Oleic Acid and Angiotensin II Induce a Synergistic Mitogenic Response in Vascular Smooth Muscle Cells

Gang Lu, Kathryn E. Meier, Ayad A. Jaffa, Steven A. Rosenzweig, Brent M. Egan

Abstract—Oleic acid and angiotensin II (Ang II) are elevated and may interact to accelerate vascular disease in obese hypertensive patients. We studied the effects of oleic acid and Ang II on growth responses of rat aortic smooth muscle cells (VSMCs). Oleic acid (50 μmol/L) raised thymidine incorporation by 50% at 24 hours and cell number by 55% at 6 days (P<.05). Ang II (10−11 to 10−6 mol/L) did not significantly increase thymidine incorporation or VSMC number. Combining Ang II and 50 μmol/L oleic acid doubled thymidine incorporation and VSMC number. Losartan, an angiotensin type I (AT1) receptor antagonist, blocked the synergistic interaction between Ang II and oleic acid, whereas the AT2 receptor antagonist PD 123319 did not. Protein kinase C inhibition and downregulation, as well as inhibition of extracellular signal-regulated kinase (ERK) activation by PD 98059, eliminated the rise of thymidine incorporation in response to oleic acid and the synergistic interaction with Ang II. However, the response to 10% fetal bovine serum was unaffected. An antisense oligodeoxynucleotide to ERK-1 and ERK-2 reduced ERK protein expression and activation by 83% and 75%, respectively. Antisense prevented the rise of thymidine incorporation in response to oleic acid and the synergy with Ang II. Antisense reduced but did not prevent increased thymidine incorporation in response to serum. The data indicate that oleic acid and Ang II exert a synergistic mitogenic effect in VSMCs and suggest an important role for the AT1 receptor, PKC, and ERK in this synergy. The observations raise the possibility that a synergistic mitogenic interaction between oleic acid and Ang II accelerates vascular remodeling in obese hypertensive patients. (Hypertension. 1998;31:978-985.)

Key Words: muscle, smooth, vascular ■ oleic acid ■ angiotensin II ■ receptors, angiotensin ■ protein kinase C ■ extracellular signal-regulated kinases ■ oligonucleotides, antisense

The excess incidence of vascular disease in subjects with the cardiovascular risk factor cluster1-3 is only partially explained by elevations of blood pressure, lipids, and glucose. Insulin resistance may contribute independently to cardiovascular disease in patients with the risk factor cluster.1 However, the mechanisms by which insulin resistance, apart from the traditional risk factors noted, leads to cardiovascular disease remain poorly defined. One possible contributor may be the defect in the capacity of insulin to suppress plasma concentrations and turnover of nonesterified fatty acids in patients with risk factor clustering.4-6 We observed that oleic acid was the most abundant fatty acid in plasma and that obese hypertensive patients had higher fasting plasma oleic acid concentrations than lean normotensive subjects.7

Cis- Unsaturated fatty acids, eg, oleic acid (18:1[cis]), can activate PKC.8,9 Activation of PKC has been implicated as a key signaling event in cell growth and proliferation in response to several mitogens.10,11 We performed experiments that demonstrated that oleic acid induced a PKC-dependent mitogenic response in rat aortic VSMCs.12 Neither stearic (18:0) nor elaidic (18:1[trans]) acids, which are weak activators of PKC,8 induced a mitogenic response. Subjects with risk factor clustering also have a more active renin-angiotensin system.13,14 Ang II enhances the response to several mitogens.15 This raises the possibility that the increased oleic acid concentrations and the enhanced activity of the renin-angiotensin axis may interact to accelerate vascular changes among high-risk subjects such as obese hypertensives. Consequently, we examined the separate and combined effects of oleic acid and Ang II on thymidine uptake and cell number in rat aortic VSMCs.

Because the signal transduction pathways by which growth factors interact could have important therapeutic implications, we attempted to define early signaling events in the mitogenic response to the oleic acid–Ang II combination. Both oleic acid and Ang II activate PKC10,12,15,16 and, in turn, ERKs. ERK activation is a common point in the signaling pathway for cell growth and/or differentiation in response to several different mitogens.17-19 We examined whether the mitogenic effects of the oleic acid–Ang II combination were dependent on activation of PKC and ERK. Furthermore, given that Ang II may promote growth via AT1 receptors and inhibit growth via AT2 receptors,20 the effects of selective blockers on the mitogenic response to oleic acid and Ang II were assessed.

