Linoleic Acid and Its Metabolites, Hydroperoxyoctadecadienoic Acids, Stimulate c-*Fos*, c-*Jun*, and c-*Myc* mRNA Expression, Mitogen-activated Protein Kinase Activation, and Growth in Rat Aortic Smooth Muscle Cells

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Abstract

Previous studies from other laboratories suggest that linoleic acid and its metabolites, hydroxyoctadecadienoic acids, play an important role in modulating the growth of some cells. A correlation has been demonstrated between hydroxyocta-decadienoic acids and conditions characterized by abnormal cell growth such as atherosclerosis and psoriasis. To determine if linoleic acid and its metabolites modulate cell growth in atherosclerosis, we measured DNA synthesis, protoonco-gene mRNA expression, and mitogen-activated protein ki-nase (MAPK) activation in vascular smooth muscle cells (VSMC). Linoleic acid induces DNA synthesis, c-*fos*, c-*jun*, and c-*myc* mRNA expression and MAPK activation in VSMC. Furthermore, nordihydroguaiaretic acid, a potent inhibitor of the lipoxygenase system, significantly reduced the growth–response effects of linoleic acid in VSMC, sug-gesting that conversion of linoleic acid to hydroperoxyocta-decadienoic acids (HPODEs) is required for these effects. HPODEs also caused significant induction of DNA synthesis, protooncogene mRNA expression, and MAPK activation in growth-arrested VSMC, suggesting that linoleic acid and its metabolic products, HPODEs, are potential mitogens in VSMC, and that conditions such as oxidative stress and lipid peroxidation which provoke the production of these substances may alter VSMC growth. (*J. Clin. Invest.* 1995. 96:842–847.) Key words: atherosclerosis • DNA synthesis • mitogen-activated protein kinases • polyunsaturated fatty acid • protooncogene expression

Introduction

Linoleic acid, a *cis*-polyunsaturated fatty acid and the precursor for the synthesis of hydroperoxyoctadecadienoic acids (HPODEs),[1] has been shown to play a role in modulating growth in various carcino-genic cell types (1, 2). In particular, linoleic acid stimulates growth of rat hepatoma cells (1) and enhances the proliferative respon-siveness of mammary epithelial cells and fibroblasts to insulin and EGF, respectively (2, 3). Conversely, other investigators have found that inhibitors of the lipoxygenase pathway exhibit antiproliferative properties in a large number of cell types (4, 5). An association between lipoxygenase-dependent metabolites of linoleic acid and psoriasis has been demonstrated (6). Together, these related findings imply that linoleic acid and its metabolites may play an important role in regulating cell growth in certain cell types.

Vascular smooth muscle cell (VSMC) growth (hyperplasia and hypertrophy) is an important component in the initiation and progression of atherosclerosis and restenosis after angio-plasty (7). Increased amounts of hydroxyoctadecadienoic acids (HODEs) and hydroxyeicosatetraenoic acids (HETEs), the li-poxygenase-dependent metabolites of linoleic and arachidonic acids, respectively, have been reported in atherosclerotic arteries (8). Because HODEs and HETEs are growth modulators in many other cell types and the concentrations of these substances are reportedly increased in atherosclerotic arteries, we hypothe-sized that these products of linoleic and arachidonic acids may be involved in the alteration of VSMC growth in atherosclerosis. We tested this hypothesis by determining the effects of linoleic acid and HPODEs on early growth-response events such as expression of c-*fos*, c-*jun*, and c-*myc* protooncogene mRNA and activation of mitogen-activated protein kinases (MAPK), and late mitogenic events such as DNA synthesis and cell num-bers in VSMC. Induced expression of c-*fos*, c-*jun*, and c-*myc* mRNA and activation of MAPK are part of the early response events to a variety of growth stimuli (9–14). In this report, we provide evidence for the first time that linoleic acid and its metabolites, HPODES, stimulate c-*fos*, c-*jun*, and c-*myc* mRNA expression, MAPK activation, and DNA synthesis induction in VSMC. Linoleic acid and HPODEs also increased VSMC numbers. These results suggest that polyunsaturated fatty acids such as linoleic acid and its metabolites, HPODEs may be im-portant in the altered growth of VSMC in atherosclerosis and other vascular diseases such as restenosis.

