OxLDL induces mitogen-activated protein kinase activation mediated via PI3-kinase/Akt in vascular smooth muscle cells

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Abstract Oxidized low-density lipoprotein (OxLDL) is a risk factor in atherosclerosis and stimulates multiple signaling pathways, including activation of phosphatidylinositol 3-kinase (PI3-K)/Akt and p42/p44 mitogen-activated protein kinase (MAPK), which are involved in mitogenesis of vascular smooth muscle cells (VSMCs). We therefore investigated the relationship between PI3-K/Akt and p42/p44 MAPK activation and cell proliferation induced by OxLDL. OxLDL stimulated Akt phosphorylation in a time- and concentration-dependent manner, as determined by Western blot analysis. Phosphorylation of Akt stimulated by OxLDL and epidermal growth factor (EGF) was attenuated by inhibitors of PI3-K (wortmannin and LY294002) and intracellular Ca^{2+} chelator (BAPTA/AM) plus EDTA. Pretreatment of VSMCs with pertussis toxin, cholera toxin, and forskolin for 24 h also attenuated the OxLDL-stimulated Akt phosphorylation. In addition, pretreatment of VSMCs with wortmannin or LY294002 inhibited OxLDL-stimulated p42/p44 MAPK phosphorylation and [^{3}H]thymidine incorporation. Furthermore, treatment with U0126, an inhibitor of MAPK kinase (MEK)1/2, attenuated the p42/p44 MAPK phosphorylation, but had no effect on Akt activation in response to OxLDL and EGF. Overexpression of p85-DN or Akt-DN mutants attenuated MEK1/2 and p42/p44 MAPK phosphorylation stimulated by OxLDL and EGF.⬛ These results suggest that the mitogenic effect of OxLDL is, at least in part, mediated through activation of PI3-K/Akt/MEK/MAPK pathway in VSMCs.—Chien, M-W., C-S. Chien, L-D. Hsiao, C-H. Lin, and C-M. Yang. **OxLDL induces mitogen-activated protein kinase activation mediated via PI3-kinase/Akt in vascular smooth muscle cells.** *J. Lipid Res.* 2003. **44: 1667–1675.**

Supplementary key words oxidized LDL • phosphatidylinositol 3-kinase • protein kinase C • Ca^{2+} • DNA synthesis • epidermal growth factor

Several lines of evidence have demonstrated that an oxidative modification of LDL increases its atherogenic potential, because oxidatively modified LDL has been shown to be more mitogenic for vascular smooth muscle cells (VSMCs) and monocytes (1). In fact, oxidized LDL (OxLDL) exerts many effects in vitro that could contribute to the progression of atherosclerosis if the oxidative process is allowed to continue, and OxLDL accumulates to higher levels in vivo (2). OxLDL induces atherosclerosis by stimulating monocyte infiltration (3) and smooth muscle cell migration and proliferation (4). It contributes to atherothrombosis by inducing endothelial cell apoptosis (5), and thus plaque erosion (6), by impairing the anticoagulant balance in endothelium, stimulating tissue factor production by smooth muscle cells, and inducing apoptosis in macrophages. Moreover, both native-LDL and OxLDL have also been shown to stimulate the proliferation of VSMCs in several species (7–9). The mitogenic effect of OxLDL may be indirectly mediated through synthesis of platelet-derived growth factor (PDGF) in macrophages and VSMCs (10) and from other cells in the vessel walls (11). Therefore, the proliferation of VSMCs caused by OxLDL in the intima of arterial wall is considered to be a critical event in the development of atherosclerotic plaque.

The mechanisms of OxLDL-induced atherosclerosis have been under intense investigation, and have been shown to share many properties of growth factors by regulating specific signal transduction pathways involved in cell proliferation (2, 12). It has been established that growth factor receptor-mediated signaling commonly involves the activation of mitogen-activated protein kinases (MAPKs), protein tyrosine kinases, and phosphatidylinositol 3-kinase (PI3-K). For example, in VSMCs, both PDGF and angiotensin II stimulated not only p42/p44 MAPK but also kinase activity of Akt (13–15) and played a pivotal role in mitogenesis. Accordingly, OxLDL has been demonstrated to activate members of the MAPK family, transcription factors such as AP-1, and immediate early growth

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genes, including *c-fos*, *c-jun*, and *c-myc*, whose products help regulate the expression of growth factors (16). Moreover, in VSMCs, OxLDL has been shown to activate the Ras/Raf/MEK/MAPK pathway through a pertussis toxin (PTX)-sensitive G-protein-coupled receptor in our previous studies and others (7, 8). In addition, increasing evidence has emerged showing that PI3-K and its downstream effector Akt play a key role in the regulation of cell survival (17). A constitutively active form of PI3-K has been demonstrated to activate MAPK by stimulating Ras (18). Interference with the PI3-K pathway, using either pharmacological inhibitors (19, 20) or expression of a dominant negative protein (18, 20) also blocks MAPK activation, suggesting that PI3-K participates in MAPK activation in these systems. However, the relationship between PI3-K/Akt and p42/p44 MAPK activation triggered by OxLDL is poorly understood in rat VSMCs.

Akt is one of the best-characterized targets of PI3-K lipid products (21, 22). Phosphorylation of Thr308 and Ser473 is critical for activation of Akt, because mutations of Thr308 and Ser473 to alanine inhibit the insulin or insulin-like growth factor-induced activation of Akt. Furthermore, several lines of evidence have shown that PI3-K is involved in the OxLDL-induced cell proliferation of macrophages (12, 23) and -inhibited apoptosis of macrophages (24). The present study was undertaken to determine the ability of OxLDL to activate PI3-K/Akt and to investigate the role of PI3-K/Akt in the activation of p42/p44 MAPK pathway associated with cell proliferation. We demonstrate that OxLDL activated the PI3-K/Akt signaling cascade in VSMCs. PI3-K inhibitors wortmannin and LY294002 inhibited OxLDL-stimulated Akt activation and DNA synthesis in these cells. In addition, we demonstrate that inhibition of PI3-K or overexpression of p85-DN or Akt-DN partially blocks activation of p42/p44 MAPK in response to OxLDL. These data are the first to elucidate that in rat cultured VSMCs, the mechanism for OxLDL-stimulated phosphorylation of p42/p44 MAPK and cell proliferation is partially mediated through activation of PI3-K/Akt.