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Materials

Individual fatty acids were purchased from Sigma Chemical Co, and sodium salts were prepared as previously described. Losartan was obtained from Dupont–Merck Pharmaceutical Co. PD 98059 and PD 123319 were purchased from RBI. Cell culture media were obtained from Dupont-Merck Pharmaceutical Co. PD 123319 and PD 123319. Thymidine incorporation in response to oleic acid, Ang II alone and together, or 10% FBS was measured as described above.

Methods

Cell Culture

Rat aortic VSMCs were cultured by procedures modified from Chamley-Campbell et al as described. 21

\[ ^{3}H \text{Thymidine Incorporation} \]

Effects of Oleic Acid and Ang II on \[{^{3}H}\text{Thymidine Incorporation} \]

VSMCs were grown to subconfluence in 10% FBS in 24-well plates and incubated in DMEM with 0.1% FBS for 48 hours. Oleic acid and Ang II alone or together were added to triplicate wells in fresh DMEM supplemented with 0.1% FBS and 100 μM fatty acid–free albumin for 24 hours. Because some fatty acids, including oleic acid, affect angiotensin receptor affinity, 22 separate experiments were performed in which Ang II was added 5 minutes before or after oleic acid, Ang II alone and together, or 10% FBS was measured as described above.

Effect of AT₁ and AT₂ Receptor Antagonists on \[{^{3}H}\text{Thymidine Incorporation} \]

Before the addition of oleic acid and/or Ang II, quiescent VSMCs were preincubated for 1 hour with 10⁻³ mol/L losartan or 10⁻³ mol/L PD 123319. Thymidine incorporation in response to oleic acid and Ang II was quantified as described above.

Effect of PKC Inhibition and Depletion on \[{^{3}H}\text{Thymidine Incorporation} \]

Before the addition of oleic acid and/or Ang II, quiescent VSMCs were preincubated for 1 hour with 4 μmol/L bisindolylmaleimide I to inhibit PKC or for 24 hours with 200 nmol/L PMA to deplete PKC as described. 12

Effect of MEK Inhibition With PD 98059 on \[{^{3}H}\text{Thymidine Incorporation} \]

Before the addition of oleic acid and/or Ang II, quiescent VSMCs were preincubated for 1 hour with 20 μmol/L PD 98059. The incorporation of \[{^{3}H}\text{thymidine} \] in response to oleic acid and Ang II was studied using the protocol described above.

Selected Abbreviations and Acronyms

- Ang II = angiotensin II
- AT₁ = angiotensin type 1 receptor
- DMEM = Dulbecco’s modified Eagle’s medium
- ERK = extracellular signal-regulated kinase
- FBS = fetal bovine serum
- JNK = jun N-terminal kinase
- MAP kinase = mitogen-activated protein kinase
- MEK = mitogen-activated protein kinase
- p38 = p38 MAP kinase
- PKC = protein kinase C
- PMA = phorbol 12-myristate 13-acetate
- VSMC = vascular smooth muscle cell
- ODN = oligodeoxynucleotide

Effect of Antisense ODN to ERKs on \[{^{3}H}\text{Thymidine Incorporation} \]

The antisense ODN, 17-mer (5’-GCGCGCGCGCGCGCAT-3’) directed against a consensus sequence for initiating the translation of rat ERK-1 and ERK-2, sense ODN (5’-ATGGGCGGGCGCGCGCGCGCGC-3’), and scrambled controls (5’-GCGCGCTGCGGCACTC-3’) were synthesized. 23–25 Transfections of rat aortic VSMCs with different concentrations of ODNs including antisense, sense, and scrambled ODNs were performed in Opti-Media (GIBCO BRL) with 10 μg/mL lipofectin. Cells were incubated for 5 hours at 37°C in 5% CO₂. The medium was replaced with lipofectin-free DMEM containing the desired ODN concentration, and incubation was continued for another 42 hours before being harvested. ERK protein content and activity were measured with immunoblots using anti-ACTIVE MAP kinase pAb (Promega Co) and anti-ERK mAb (Transduction Laboratories), respectively. The effect of antisense and control ODNs on \[{^{3}H}\text{thymidine incorporation} \] in response to oleic acid, Ang II alone and together, or 10% FBS was measured as described above.