Methods

Materials. Aprotinin, benzamidine, calmidazolium, EDTA, EGTA, β-glycerophosphate, leupeptin, linoleic acid, myelin basic protein, pep-statin A, PMSF, and sodium orthovanadate were obtained from Sigma Chemical Co. (St. Louis, MO). [α^{32}P]-dCTP (3,000 Ci/mmol) and [methyl^{3}H]thymidine (20 Ci/mmol) were from New England Nuclear (Boston, MA). [γ-^{32}P]ATP (5,000 Ci/mmole) was obtained from Am-ersham Corp. (Arlington Heights, IL). ERK-1 and ERK-2 anti–rabbit antibodies were bought from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Hydroperoxyoctadecadienoic acids (HPODEs) were from Cayman Chemical Company (Ann Arbor, MI).

Cell culture. VSMC were isolated from the thoracic aortae of 200–250 gram/male Sprague-Dawley rats by enzymatic dissociation as de-

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1. *Abbreviations used in this paper:* AP-1, activator protein-1; HPODEs, hydroperoxyoctadecadienoic acids; MAPK, mitogen-activated protein kinases; NDGA, nordihyroguaiaretic acid; VSMC, vascular smooth muscle cells.

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scribed elsewhere (15). Cells were grown in DME supplemented with 10% (vol/vol) heat-inactivated calf serum, 200 U/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained in humidified 95% air/ 5% CO_2 at 37°C by passage of 1–3 × 10^5 cells/ml on reaching confluence. For all experiments, cells at 70–80% confluence were made quiescent by incubation for 72 h in fresh DME containing 0.1% calf serum. Cells were used at passage numbers 8–18.

DNA synthesis. Quiescent VSMC were labeled in the presence of 0.1% calf serum with 1 μCi/ml [methyl-^{3}H]thymidine for 24 h with and without agent of interest. After labeling, cells were washed with cold PBS, trypsinized, and collected by centrifugation (150 g for 5 min). The cell pellet was suspended in cold 10% TCA and vortexed vigorously to lyse the cells. After standing on ice for 20 min, the cell lysate was passed through glass fibers and washed with cold 5% TCA and 70% ethanol. The filter was then dried. Incorporated [^{3}H]thymidine was measured in a liquid scintillation counter (LS 5000 TD; Beckman Instruments Inc., Fullerton, CA).

Cell number. Growth-arrested VSMC (35-mm dishes) were exposed to 20 μM linoleic acid and/or 1 μM 9-, or 13-HPODE for 96 h. Cell numbers were then determined by trypan blue dye exclusion assay using a hemocytometer.

RNA blot analysis. Total cellular RNA from quiescent and agent-treated VSMC was isolated by the guanidine isothiocyanate-cesium chloride protocol of Chirgwin et al. (16). An equal amount of total cellular RNA (20 μg) from quiescent and agonist-treated cells was size fractionated on 1% (wt/vol) agarose gel in 25 mM Mops buffer (pH 7.8) containing 1 mM EDTA and 2% (wt/vol) formaldehyde. RNA was transferred to a Nytran membrane (Schleicher & Schuell, Inc., Keene, NH) according to the method of Thomas (17). RNA was cross-linked to the membrane using ultraviolet irradiation (Stratalinker; Stratagene Inc., La Jolla, CA). The cDNAs (an EcoRI and SstI fragment of 1.2-kb mouse c-*fos* cDNA; HindIII and NotI fragment of rat c-*jun* cDNA; and a full-length human c-*myc* and rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNAs were labeled with [α-^{32}P]-dCTP using a random primers labeling kit using the manufacturer's protocol (GIBCO BRL, Gaithersburg, MD). After a 4-h prehybridization in 50% (vol/vol) formamide, 5× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate), 5× Denhardt's (1× Denhardt's = 0.02% (wt/vol) each of Ficoll, polyvinyl pyrrolidone, and bovine serum albumin), 50 mM sodium phosphate (pH 6.5) and 250 μg/ml of sheared salmon sperm DNA at 42°C, the Nytran membrane was hybridized in the above buffer containing 10% (wt/vol) dextran sulfate and 1 × 10^6 cpm/ml of probe for 16 h at 42°C. The Nytran membrane was washed three times in 2× SSC, 0.2% SDS (15 min at 25°C) and twice in 0.1× SSC, 0.1% SDS (30 min at 60°C). The membrane was exposed to x-ray film (X-Omat R; Eastman Kodak Co., Rochester, NY) with an intensifying screen at −70°C for 4–6 h. Densitometric analysis of the autoradiograms exposed in the linear range of film density was made using a LaCie scanner with National Institute of Health Image software.

