BB (20 ng ml^{-1}) for 10 min. The cell lysates were subjected to 10% SDS–PAGE and transferred to nitrocellulose membrane. Western blot analysis was performed using an antiserum reactive with the anti-phospho-MEK1/2, anti-phospho-p42/p44 MAPK, and total p42 MAPK (as a control) polyclonal antibody. Bands were visualized by an ECL method. $*P<0.05$; $\#P<0.001$, compared with respective control.

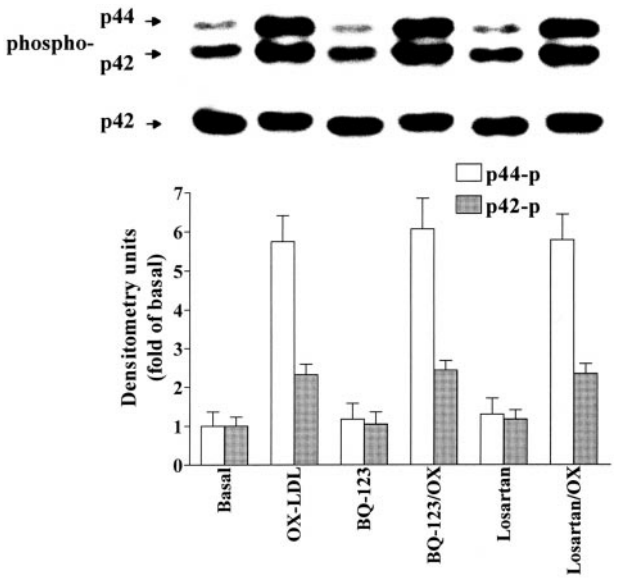

Figure 11 Effects of BQ-123 and losartan on OX-LDL-stimulated p42/p44 MAPK phosphorylation in VSMCs. The cells were grown to confluence, made quiescent by serum-deprivation for 24 h. The cells were preincubated with 10 μM BQ-123 or 10 μM losartan for 1 h, and then stimulated with vehicle or 100 μg ml^{-1} OX-LDL for 5 min. The cell lysates were subjected to 10% SDS–PAGE and transferred to nitrocellulose membrane. Bands were visualized by an ECL method as described in Figure 2. Data are expressed as the mean$\pm$s.e.m. of three separate experiments.

which were coupled to VSMC proliferation and activation of MAPK. This seemed not to be the case, since these cellular responses were not blocked by the endothelin-1 receptor antagonist BQ-123 or the angiotensin II receptor antagonist losartan (Figure 11).

To assess possible mechanisms that might mediate the mitogenic action of OX-LDL, we attempted to analyse some potentially participating pathways. The activation of VSMCs by OX-LDL is linked to stimulation of phosphoinositide hydrolysis, which produces two second messengers, DAG and IP_3 (Resink *et al*., 1992). DAG is known to activate PKC and IP_3 to release Ca^{2+} from intracellular stores. The activation of PKC and increase in $[Ca^{2+}]_i$ seems to account for the effect of growth-promoting agents as revealed by [^{3}H]-thymidine incorporation (Natarajan *et al*., 1995; Auge *et al*., 1995; Heery *et al*., 1995). In this study, the mitogenic effect of OX-LDL may be correlated with its ability to stimulate IP accumulation and Ca^{2+} mobilization. The mitogenic effect of OX-LDL in VSMCs required the presence of Ca^{2+}. This hypothesis was supported by the results that removal of Ca^{2+} with BAPTA/EGTA significantly attenuated p42/p44 MAPK activation and [^{3}H]-thymidine incorporation in rat cultured VSMCs. In addition, thapsigargin depleted the internal Ca^{2+} pools and completely attenuated [^{3}H]-thymidine incorporation induced by OX-LDL. These results indicated that release of internal Ca^{2+} may play an important role in the mitogenic effect of OX-LDL.

Activation of p42/p44 MAPK is known to require both tyrosine and threonine phosphorylations by the dual specificity MEK1/2. Several lines of evidence indicate the complexity in the mechanisms for agonist stimulation of MAPK activities in VSMCs including the possible involvement of tyrosine kinase upstream of MAPK kinase (Blenis, 1993; Post & Brown, 1996). In this study, we have shown that stimulation with OX-LDL results in activation of p42/p44 MAPK in a time- and concentration-dependent manner. These results are consistent with the findings that MAPK isoforms are activated by agonists in several cell types (Blenis, 1993; Lange-Carter *et al*., 1993; Post & Brown, 1996; Pouyssegur & Seuwen, 1992). Furthermore, we also investigated the involvement of a tyrosine kinase in the MAPK cascade of VSMCs stimulated by OX-LDL, using tyrosine kinase inhibitors genistein and herbimycin A. The results with the tyrosine kinase inhibitors showed that the OX-LDL-induced DNA synthesis was mediated through the activation of tyrosine kinase. These results may imply that the mitogenic response to OX-LDL was dependent on a tyrosine kinase, with the demonstration that [^{3}H]-thymidine incorporation was completely inhibited by tyrosine kinase inhibitors. However, the activation of p42/p44 MAPK by OX-LDL was slightly inhibited by genistein and herbimycin A. These results suggest that activation of p42/p44 MAPK induced by OX-LDL may be not mediated through a tyrosine kinase pathway in VSMCs.