MATERIALS AND METHODS

Materials

DMEM, Lipofectamine Plus reagent, and fetal bovine serum (FBS) were purchased from Gibco BRL (Gaithersburg, MD). [³H]methyl thymidine, Hybond C membrane, and enhanced chemiluminescence (ECL) Western blotting detection system were from Amersham (Buckinghamshire, UK). PhosphoPlus p42/p44 MAPK, phosphoPlus MEK1/2 and phosphoPlus Akt antibody kits were from New England Biolabs (Beverly, MA). p42 MAPK and Akt antibodies were from Santa Cruz (Santa Cruz, CA). GAPDH was from Biogenesis (Bournemouth, UK). Genistein, BAPTA/AM, U0126, daidzein, and wortmannin were from Biomol (Plymouth Meeting, PA). BCA protein assay kit was from Pierce (Rockford, IL). Enzymes and other chemicals were from Sigma (St. Louis, MO).

Preparation of LDL and OxLDL

Human LDL (*d* 1.019–1.063 g/ml) was prepared by sequential ultracentrifugation (4°C) of plasma from healthy blood (25).

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. (26). The OxLDL 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, lipid peroxides, and conjugated dienes using the method described by Morel, Dicorleto, and Chisolm (27). The OxLDL preparation was filtered through 0.22 μm filters and stored at 4°C. The protein content of OxLDL was determined by BCA reagents (Pierce).

VSMCs

VSMCs were isolated and cultured from Sprague-Dawley rats as previously described (8). The cells were plated onto (0.5 ml/well) 24-well and (2 ml/well) 6-well culture plates for [³H]thymidine incorporation and Akt and MAPK phosphorylation, respectively.

[³H]thymidine incorporation

Cells were growth-arrested by incubation in serum-free DMEM for 24 h. The cells were stimulated with OxLDL at a concentration of 100 μg/ml. When inhibitors were used, they were applied 1 h prior to the addition of OxLDL. [³H]thymidine incorporation and OxLDL preparation were performed as previously described (8).

Plasmids and transfection

The plasmids encoding p85-DN and Akt-DN (dominant negative mutants of p85 and Akt) were kindly provided by Dr. Richard D. Ye (Department of Pharmacology, University of Illinois at Chicago). All plasmids were prepared by using QIAGEN plasmid DNA preparation kits.

VSMCs were plated at 3×10^5 cells/ml (2 ml/well) in 6-well culture plates for 24 h, reaching ~80% confluence. Cells were washed once with PBS and 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 the instructions of the manufacturer (GIBCO-BRL). The amount of plasmid was kept constant (2 μg of Akt-DN for each well). The DNA PLUS-Lipofectamine reagent complex (0.2 ml) was added to each well and incubated at 37°C for 5 h, and then 1 ml of OPTI-MEM I medium containing 20% FBS was 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 OxLDL or epidermal growth factor (EGF).

Preparation of cell extracts and Western blot analysis

SDS-PAGE and Western blotting were performed as previously described (8). The membrane was incubated successively at room temperature with 5% (w/v) BSA in TTBS for 1 h, and then incubated overnight at 4°C with the anti-phospho-MAPK kinase (MEK)1/2, anti-phospho-p42/p44 MAPK, or anti-phospho-Akt polyclonal antibody used at a dilution of 1:1000 in TTBS. Total p42 MAPK and Akt antibodies were used as positive controls. Membranes were washed with TTBS four times for 5 min each and 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

Data are expressed as means ± SEM. 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 symbols.

RESULTS

Akt activation by OxLDL

In our previous studies, we have found that OxLDL stimulated p42/p44 MAPK phosphorylation mediated through a Ras/Raf/MEK pathway in VSMCs (7, 8). In searching for alternative pathway involvement in p42/p44 MAPK activation, the PI3-K pathway was investigated. To evaluate the role of PI3-K in the OxLDL-mediated signaling pathway, we examined whether OxLDL stimulated Akt phosphorylation by immunoblotting cell lysates with a phospho-specific antibody for activated Akt that recognized Akt only when phosphorylated at Ser-473 (28). As shown in **Fig. 1A**, OxLDL stimulated a marked phosphorylation of Akt at 1 min, reached a peak at 3 min, and then gradually declined close to the basal level within 6 h. In addition, OxLDL evoked Akt phosphorylation in a concentration-dependent manner, and a maximal effect was achieved with 100 µg/ml OxLDL (Fig. 1B). Total Akt proteins were not changed by treatment with OxLDL during the period of observation.

Effects of PI3-K inhibitors on Akt activation

Because Akt functions as a downstream component of PI3-K (28), we next examined the effect of PI3-K inhibitors wortmannin and LY294002 on OxLDL- and EGF-induced Akt activation. As shown in **Fig. 2**, pretreatment of VSMCs with either wortmannin or LY294002 significantly attenuated the OxLDL- and EGF-stimulated Akt activation. These results suggest that PI3-K is involved in the OxLDL- and EGF-stimulated Akt activation in VSMCs.