Western blot analysis. Growth-arrested VSMC were incubated at 37°C for various times in the presence and absence of an appropriate agent. After incubation, medium was aspirated, cells rinsed with cold PBS, and frozen immediately in liquid nitrogen. Cells were then thawed in 250 μl of lysis buffer (50 mM Hepes, pH 7.4, containing 50 mM sodium pyrophosphate, 50 mM sodium fluoride, 50 mM sodium chloride, 5 mM EDTA, 5 mM EGTA, 2 mM sodium orthovanadate, 0.5 mM PMSF, 10 μg/ml leupeptin, and 0.01% Triton X-100) and sonicated. Cell lysates were cleared by centrifugation at 14,000 rpm for 20 min in a microfuge and protein content was determined using a reagent kit (Bradford; Bio-Rad Laboratories, Richmond, CA). Cell lysates containing equal amount of protein (50 μg) from each condition were separated by SDS-PAGE (18) and transferred electrophoretically to nitrocellulose membranes. The membranes were probed with 1 μg/ml each of ERK-1 and ERK-2 anti–rabbit primary antibodies. After treating the membrane with peroxidase-conjugated goat anti–rabbit secondary antibodies, peroxidase activity was detected using ECL reagents (Amersham Corp.).

Immunocomplex MAP kinase assay. Cell lysates containing equal amounts of proteins (500 μg) from each condition were incubated with
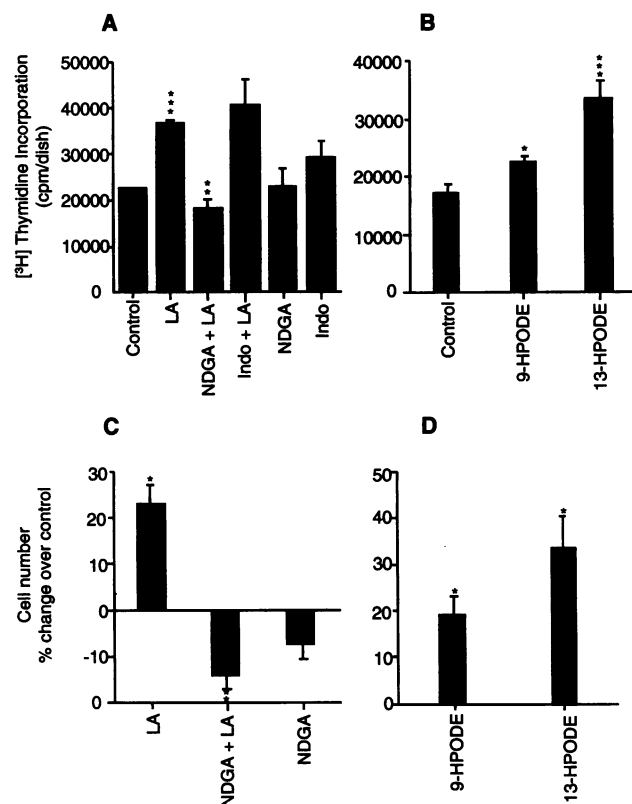
Figure 1. Linoleic acid and HPODEs induce VSMC growth. Thymidine incorporation (*A* and *B*): Growth-arrested VSMC were treated with linoleic acid (20 μM) or HPODEs (1 μM) in the presence of 1 μCi/ ml of [^{3}H]thymidine and DNA synthesis was measured as trichloroacetic acid precipitable counts per min. Cell number (*C* and *D*): Growth-arrested VSMC were exposed to either linoleic acid (*LA*) or HPODEs and 96 h later the cell number was determined by hemocytometer. Wherever appropriate, inhibitors were added 20 min before the addition of linoleic acid. Results are expressed as means±SD of three different experiments performed in triplicate in each experiment. *** $P < 0.01$ vs control; ** $P < 0.01$ vs LA alone; * $P < 0.05$ vs control.