Effects of Oleic Acid and Ang II on VSMC Number

In separate experiments, rat aortic VSMCs were seeded at 10 000 cells per well, grown to subconfluence, and growth-arrested by serum deprivation. These cells were incubated with oleic acid and/or Ang II, or with or without 4 μmol/L bisindolylmaleimide I, for 6 days with a change of media after 72 hours. Elaidic and stearic acids were used to control for potential nonspecific effects of oleic acid on cell growth. 12 Cells were resuspended with 0.3 mL trypsin/EDTA (0.05%/0.5 mmol/L), and cell number was determined using a hemocytometer.

ERK Activity Assay

Confluent VSMCs in 100-mm Petri dishes were incubated in 0.1% FBS DMEM for 48 hours. The monolayer was exposed to oleic acid, Ang II, or 10% FBS in serum-free DMEM for 10 minutes under 5% CO₂ at 37°C. The incubation was stopped by adding ice-cold PBS. Cells were scraped into PBS and pelleted by centrifugation for 20 seconds at 2000g. The pellet was suspended in cold lysis buffer. The suspended pellet was sonicated for 5 seconds and centrifuged at 100 000g at 4°C. The supernatant was used as the enzyme preparation for assay of activated MAP kinase. ERK activity in cell lysates was assessed using \[ ^{3}P \text{phosphorylation} \] of myelin basic protein. 17 ERK activity was corrected for protein and expressed as a percentage of the activity in untreated cells.

Immunoblots using anti-ACTIVE MAP kinase pAb 26–28 and anti-ERK mAb were performed to corroborate findings from the ERK activity assay. Proteins in whole cell extracts from cells treated with/without PD98059 or antisense ODNs were resolved by SDS–polyacrylamide gel electrophoresis. 12

Effects of Oleic Acid on Activation of JNK and p38

Quiescent VSMCs were treated with 50 μmol/L oleic acid and/or Ang II and serum for 30 minutes or UV irradiation. For the UV experiments, cells were washed with ice-cold PBS and irradiated (254 nmol/L, UV-C) for 40 seconds. Media were added immediately after the irradiation, and the cells were harvested after 30 minutes. Proteins in whole cell extracts were resolved by SDS–polyacrylamide gel electrophoresis, electrophoretically transferred to polyvinylidene membranes, and then immunoblotted with anti–phosphospecific p38, anti-p38, anti–phospho-specific SAPK/JNK (New England Biolab, Inc), and anti-SAPK/JNK antibodies (generated and provided by S.A.R.).

Statistical Analysis

Data are presented as mean±SEM. Data were analyzed with SPSS 6.0 (SPSS Inc). One-way ANOVA followed by Duncan’s multiple range test was used to compare the cell number and ERK activity
changes between treatment and control groups. Values of $P<.05$ were considered statistically significant.

Results

Effects of Oleic Acid and Ang II Alone and Together on $[^{3}H]$Thymidine Incorporation in VSMCs

Ang II from $10^{-10}$ mol/L to $10^{-8}$ mol/L increased thymidine incorporation from 111% to 121% of control ($P=NS$). Oleic acid (50 μmol/L) increased thymidine incorporation to 150% of control ($P<.05$). When 50 μmol/L oleic acid was added to the media 5 minutes before Ang II, the combination of oleic acid with $10^{-10}$ mol/L to $10^{-8}$ mol/L Ang II synergistically enhanced thymidine incorporation from 206% to 225% of control. The synergistic interaction between oleic acid and Ang II was abolished by PKC inhibition with 4 μmol/L bisindolylmaleimide I or by PKC depletion with PMA (Fig 1). When Ang II was added 5 minutes before oleic acid, the synergistic effect between oleic acid and Ang II on thymidine uptake was observed at $10^{-11}$ mol/L Ang II (Fig 1). When the sequence was reversed, $10^{-11}$ mol/L Ang II did not synergistically enhance the response to oleic acid. The order of addition did not affect thymidine incorporation at Ang II concentrations of $10^{-10}$ mol/L and greater.