Effect of PTX, cholera toxin, or forskolin on Akt activation

To determine whether OxLDL-stimulated Akt activation was mediated by a PTX-sensitive G protein, the cells were pretreated with 100 ng/ml PTX for 24 h and then stimulated with 100 µg/ml OxLDL or 5 ng/ml EGF for 3 min. As expected, the OxLDL-stimulated Akt activation was significantly attenuated by pretreatment with PTX (**Fig. 3**). To investigate whether PTX-insensitive G_s protein might also play a role in the activation of the PI3-K/Akt pathway, VSMCs were pretreated with cholera toxin (CTX) (10 µg/ml) for 24 h. The effect of OxLDL on Akt activation was significantly inhibited by CTX treatment (Fig. 3). In addition, treatment of VSMCs with forskolin also prevented the activation of Akt by OxLDL (Fig. 3). Because cAMP has been reported to inhibit Akt activation in several cell types (29, 30), we conclude that the permanent activation of adenylate cyclase by CTX and forskolin
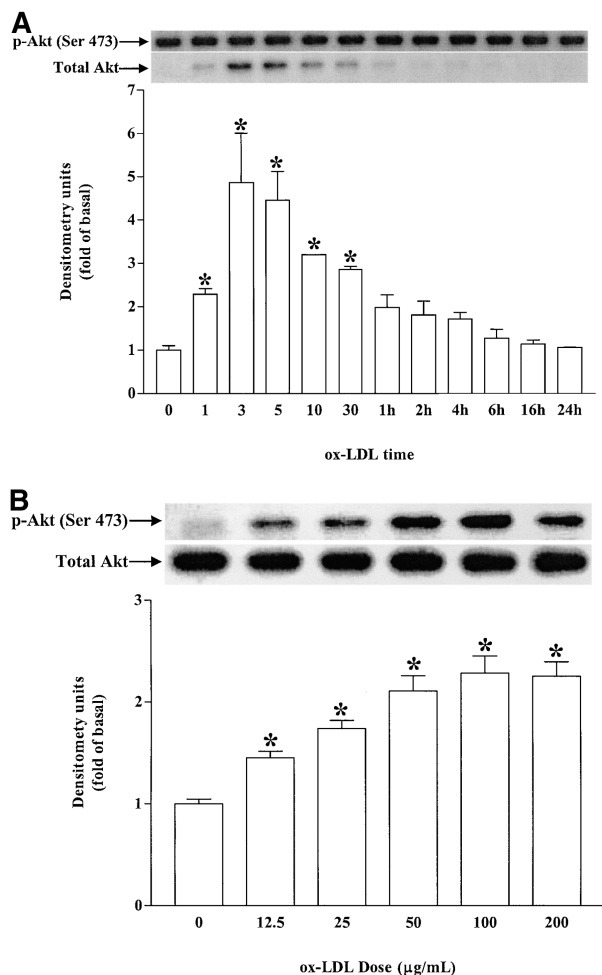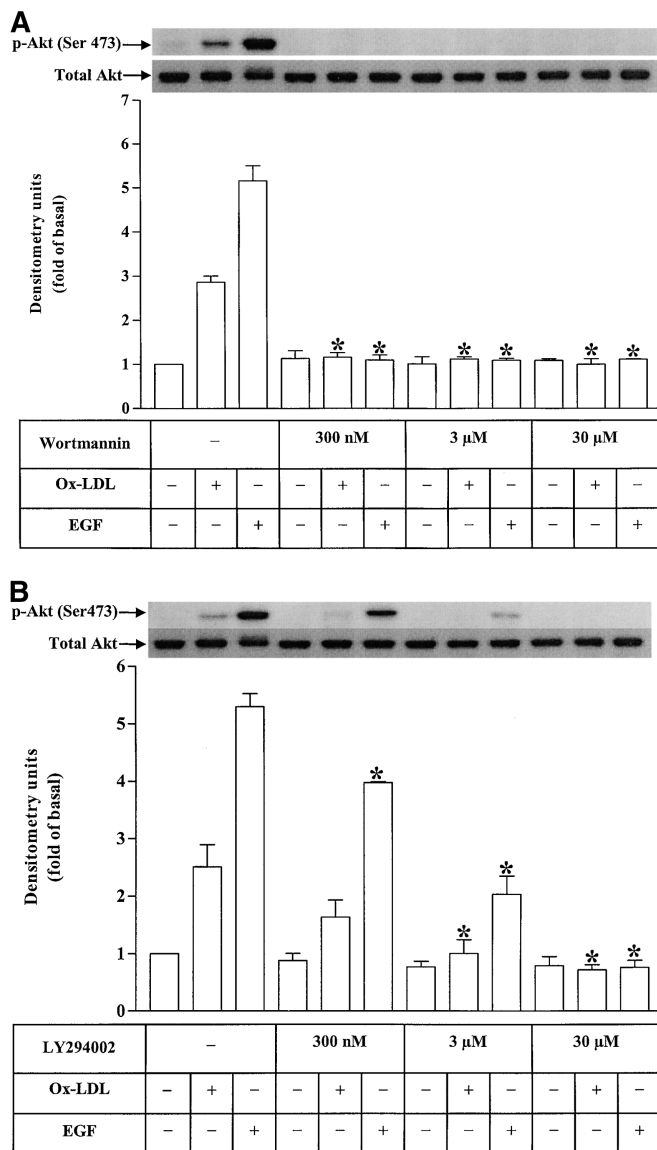

Fig. 1. Oxidized LDL (OxLDL)-stimulated Akt phosphorylation in vascular smooth muscle cells (VSMCs). A: Time-dependence of OxLDL-stimulated Akt phosphorylation. Cells were grown to confluence, made quiescent by serum deprivation for 24 h, and incubated with 100 µg/ml OxLDL for various times (1 min to 24 h). B: Concentration dependence of OxLDL-stimulated Akt phosphorylation. Cells were treated with various concentrations of OxLDL (6.25 to 200 µg/ml) for 3 min. After incubation, 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-Akt polyclonal antibody for activated Akt and anti-Akt antibody for the total Akt as an indicator of protein loading in each well. Bands were visualized by an enhanced chemiluminescence (ECL) method. Data are expressed as the mean ± SEM of three separate experiments. * $P < 0.05$, compared with the respective basal levels.

counteracts the stimulation of Akt activity in response to OxLDL. In contrast, treatment of VSMCs with these reagents did not significantly attenuate the EGF-stimulated Akt activation.