10 μl each of ERK-1 (C-16) and ERK-2 (C-14) antiserum and 20 μl of 50% (wt/vol) protein A-Sepharose beads overnight at 4°C. The immunoprecipitates were washed three times with lysis buffer and resuspended in lysis buffer. Reactions were carried out in a final volume of 50 μl containing 50 mM β-glycerophosphate (pH 7.3), 1.5 mM EGTA, 0.1 mM sodium orthovanadate, 1 mM DTT, 10 μM calmidazolium, 10 mM $MgCl_2$, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 μg/ml pepstatin A, 1 mM benzamidine, 0.3 mM [γ^{32}P]ATP (2,000 cpm/pmol) and 0.5 mg/ml myelin basic protein for 30 min at 37°C (19). Reactions were terminated by the addition of 50 μl of cold 20% TCA. 50 μl of the mix was then spotted on P-81 phosphocellulose filter. The filter was washed four times (5 min each) with 0.5% phosphoric acid and once with absolute ethanol. The filter was dried and the radioactivity was measured.

Statistics. Treatment effects were analyzed by Student's *t* test. P values < 0.05 were considered to be significant.

Results

To determine the mitogenic effect of linoleic acid, VSMC were growth arrested and stimulated with linoleic acid (20 μM) in the presence of 1 μCi/ml [^{3}H]thymidine for 24 h. Incorporation of [^{3}H]thymidine into VSMC DNA was then measured. Linoleic acid increased VSMC DNA synthesis by 70% over control (Fig. 1 *A*). Since linoleic acid metabolizes to HPODEs by the

lipoxygenase pathway (20), to determine whether conversion of linoleic acid to HPODEs is required for induction of DNA synthesis, growth-arrested VSMC were stimulated with linoleic acid in the presence and absence of nordihydroguaiaretic acid (NDGA) (10 μM), a potent inhibitor of the lipoxygenase system (21) and DNA synthesis was measured as described above. NDGA completely blocked the linoleic acid–induced DNA synthesis in VSMC. NDGA had no significant effect on serum-induced VSMC DNA synthesis (control, 17,721±2,135 cpm/dish; serum, 209,329±963; NDGA + serum, 180,588±1,197; NDGA, 15,314±1,910; values are expressed as mean±SD), suggesting that its effect on linoleic acid–induced VSMC DNA synthesis is specific. Indomethacin (10 μM), an inhibitor of the cyclooxygenase pathway (21), had no effect on linoleic acid–stimulated VSMC DNA synthesis. These results indicate that conversion of linoleic acid to HPODEs via the lipoxygenase system may be required for its observed effects on VSMC DNA synthesis. To determine if linoleic acid–induced DNA synthesis is associated with growth, VSMC numbers were measured 96 h after adding linoleic acid to growth-arrested cells. Consistent with thymidine incorporation data, linoleic acid treatment resulted in a 22% increase in VSMC number compared to untreated cells (Fig. 1 C). As expected, increased numbers of VSMC in the presence of linoleic acid were suppressed by NDGA. Since this drug, besides inhibiting linoleic acid–induced VSMC growth, also blocked the basal growth of these cells to some extent, it is intriguing to suspect that lipoxygenase products of endogenous linoleic acid or arachidonic acid are modulators of basal growth in these cells. This observation is in agreement with the reported findings that lipoxygenase inhibitors exhibit antiproliferative activity (4, 5).

Since linoleic acid induced VSMC DNA synthesis and NDGA blocked this effect, we hypothesized that HPODEs, the lipoxygenase-dependent metabolites of linoleic acid, mediate this event. We tested this possibility by treating growth-arrested VSMC with 9-, or 13-HPODEs (1 μM) for 24 h and determining VSMC DNA synthesis by [^{3}H]thymidine incorporation. Both 9-, and 13-HPODEs increased VSMC DNA synthesis by 30 and 90%, respectively (Fig. 1 B). Cell counts showed an 18% and 32% increase by 9-, and 13-HPODEs, respectively (Fig. 1 D). Together, these findings indicate that HPODEs mediate the effects of linoleic acid on VSMC growth.