Effect of Oleic Acid and Ang II Alone and Together on VSMC Number

After 48 hours of growth arrest with 0.1% FBS DMEM, there were $3.51 \pm 0.33 \times 10^6$ cells per well (n=6). Under basal conditions (ie, a 6-day incubation with 0.1% FBS DMEM and 100 μmol/L fatty acid–free albumin), there were $5.79 \pm 0.24 \times 10^6$ cells per well (n=6). Addition of $10^{-10}$ mol/L to $10^{-8}$ mol/L Ang II for 6 days did not increase cell number compared with the control condition (Table). Addition of 50 μmol/L oleic acid increased cell number to 155% of control, while the combination of oleic acid and Ang II from $10^{-10}$ mol/L to $10^{-8}$ mol/L increased cell number to 188% to 214% of control ($P<.05$) compared with oleic acid alone. In contrast to oleic acid, the same concentrations of stearic acid and elaidic acid did not increase VSMC number after 6 days (102% and 114% of control, respectively).

Effect of AT$_1$ and AT$_2$ Receptor Antagonists on Thymidine Incorporation in Response to the Oleic Acid–Ang II Combination

Oleic acid (50 μmol/L) increased thymidine incorporation to 147% of control (Fig 2). The combination of 50 μmol/L oleic acid and $10^{-8}$ mol/L Ang II induced a more marked increase to 219% of control. When cells were pretreated with $10^{-5}$ mol/L losartan, an AT$_1$ receptor antagonist, values for thymidine incorporation in response to the combination of oleic acid and Ang II were not different from those for oleic acid alone. In contrast, blocking AT$_2$ receptors with $10^{-5}$ mol/L PD 123319 did not prevent the synergistic interaction between oleic acid and Ang II on thymidine incorporation. Neither $10^{-5}$ mol/L losartan nor $10^{-5}$ mol/L PD 123319 altered basal thymidine incorporation or the response to oleic acid (not shown).

Effect of ERK Kinase (MEK) Inhibition on ERK Activity and Thymidine Incorporation in Response to Oleic Acid and Ang II

When VSMCs were preincubated for 1 hour with 20 μmol/L PD 98059, a specific MEK inhibitor, basal ERK activity was unchanged. However, stimulation of ERK activity in response to oleic acid, Ang II, and serum was reduced by $\approx75\%$ to 95% by PD 98059 (Fig 3A). The findings with the

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ERK activity assay using myelin basic protein as the substrate were confirmed in experiments using an antibody specific for active MAP kinase and ERKs (Fig 3A). Although active ERK-1 (≈44 kD) was visible only after a longer exposure time than for active ERK-2 (≈42 kD), the patterns of immunoreactivity for these two active ERK isoforms in response to oleic acid and Ang II were similar (not shown). Control experiments using anti-ERK confirmed that equal amounts of total ERK protein were present in each of the conditions. PD 98059 reduced basal thymidine incorporation by ≈50% and abolished the rise of thymidine incorporation in response to 50 μmol/L oleic acid and 10⁻⁸ mol/L Ang II both singly and combined (Fig 3B). In contrast, 20 μmol/L PD 98059 did not significantly blunt thymidine incorporation in response to serum.

### Effect of Antisense ODN on [³H]Thymidine Incorporation in Response to Oleic Acid

Antisense experiments were performed using a 17-mer ODN targeting a consensus sequence at the initiation site for ERK-1 and ERK-2 mRNA. Immunoblotting demonstrated that the antisense ODNs induced a concentration-dependent decrease in ERK protein 48 hours after liposomal transfection (Fig 4A). Lipofectin alone and together with sense and scrambled ODNs did not affect ERK protein. Antisense did not affect the expression of PKC α, JNK, and p38 (867±76, 344±32, and 1024±223 arbitrary units for antisense ODN–treated cells versus 913±121, 359±64, and 996±89 arbitrary units for cells treated with lipofectin, respectively). Immunoblots for phospho-MAP kinase (Fig 4B) also showed that treatment with antisense ODNs significantly inhibited activation of the p42 and p44 MAP kinase isoforms in response to 100 μmol/L oleic acid in a dose-dependent manner. Microscopic observation revealed a normal morphology and viability in antisense ODN–treated cells. Antisense ODNs reduced basal thymidine incorporation ≈35% and prevented a significant rise of thymidine incorporation in response to 50 μmol/L oleic acid and Ang II 10⁻⁸ mol/L alone and together (Fig 5). Antisense ODNs reduced but did not eliminate the significant increase of thymidine incorporation in response to serum. Sense ODNs did not affect thymidine incorporation in response to oleic acid or serum.