Involvement of Ca^{2+} in Akt activation

It has been shown that OxLDL stimulates phosphoinositide hydrolysis (31) and increases intracellular Ca^{2+} mobilization (32). To evaluate the involvement of intracellular Ca^{2+} mobilization in OxLDL-mediated Akt activation, we pretreated VSMCs with either the intracellular Ca^{2+} chelator BAPTA-AM plus EDTA or nifedipine for 1 h

A

Wortmannin		–			300 nM			3 μM			30 μM	
Ox-LDL	–	+	–	–	+	–	–	+	–	–	+	–
EGF	–	–	+	–	–	+	–	–	+	–	–	+

B

LY294002		–			300 nM			3 μM			30 μM	
Ox-LDL	–	+	–	–	+	–	–	+	–	–	+	–
EGF	–	–	+	–	–	+	–	–	+	–	–	+

Fig. 2. Effects of phosphatidylinositol 3-kinase (PI3-K) inhibitors on Akt phosphorylation stimulated by OxLDL and epidermal growth factor (EGF). VSMCs were incubated with (A) wortmannin or (B) LY294002 (ranging from 300 nM to 30 μM) for 1 h and then stimulated with 100 μg/ml OxLDL or 5 ng/ml EGF for 3 min. After incubation, the cell lysates were subjected to 10% SDS-PAGE and transferred to nitrocellulose membrane. Phosphorylation of Akt was determined as described in the legend to Fig. 1. Data are expressed as the mean ± SEM of three separate experiments. * $P <$ 0.05, compared with the respective basal levels.

and then stimulated them with 100 μg/ml OxLDL or 5 ng/ml EGF for 3 min. We found that BAPTA-AM/EDTA inhibited both OxLDL- and EGF-mediated Akt activation in a concentration-dependent manner (**Fig. 4A**). Moreover, to investigate the source of external Ca^{2+} involved in Akt phosphorylation, VSMCs were pretreated with the Ca^{2+} channel blocker nifedipine before exposure to OxLDL or EGF. Nifedipine had no effect on OxLDL-induced Akt phosphorylation (Fig. 4B). These results suggest that release of Ca^{2+} from internal stores was involved in OxLDL-stimulated Akt activation in VSMCs.

| Treatment		–			PTX			CTX			FKL	
Ox-LDL	–	+	–	–	+	–	–	+	–	–	+	–
EGF	–	–	+	–	–	+	–	–	+	–	–	+
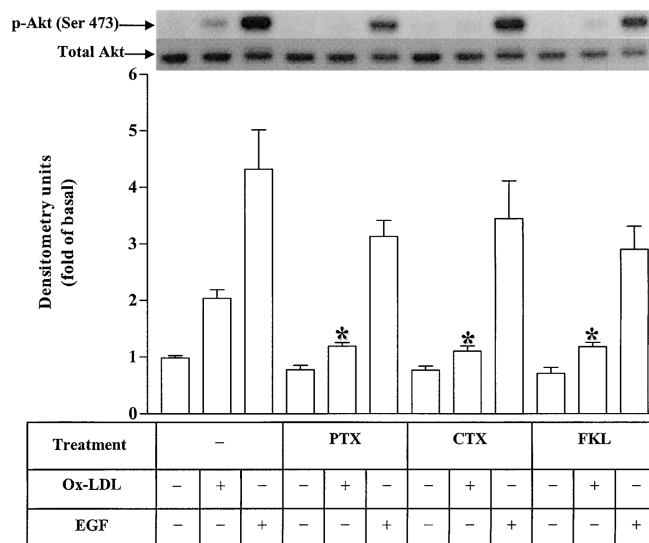
Fig. 3. Effects of pertussis toxin (PTX), cholera toxin (CTX), and forskolin on Akt phosphorylation stimulated by OxLDL and EGF. VSMCs were incubated with or without PTX (100 ng/ml) or CTX (10 μg/ml) for 24 h, or forskolin (100 μM) for 1 h and then stimulated with 100 μg/ml OxLDL or 5 ng/ml EGF for 3 min. After incubation, the cell lysates were subjected to 10% SDS-PAGE and transferred to nitrocellulose membrane. Phosphorylation of Akt was determined as described in the legend to Fig. 1. Data are expressed as the mean ± SEM of three separate experiments. * $P <$ 0.05, compared with the respective basal levels.

PI3-K/Akt is involved in p42/p44 MAPK activation in response to OxLDL

The downstream effect of PI3-K/Akt on the activation of p42/p44 MAPK pathway in response to OxLDL was investigated by determining the phosphorylation of p42/p44 MAPK using a phospho-specific antibody against these proteins. As shown in **Fig. 5A, B**, pretreatment of VSMCs with either wortmannin or LY294002 significantly inhibited p42/p44 MAPK phosphorylation stimulated by OxLDL, but had no significant effect on EGF responses. [³H]thymidine incorporation induced by OxLDL was also inhibited by wortmannin (Fig. 5C). In contrast, pretreatment of these cells with the MEK1/2 inhibitor U0126 significantly attenuated the p42/p44 MAPK phosphorylation stimulated by OxLDL and EGF, but there was no effect on Akt activation in response to these agents (**Fig. 6**). These results suggest that PI3-K is an upstream component and critical for p42/p44 MAPK activation and DNA synthesis stimulated by OxLDL in VSMCs.