Induced expression of c-*fos*, c-*jun*, and c-*myc* protooncogene mRNA is an early response to various growth stimuli (9–12). These protooncogenes encode for nuclear binding transcriptional factors (9–12, 22) and play an obligatory role in growth factor–induced cell division (23, 24). A recent study demonstrated that a null mutation at the c-*jun* locus causes embryonic lethality and retarded cell growth (25). To determine if these protooncogenes are also involved in linoleic acid–induced VSMC growth, we measured mRNA levels of these protooncogenes in VSMC in the presence of linoleic acid. Growth-arrested VSMC were stimulated with linoleic acid (20 μM) for various times and total cellular RNA was isolated. 20 μg of total cellular RNA from linoleic acid–stimulated and nonstimulated VSMC was then analyzed for c-*fos*, c-*jun*, and c-*myc* transcripts by Northern blotting using the respective [^{32}P]-labeled cDNA probes. Linoleic acid stimulated expression of mRNAs of all three protooncogenes tested (Fig. 2). Maximal increases in c-*fos* (40-fold), c-*jun* (20-fold), and c-*myc* (3-fold) mRNA levels occurred at 1 h of linoleic acid treatment. Expression of c-*fos* and c-*jun* mRNAs in response to linoleic acid was found to be transient. Linoleic acid–induced expression of c-*myc*
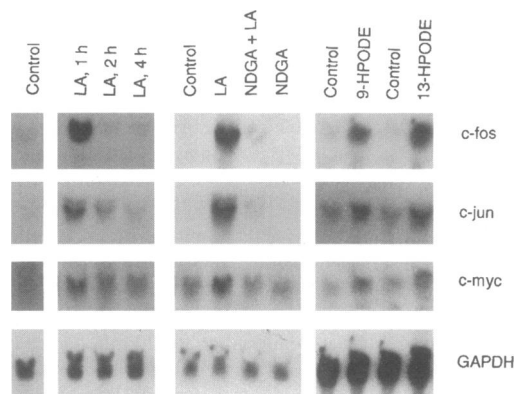

Figure 2. Linoleic acid (*LA*) and HPODEs stimulate expression of protooncogene mRNAs in VSMC. Growth-arrested VSMC were treated with indicated agents for 1 h (or for the time periods shown) and total cellular RNA was isolated. 20 μg of total cellular RNA from each condition was then analyzed by Northern blotting for c-*fos*, c-*jun*, and c-*myc* transcripts using the respective [^{32}P]-labeled cDNA probes. Analysis of total cellular RNA for each condition from three separate experiments provided similar results. Results of one independent experiment are shown above.

mRNA, on the other hand, was persistent for at least 4 h, although at lower levels than those at 1 h (Fig. 2).

Since linoleic acid–induced thymidine incorporation and cell numbers were sensitive to NDGA, we tested to see whether expression of c-*fos*, c-*jun*, and c-*myc* mRNA by linoleic acid was also blocked by NDGA. Growth-arrested VSMC were treated with linoleic acid (20 μM) for 1 h in the presence and absence of NDGA (10 μM) and total cellular RNA was isolated and analyzed for c-*fos*, c-*jun*, and c-*myc* mRNA levels as described above. NDGA completely blocked the linoleic acid–induced expression of these protooncogenes in VSMC (Fig. 2), a result which strengthens the argument that linoleic acid conversion via the lipoxygenase system is required to induce growth factor–like events in VSMC. In fact, stimulation for 1 h of growth-arrested VSMC with 9- and 13-HPODEs (1 μM) the primary lipoxygenase products of linoleic acid, resulted in similar increases in the mRNA levels of these protooncogenes (Fig. 2).