### Effect of Oleic Acid on Activation of p38 and JNK

As shown in Fig 6, serum induced activating phosphorylation of both p38 and JNK within 30 minutes. UV irradiation activated JNK only, whereas oleic acid and Ang II alone or together failed to activate either of these kinases.

### Discussion

The principal finding of this study is that oleic acid and Ang II induce a synergistic mitogenic effect in rat aortic VSMCs (Fig 1, Table 1). While Ang II alone did not significantly increase either thymidine incorporation or cell number, it approximately doubled the increase of thymidine incorporation and cell number in response to oleic acid. Subjects with the cardiovascular risk factor cluster have an increase of plasma nonesterified fatty acids and a more active renin-angiotensin system. The data raise the possibility that oleic acid and Ang II combine to accelerate vascular remodeling among high-risk subjects such as obese hypertensive patients.

The potential relevance of the synergistic mitogenic interaction between oleic acid and Ang II and vascular disease among insulin-resistant subjects with the risk factor cluster is heightened by two points. First, the synergistic interaction occurs at physiological concentrations of these two growth factors. Although the 50-μmol/L concentration of oleic acid is significantly lower than mean plasma values measured in obese hypertensive patients (172±11 μmol/L), nonesterified fatty acids in the extracellular fluid are predominantly bound to albumin. Albu-
min concentrations in the interstitial fluid are approximately one quarter of those in plasma. Thus, we estimate that interstitial fluid concentrations of oleic acid surrounding VSMCs in obese hypertensive patients would be approximately 40 to 50 μmol/L.

Second, when Ang II was added to the media before oleic acid, Ang II concentrations at the very low end of the physiological range (10⁻¹¹ mol/L) augmented thymidine incorporation in combination with oleic acid. If very low concentrations of Ang II elicit most of the synergistic mitogenic effect, then the higher Ang II in subjects with risk factor clustering ¹³,¹⁴ would have less impact. However, some fatty acids, including oleic acid, decrease affinity of Ang II for its receptor. ²² When oleic acid was added to the media before Ang II, a synergistic mitogenic effect was seen at 10⁻¹⁰ mol/L Ang II and greater but not at 10⁻¹¹ mol/L (Fig 1). The greater activity of the renin-angiotensin system in patients with risk factor clustering ¹³,¹⁴ may be essential in eliciting a synergistic mitogenic effect on VSMCs in the presence of physiological concentrations of nonesterified fatty acids.

![Figure 3](http://hyper.ahajournals.org/)

**Figure 3.** Effects of MEK inhibition on ERK activation and thymidine incorporation in response to oleic acid (OA) and Ang II (AlI) alone and together. A, Oleic acid (50 μmol/L) and 10⁻⁸ mol/L Ang II alone or together and 10% FBS were added to rat aortic VSMCs with/without 1-hour preincubation in 20 μmol/L PD 98059 for 10 minutes. Cells were harvested and cytosolic extracts prepared as described for the ERK assay using the incorporation of ³²P into myelin basic protein. Values shown are the mean±SD from three experiments. For comparison, whole cell lysates were immunoblotted with active MAP kinase and ERK antibodies. B, Oleic acid or Ang II were added to quiescent rat aortic VSMCs pretreated for 1 hour with PD 09859. The cells were then incubated for 24 hours at 37°C. [³H]Thymidine incorporation was measured 6 hours after addition of [³H]thymidine. Data are presented as mean±SD for three independent experiments in triplicate. *P<.05 vs control.