PI3-K is activated following tyrosine phosphorylation of its p85 subunit (33). Akt, the downstream component of PI3-K, is activated subsequent to phosphorylation of its Ser-473 and Thr-308 residues. To further assess the role of PI3-K/Akt in p42/p44 MAPK activation in response to OxLDL and EGF, dominant negative constructs of p85-DN and Akt-DN were transfected into these cells. Similar to the results with PI3-K inhibitors, transfection of VSMCs with either p85-DN or Akt-DN plasmid significantly attenuated both MEK1/2 and p44 MAPK phosphorylation stimulated by OxLDL and EGF, but there was no significant effect on p42 MAPK, as compared with those of control cells (**Fig. 7**). Taken to-

A

B
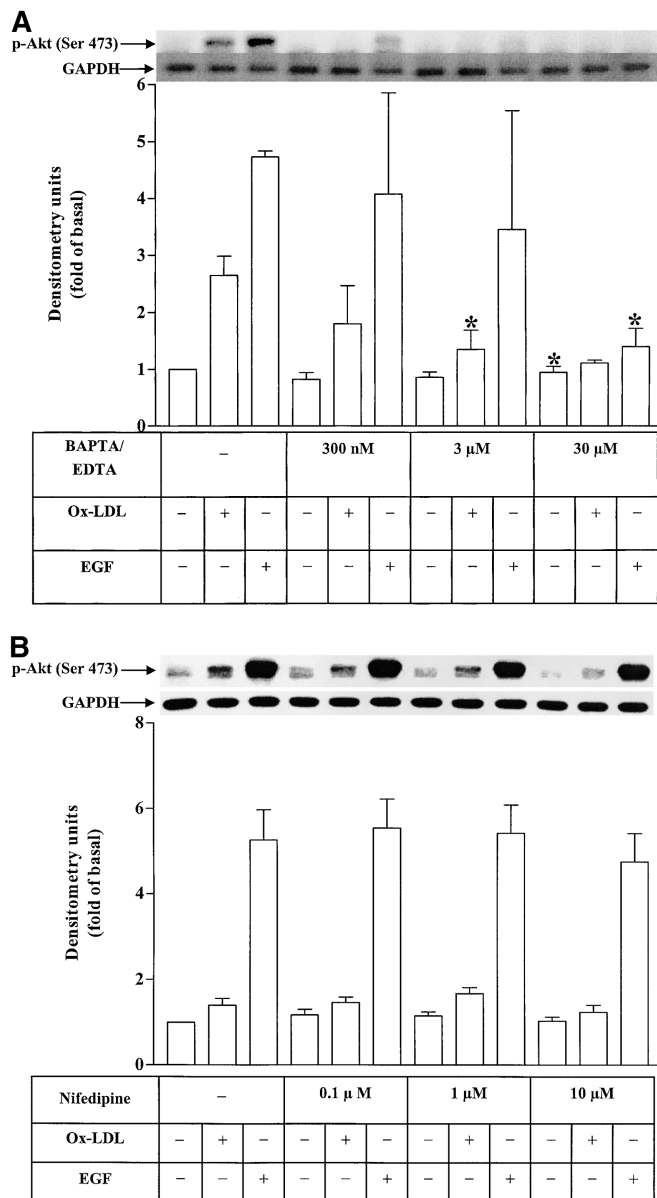
Fig. 4. Effect of Ca^{2+} on Akt phosphorylation stimulated by OxLDL and EGF. VSMCs were incubated with (A) BAPTA-AM (ranging from 300 nM to 30 μM) plus 1 mM EDTA and (B) nifedipine (ranging from 0.1 to 10 μM) for 1 h and then stimulated with 100 μg/ml OxLDL or 5 ng/ml EGF for 3 min. After incubation, the cell lysates were subjected to 10% SDS-PAGE and transferred to nitrocellulose membrane. Phosphorylation of Akt was determined as described in the legend to Fig. 1. Then the membrane was stripped and reprobed with GAPDH antibody to serve as an indicator of protein loading in each well. Data are expressed as the mean ± SEM of three separate experiments. * $P < 0.05$, compared with the respective basal levels.

gether, these results suggest that PI3-K/Akt, at least in part, acts as an upstream component in MEK/MAPK activation in response to OxLDL and EGF in VSMCs.

DISCUSSION

It has been shown that OxLDL displays a range of biological activities including: modulation of gene expression of growth factors, adhesion molecules, and tissue factors; and alteration of the motility of monocytes and macrophages. It is also mitogenic for VSMCs (2, 7–9). Several lines of evidence indicate that OxLDL-mediated VSMC proliferation is an important contributor to the development of atherosclerosis. Our data and those of others have shown previously that OxLDL-stimulated p42/p44 MAPK activation associated with cell proliferation might be mediated through Ras/Raf/MEK pathway in cultured rat VSMCs (8, 9). However, several extracellular stimuli can activate more than one pathway, and different pathways may converge to activate a common signaling pathway in various cell types. For example, in VSMCs, both PDGF and angiotensin II stimulated not only p42/p44 MAPK but also kinase activity of Akt (13, 14) and played a pivotal role in mitogenesis. In addition, a constitutively active form of PI3-K has been demonstrated to activate MAPK by stimulating Ras (18). Interference with the PI3-K pathway, using either pharmacological inhibitors (19, 20) or expression of a dominant negative protein (18, 20), also blocks MAPK activation, suggesting that PI3-K is involved in MAPK activation in these systems. However, the relationship between PI3-K/Akt and p42/p44 MAPK activation triggered by OxLDL is poorly understood in rat VSMCs. The present study demonstrates that OxLDL-stimulated p42/p44 MAPK activation might be partially mediated through PI3-K/Akt pathway, which is consistent with results obtained in several cell types (18–20, 34). Moreover, activation of PI3-K/Akt stimulated by OxLDL was modulated by Ca^{2+} via a PTX-sensitive G protein-coupled receptor in rat VSMCs.