Recent results from several laboratories have elucidated how MAPK plays a critical role in the transmission of growth stimuli–elicited mitogenic signals in various cell types (13, 14). In the mitogenic signaling pathway, activation of these kinases occurs before the induction of expression of c-*fos*, c-*jun*, and c-*myc* mRNAs. These findings stimulated our examination of the activation of these kinases by linoleic acid and its metabolites in VSMC. Growth-arrested VSMC were stimulated with linoleic acid (20 μM) for varying times and cell lysates were prepared. Equal amounts of protein from linoleic acid–treated and untreated samples were then assayed for MAPK activities. MAPK activation was determined by gel-shift and immunocomplex kinase assays. The gel-shift assay was selected because phosphorylated and activated MAPK migrate slower on SDS-PAGE than the nonphosphorylated inactive form (26). In the immunocomplex kinase assay, MAPK are immunoprecipitated and their activities are measured using myelin basic protein as a substrate in these immunocomplexes. As shown in Fig. 3, *A* and *B*, linoleic acid activated MAPK in VSMC, which was apparent at 5 min, peaked at 10 min (threefold) and dropped

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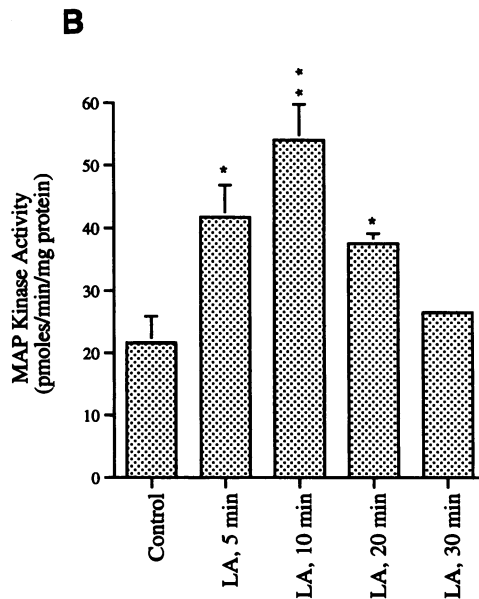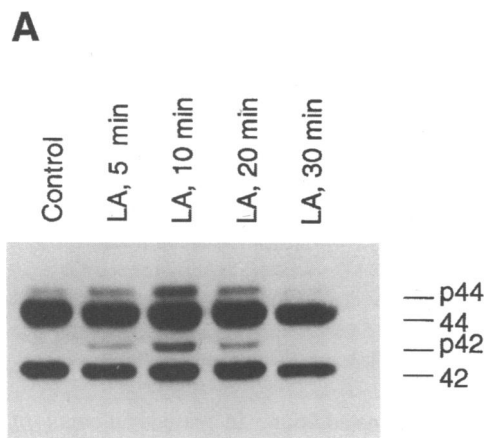
Figure 3. Linoleic acid (*LA*) activates MAPK in VSMC. Growth-arrested VSMC were treated with linoleic acid (20 μM) for varying times and cell lysates were prepared. MAPK were detected in cell lysates by Western blotting (*A*) as described in Methods. Phosphorylated and activated MAPK migrate slower on SDS-PAGE than the nonphosphorylated inactive form. p42 and p44 are phosphorylated forms of 42 and 44 kD MAPK, respectively. MAPK activity was measured in cell lysates of VSMC treated identically to those in *A*, using myelin basic protein as substrate (*B*). (*A*) Results are representative of one experiment and these results were reproduced in two separate experiments. Results in *B* are means$\pm$SD of three separate experiments performed in duplicates each time. ** $P < 0.01$ and * $P < 0.05$ vs control.

thereafter, nearly reaching baseline by 30 min. Furthermore, linoleic acid–induced MAPK activation was found to be sensitive to NDGA (Fig. 4, *A* and *B*). Treatment of growth-arrested VSMC with 9- and/or 13-HPODE (1 μM) for 10 min also activated these kinases in VSMC (Fig. 5, *A* and *B*).