Although mitogenic signals from the activation of specific tyrosine kinase-coupled growth factors have been well characterized, the mechanism by which the G protein-coupled

receptors activate the components of MAPK pathway is not completely understood. PD98059, a synthetic and highly specific MEK1/2 inhibitor, has been shown to inhibit the activation of p42/p44 MAPK by several stimuli (Dudley *et al*., 1995). Because activation of components in the MAPK cascade originates from stimulation of cells by growth factors, it has been proposed that transmission of the signal along this pathway is required for the induction of mitogenesis. In support of this hypothesis, inhibition of MEK1/2 by PD98059 has been associated with a decrease in PDGF-stimulated [^{3}H]-thymindine incorporation in 3T3 cells (Dudley *et al*., 1995). In the current study, pretreatment with PD98059 attenuated the OX-LDL-induced activation of p42/p44 MAPK and DNA synthesis in VSMCs, revealing that stimulation of MEK1/2 was required for OX-LDL-induced responses in these cells. In addition, SB203580 showed an inhibitory effect on [^{3}H]-thymidine incorporation similar to that of PD98059, but only displayed an inhibitory effect on p42/p44 MAPK activation at high concentration, indicating that an alternate pathway such as p38 MAPK may also be involved in the mitogenic effect of OX-LDL in rat cultured VSMCs.

It is well established that growth factors activate phosphorylation of cascade of protein kinases including tyrosine kinases, Ras, Raf-1, MEK and MAPK (Blenis, 1993; Post & Brown, 1996). Several studies have proposed that MAPK could be a convergence point for growth signals originating from tyrosine kinase receptors, G protein-coupled receptors, and cytokines (Blenis, 1993; Post & Brown, 1996). In VSMCs, it has been reported that MAPK activation is important for PDGF and OX-LDL-induced cell proliferation (Heery *et al*., 1995; Auge *et al*., 1995; Marrero *et al*., 1997; Weber *et al*., 1997). Ras, the small G protein, regulates a variety of cellular processes including growth and differentiation of many cell types (Kerkhoff & Rapp, 1998). Several lines of evidence have suggested that Ras is activated by various stimuli for growth and differentiation and that the activated Ras evokes the phosphorylation cascade of protein kinases including Raf-1, MEK1/2, and MAPK (Blenis, 1993; Post & Brown, 1996). It has been demonstrated that PDGF-BB-induced cell proliferation may be mediated through the activation of Ras in VSMCs (Marrero *et al*., 1997; Weber *et al*., 1997). In this study, to elucidate whether Ras was required for OX-LDL- and PDGF-BB-induced activation of MAPKs, VSMCs were transfected with H-Ras-15A that preferentially interacted with guanine nucleotide exchange factors and inhibited Ras functions (Chen *et al*., 1994; Cepko *et al*., 1984). We found that OX-LDL- and PDGF-BB-induced p42/p44 MAPK activation were suppressed by transfection with the dominant negative mutant of Ras (H-Ras-15A) in VSMCs, as previously reported in other cell types (Chen *et al*., 1994; Abellatif *et al*., 1998). Several studies have also shown that activated Ras binds and activates Raf-1, resulting in the activation of MEK and MAPK (Blenis, 1993; Satoh *et al*., 1992; Brtva *et al*., 1995). Consistent with these reports, we demonstrated that in VSMCs, OX-LDL- and PDGF-BB-induced MAPK activation are suppressed by transfection with a dominant negative mutant of Raf-1 (Raf-N4), suggesting that Raf-1 plays a key role in OX-LDL- and PDGF-BB-induced activation of MEK/MAPK cascade in VSMCs.

In conclusion, we report here that the mitogenic effect of OX-LDL is mediated through a PTX-sensitive G protein-coupled receptor that involves the activation of the Ras/Raf/MEK/MAPK pathway similar to that of PDGF-BB in rat cultured VSMCs. These results conclude that OX-LDL stimulates activation of MAPK pathway that is regulated by PKC, Ca^{2+}, and tyrosine kinase. These results raise the possibility that OX-LDL may play an important role in the pathogenesis of atherosclerosis as well as structural changes seen in cardiovascular disease.

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