We defined PI3-K/Akt as an upstream activator of p42/p44 MAPK in response to OxLDL, consistent with the results obtained in other signaling systems (18–20, 34). Our results also contrast with those obtained with human coronary artery endothelial cells (35). In that study, OxLDL was shown to reduce the phosphorylation of protein kinase B/Akt in human coronary artery endothelial cells. The apparent discrepancy between these two studies most likely reflects fundamental differences in the cell types used. Several studies have suggested that G_i protein-coupled receptor- and $G_{\beta\gamma}$-stimulated MAPK activation is attenuated by the PI3-K inhibitors wortmannin and LY294002 (36). PI3-K was also identified as the target of $G_{\beta\gamma}$ complexes from PTX-sensitive G proteins and was suggested to link G_i protein-coupled receptors to the MAPK pathway (37, 38). Furthermore, the PI3-K/Akt pathway itself plays an important role in cellular proliferation and survival (17, 39) and may thus provide essential signals for cell growth in response to extracellular stimuli. In the present study, Akt, a downstream component of PI3-K, is phosphorylated in response to OxLDL and EGF in VSMCs (Fig. 2). Activation of Akt was attenuated by the PI3-K inhibitors wortmannin and LY294002 in a concentration-dependent manner, suggesting the implication of PI3-K in Akt activation stimulated by OxLDL and EGF (Fig. 2). However, it should be noted that the EGF-stimulated response was not significantly affected by PI3-K inhibitors and genistein, possibly because different PI3-K isoforms were activated by EGF and OxLDL, respectively (38).
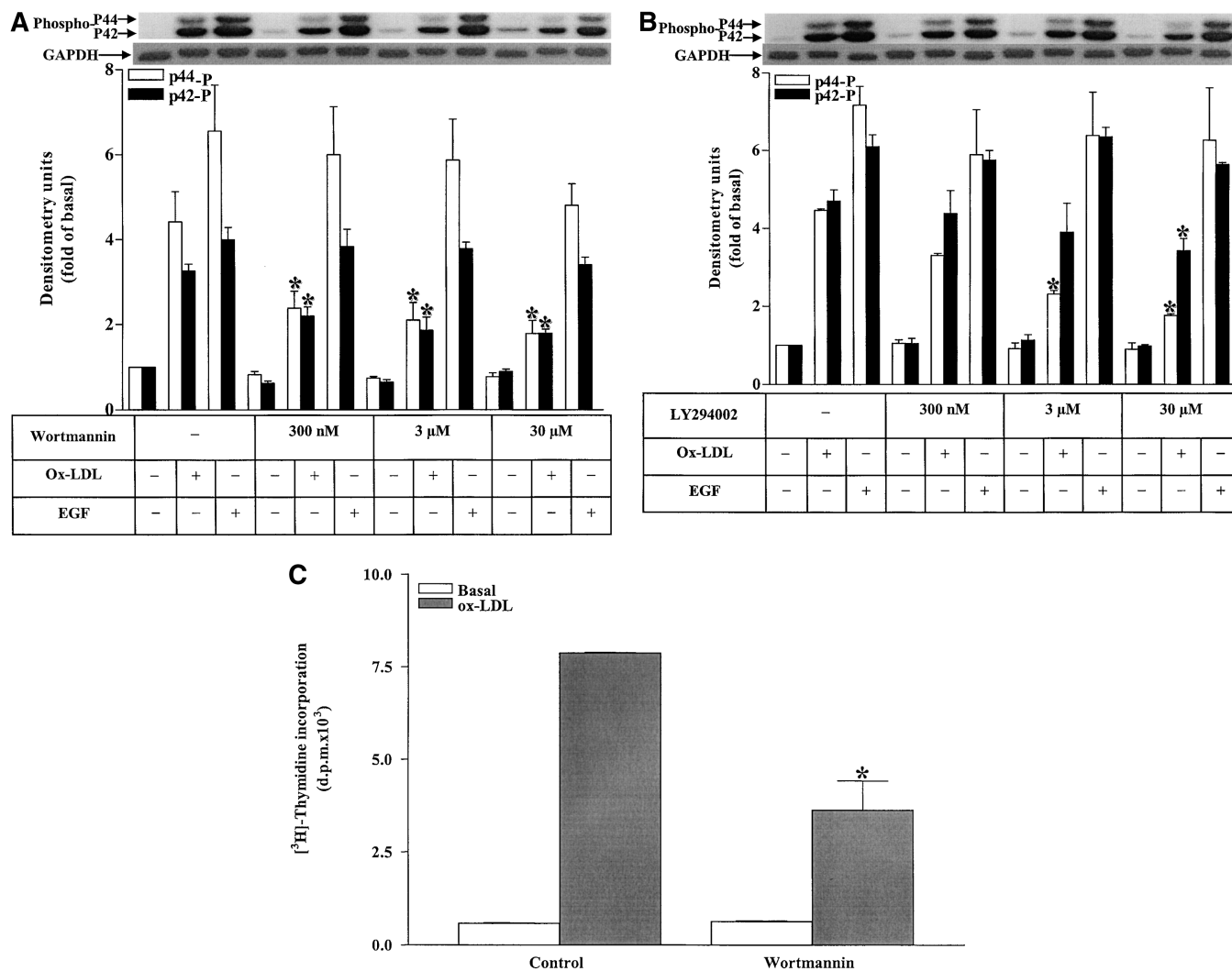
Fig. 5. Involvement of PI3-kinase in p42/p44 p44 mitogen-activated protein kinase (MAPK) phosphorylation and DNA synthesis stimulated by OxLDL. VSMCs were preincubated with PI3-K inhibitors. For the MAPK experiment, after incubation with either wortmannin (A) or LY294002 (B) for 1 h, the cells were stimulated with 100 µg/ml OxLDL or 5 ng/ml EGF for 3 min, and 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-GAPDH antibody as an indicator of protein loading in each well. C: For DNA synthesis, after incubation with wortmannin for 1 h, cells were labeled with 1 µCi/ml [^{3}H]thymidine for another 16 h in the continuous presence of 100 µg/ml OxLDL. The incorporation of [^{3}H]thymidine was determined as described in Materials and Methods. Bands were visualized by an ECL method. Data are expressed as the mean ± SEM of three separate experiments. * $P < 0.05$, compared with the respective basal levels.
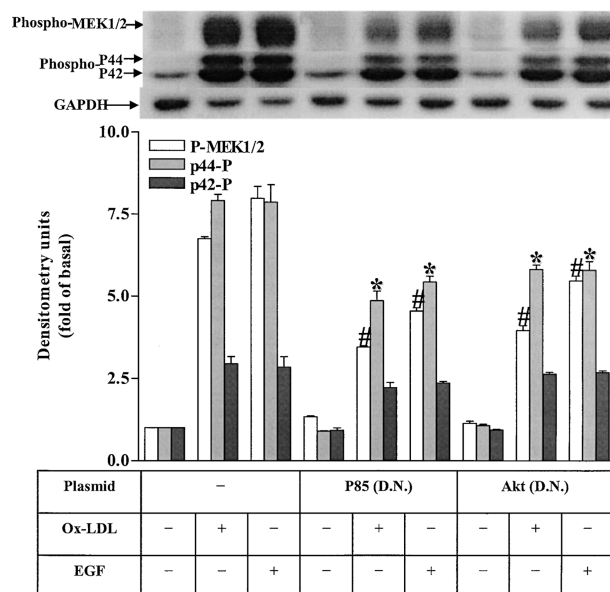
Several studies have demonstrated that activation of PI3-K/Akt was mediated through a PTX-sensitive G protein signaling in various cell types (15, 36). In the present study, OxLDL-stimulated Akt activation was blocked by PTX treatment in VSMCs (Fig. 3). These results implied that a G protein of the G$_i$ subfamily is involved in OxLDL-mediated Akt activation. In addition, OxLDL may be capable of interacting with multiple G proteins, including G$_s$. If the effect of OxLDL on Akt activation is stimulated by βγ complexes released from a G$_s$ protein, as demonstrated for the β-adrenoceptor (40), permanent activation of G$_s$ by CTX may stimulate or potentiate Akt activation in response to OxLDL. Surprisingly, pretreatment with CTX markedly attenuated OxLDL-stimulated Akt activation (Fig. 3). Furthermore, the OxLDL-stimulated Akt activa-