Discussion

The main significance of this study is that linoleic acid is a mitogen for VSMC. Several observations support this conclusion. First, linoleic acid induced VSMC DNA synthesis and its number. Second, linoleic acid stimulated early growth–response events such as induced expression of c-*fos*, c-*jun*, and c-*myc* mRNA and activation of MAPK. Linoleic acid–induced

growth events in VSMC appeared to be mediated by HPODEs as these activities were suppressed by NDGA, a potent inhibitor of the lipoxygenase system and were stimulated by HPODEs, the lipoxygenase products of linoleic acid. These findings combined with the fact that linoleic acid and its metabolites stimulate growth in hepatoma cells (1), and enhance the mitogenic effect of EGF in mammary epithelial cells and fibroblasts (2, 3) imply that these lipid molecules act as growth modulators of several cell types, now including VSMC. The ability of linoleic acid and its metabolites to stimulate early growth response events such as induced expression of c-*fos*, c-*jun*, and c-*myc* mRNA and activation of MAPK strongly suggest their role in regulating VSMC growth. Although the exact mechanism by which linoleic acid and its metabolites transmit mitogenic sig-

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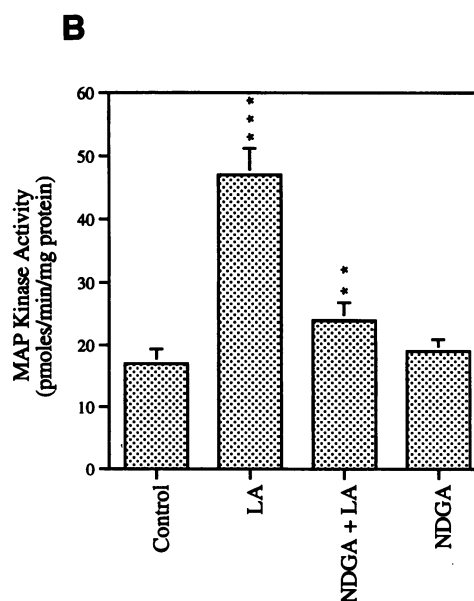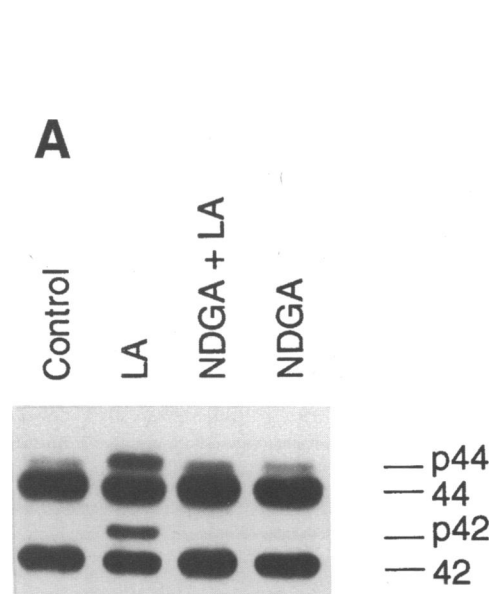
Figure 4. NDGA blocks linoleic acid (*LA*)–induced activation of MAPK in VSMC. Growth-arrested VSMC were exposed to 20 μM linoleic acid for 10 min in the presence and absence of 10 μM NDGA and cell lysates were prepared and analyzed for MAPK activities as described in Fig. 3. (*A*) Results are representative of one experiment and these results were reproduced in two separate experiments. Results in *B* are means$\pm$SD of three separate experiments performed in duplicates each time. *** $P < 0.01$ vs control; ** $P < 0.05$ vs LA alone.