tion was also inhibited by treatment with forskolin (Fig. 3), which activates adenylate cyclase independently of the G$_s$ protein. It may therefore be assumed that the inhibitory effect of CTX on the OxLDL-stimulated Akt activation is due to an increase of cyclic AMP level induced by this toxin in VSMCs. Because cAMP has been reported to inhibit Akt activation in several cell types (29, 30), we speculate that the permanent activation of adenylate cyclase by CTX counteracts the stimulation of Akt activity in response to OxLDL. However, pretreatment with these reagents had no significant effect on EGF-induced responses, possibly due to the activation of Akt by OxLDL, and EGF was mediated through different signaling pathways. In addition, the detailed mechanisms of CTX and forskolin action on these responses should be investigated further.
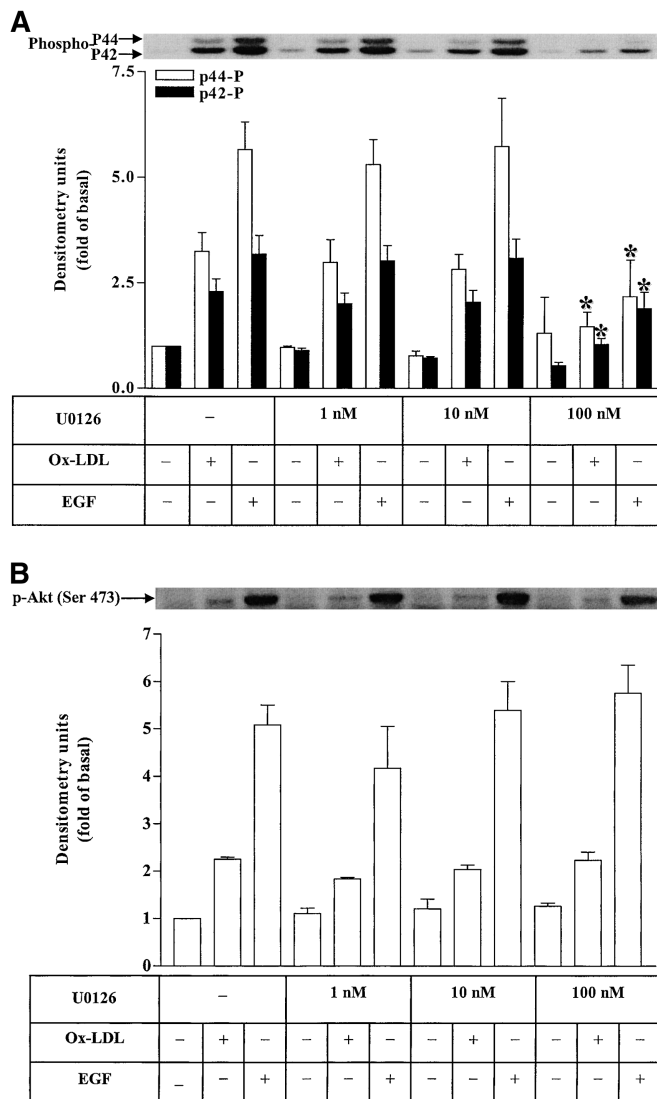
Fig. 6. Effect of MAPK kinase (MEK)1/2 inhibitor on p42/p44 MAPK and Akt phosphorylation stimulated by OxLDL and EGF. VSMCs were incubated with or without U0126 (ranging from 10 nM to 1 μM) for 1 h and then stimulated with 100 μg/ml OxLDL or 5 ng/ml EGF for 3 min. After incubation, the cell lysates were subjected to 10% SDS-PAGE and transferred to nitrocellulose membrane. Phosphorylations of p42/p44 MAPK (A) and Akt (B) were determined as described in the legends to Figs. 5 and 1. Data are expressed as the mean ± SEM of three separate experiments. * $P <$ 0.05, compared with the respective basal levels.

Fig. 7. Requirement of Akt for OxLDL- and EGF-stimulated activation of MEK1/2 and p42/p44 MAPK in VSMCs. Cells were transfected with plasmid encoding dominant negative constructs of p85-DN and Akt-DN, and then stimulated with 100 μg/ml OxLDL or 5 ng/ml EGF for 3 min. The cell lysates were subjected to 10% SDS-PAGE and transferred to nitrocellulose membrane. Phosphorylations of MEK1/2 and p42/p44 MAPK were determined as described in the legend to Fig. 5. Bands were visualized by an ECL method. * $P <$ 0.05, # $P <$ 0.01, compared with respective controls.

ported by the results that removal of intracellular Ca^{2+} by BAPTA/AM plus EDTA almost completely inhibited Akt activation by OxLDL and EGF in VSMCs (Fig. 4A). In addition, nifedipine, a Ca^{2+} channel blocker, which blocked Ca^{2+} influx, had no effect on Akt phosphorylation stimulated by OxLDL and EGF (Fig. 4B). These results suggest that release of Ca^{2+} from internal stores may be required for the OxLDL-induced responses.

It has been established that growth factors such as PDGF and EGF have been shown to stimulate Akt activation, which is inhibited by wortmannin, LY294002, or overexpression of the dominant mutant of PI3-K (14, 42, 43). Thus, PI3-K may be necessary and sufficient for growth factor-stimulated Akt activation. However, an additional pathway for activation of Akt that is independent of PI3-K could exist, because cellular stress such as heat shock and hyperosmolarity and β_3-adrenoceptor stimulation have been shown to activate Akt through a pathway independent of PI3-K (44, 45). In this study, we also demonstrate that PI3-K/Akt partially contributes to OxLDL-induced p42/p44 MAPK activation in VSMCs using these pharmacological inhibitors and dominant negative p85-DN and Akt-DN constructs. Pretreatment of VSMCs with the PI3-K inhibitors wortmannin and LY294002 almost completely inhibited OxLDL- and EGF-stimulated Akt, but only partially attenuated p42/p44 MAPK activation and [³H]thymidine incorporation (Fig. 5), suggesting alternative actions of PI3-K inhibitors or alternative pathways involved in regulating p42/p44 MAPK phosphorylation in response to OxLDL. In contrast, treatment with the MEK1/2

To assess possible mechanisms that might mediate the effect of OxLDL on Akt activation, we attempted to analyze some potentially participating pathways. The activation of VSMCs by OxLDL is linked to stimulation of phosphoinositide hydrolysis, which produces two second messengers, DAG and IP_3 (31). DAG is known to activate PKC and IP_3 to release Ca^{2+} from intracellular stores. The activation of PKC and increase in $[Ca^{2+}]_i$ seem to account for the effects of growth-promoting agents (7–9, 41). In this study, the effect of OxLDL may be correlated with its ability to stimulate Ca^{2+} mobilization. The stimulatory effects of OxLDL and EGF on Akt activation in VSMCs required the presence of Ca^{2+}. This hypothesis was sup-

inhibitor U0126, at a concentration of 100 nM [close to its IC$_{50}$ value for MEK1/2 (46)], almost completely inhibited OxLDL- and EGF-stimulated p42/p44 MAPK phosphorylation, but there was no effect on Akt activation (Fig. 6). These results showed that U0126 attenuated p42/p44 MAPK phosphorylation in response to OxLDL and EGF, but there was no effect on Akt phosphorylation. Although the higher concentration was not used in this study, U0126 appeared as a potent inhibitor of MEK1/2 in VSMCs. These results suggest that PI3-K/Akt is one of the upstream activators of the p42/p44 MAPK pathway in VSMCs. This hypothesis was further confirmed by the results that transfection of VSMCs with either p85-DN or Akt-DN also significantly inhibited OxLDL- and EGF-stimulated MEK1/2 and p42/p44 MAPK phosphorylation (Fig. 7). However, it should be noted that treatment with either wortmannin or LY294002 did not significantly inhibit p42/p44 MAPK phosphorylation stimulated by EGF, implying that the different PI3-K isoforms activated by EGF and OxLDL may be converged to p42/p44 MAPK activation in VSMCs (38). In addition, the results obtained with overexpression of dominant negative plasmids did not completely inhibit p42/p44 MAPK phosphorylation, possibly due to the transfection efficiency.

In conclusion, the intracellular signaling events involved in OxLDL-stimulated p42/p44 MAPK phosphorylation linked to cell proliferation of VSMCs have been further explored. In addition to mediating through Ras/Raf pathway, OxLDL activates PI3-K/Akt pathway. PI3-K may act as an upstream activator and contribute to p42/p44 MAPK activation. It is apparent that cross-talk between Akt and MAPK pathways may play potential roles in cell regulation, including cell survival and mutagenesis. Our results further clarify an additional pathway of p42/p44 MAPK activation induced by OxLDL in VSMCs.

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