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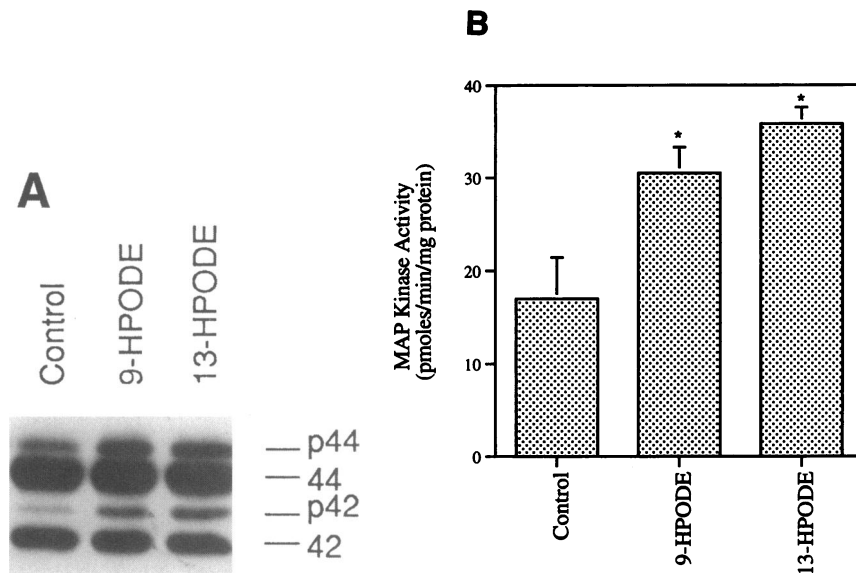
Figure 5. HPODEs stimulate activation of MAPK in VSMC. Conditions were the same as described in Fig. 3 except that cells were treated with 1 μM 9- or 13-HPODE for 10 min. (*A*) Results are representative of one experiment and these results were reproduced in two separate experiments. Results in *B* are means$\pm$SD of three separate experiments performed in duplicates each time. * $P < 0.05$ vs control.

nals in VSMC is unclear, our findings provide initial evidence that these molecules possess the ability to induce expression of c-*fos*, c-*jun*, and c-*myc* mRNA and stimulate activation of MAPK in VSMC; events that are required for cells to grow in response to growth stimuli (9–15, 22–25). Previous studies have demonstrated a requirement for activator protein-1 (AP-1) activity in modulating growth in vivo and in vitro (25). Since linoleic acid and HPODEs were found to be modest inducers of c-*fos*, and c-*jun* mRNAs, the protein products of which constitute the transcriptional factor AP-1, it is likely that AP-1 plays a role in the growth stimulated by these agents. In fact, linoleic acid and HPODEs induced AP-1 activity markedly in VSMC (Rao, G. N., unpublished observations). Future studies on the role of AP-1 in linoleic acid and HPODEs-induced gene transactivation should address whether AP-1 mediates the growth events induced by these lipid molecules.

A large body of evidence indicates that MAPK play an important role in transmitting mitogenic signals in response to growth factors (13, 14, 27–31). MAPK phosphorylate and activate nuclear binding proteins such as c-*fos*, c-*jun*, and c-*myc*, which, in turn modulate transcription of target genes (32–34). Since linoleic acid and HPODEs are capable of activating MAPK in VSMC, it is possible that these kinases are involved in lipid molecule-induced propagation of mitogenic signals. Activation of MAPK by growth factors requires an upstream kinase cascade of activities such as "Ras-Raf-MEK" in this order (13, 14, 27–31). Mitogenically active lipids such as phosphatidic acid, arachidonic acid, and linoleic acid can reportedly activate ras via inhibition of guanosine triphosphatase activating protein activity (35). Recently we reported that arachidonic acid activates MAPK and protein kinase C (PKC) mediates this effect (36). PKC has also been shown to activate ras (37). Together these findings lead us to speculate that linoleic acid and HPODEs-induced activation of MAPK may be mediated by a kinase cascade that includes ras and PKC. Future studies, however, are required to elucidate the exact upstream kinases associated with linoleic acid and HPODEs-induced activation of MAPK and growth in VSMC.

The present findings have important implications in the pathogenesis of atherosclerosis. Steinberg and associates have demonstrated that 15-lipoxygenase protein deposits in athero-

sclerotic plaque (38). In addition, increased amounts of HODEs, the lipoxygenase products of linoleic acid, have also been reported in atherosclerotic arteries. Because HPODEs appear to be mitogenic to VSMC, these molecules could account, at least partially, for the observed growth of VSMC in atherosclerosis. In conclusion, our results provide the first molecular evidence for the mitogenic role of linoleic acid and its metabolites, HPODEs, in VSMC. Therefore, strategies targeting the inhibition of the oxidative metabolism of polyunsaturated fatty acids such as linoleic acid may be therapeutically beneficial in modifying the atherogenic process.

Acknowledgments

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