![Figure 4](http://hyper.ahajournals.org/)

**Figure 4.** Effects of antisense ODNs on MAP kinase (ERK) protein content and MAP kinase (ERK) activity. Forty-eight hours after liposomal transfection of ODNs, cells were treated with DMEM or 100 μmol/L oleic acid for 10 minutes, harvested, subjected to SDS-polyacrylamide gel electrophoresis, and immunoblotted for ERKs. A, top, Representative immunoblot for total ERK-1 and ERK-2; bottom, densitometric quantification of immunoblots for total ERK-1 and ERK-2. *P<.05 vs control (C; lipofectin alone), sense, and scrambled ODNs. B, top, Representative immunoblot of phospho-MAP kinase; bottom, densitometric quantification of immunoblots for phospho-MAP kinase. *P<.05 vs control (lipofectin alone), sense, and scrambled ODNs.
The diacylglycerol and elevated intracellular Ca\textsuperscript{2+} activates phospholipase C, which hydrolyzes phosphatidylinositol to diacylglycerol and inositol trisphosphate. Ang II, acting via its G protein–coupled AT\textsubscript{1} receptor, PKC, MEK, and ERK in effects at multiple points that converge at MEK. Therefore, PKC inhibition and depletion eliminated the synergistic mitogenic effect of the oleic acid–Ang II combination (Fig 1). The effects of PKC inhibition and depletion on thymidine incorporation did not reflect nonspecific toxicity, since basal thymidine incorporation and the increase in response to 10% FBS were unaffected.\textsuperscript{12} These data confirm that the mitogenic response to oleic acid is PKC dependent\textsuperscript{12} and indicate that PKC is also required for the synergistic interaction between oleic acid and Ang II.

We previously reported that rat aortic VSMCs contain the \( \alpha \), \( \mu \), \( \tau \), and \( \zeta \) PKC isoforms.\textsuperscript{12} Although \( \tau \) and \( \zeta \) do not have a phorbol ester binding site, these two isoforms were downregulated by PMA after 24 hours. Activation of PKC, which had been partially purified from rat VSMCs, in response to oleic acid was almost entirely Ca\textsuperscript{2+} independent.\textsuperscript{12} This is consistent with previous reports indicating that oleic acid more potently and completely activates the Ca\textsuperscript{2+}-independent and atypical PKC isoforms.\textsuperscript{8,9} PKC \( \zeta \) has been shown to activate ERK and has been associated with mitogenesis in various cell lines.\textsuperscript{35} In contrast, PKC \( \alpha \), a Ca\textsuperscript{2+}-dependent isoform, is less potently stimulated by oleic acid\textsuperscript{8} and has been associated with differentiation rather than proliferation in VSMCs.\textsuperscript{35} The novel and/or atypical PKC isoforms may explain the mitogenic response to oleic acid and the synergistic interaction with Ang II.

MEK inhibition with PD 98059 essentially blocked ERK activation in response to oleic acid and Ang II singly and combined (Fig 3). PD 98059 and the antisense ODN prevented the significant rise of thymidine incorporation in response to oleic acid alone and the synergistic interaction with Ang II. However, PD 98059 did not significantly reduce thymidine incorporation in response to serum, which indicates that the cells were still capable of synthesizing DNA. The data also suggest that activation of MEK and ERK are important in the mitogenic response to oleic acid and the synergistic interaction with Ang II. Nevertheless, the findings could still reflect a nonspecific or toxic effect of PD 98059 on cell signaling that affected the response to oleic acid and Ang II more than to serum.

To address this concern, experiments were conducted using antisense ODNs to ERK-1 and ERK-2 on \([^{3}H]\)thymidine incorporation in response to serum and to oleic acid (OA) and Ang II (AII) either alone or together. Rat aortic VSMCs were transfected with antisense, sense, and scrambled ODNs and then treated with oleic acid, Ang II, and 10% FBS for 24 hours. Thymidine incorporation was measured 6 hours after addition of \([^{3}H]\)thymidine. Data are presented as mean \(\pm\) SD for three independent experiments in triplicate. *\( P<.05\) vs lipofectin plus antisense ODN.

Signaling Events in the Mitogenic Interaction Between Oleic Acid and Ang II

Ang II, acting via its G protein–coupled AT\textsubscript{1} receptor, activates phospholipase C, which hydrolyzes phosphatidylinositol to diacylglycerol and inositol trisphosphate. The diacylglycerol and elevated intracellular Ca\textsuperscript{2+} activate PKC. Oleic acid increases predominantly cytosolic Ca\textsuperscript{2+}– independent PKC activity.\textsuperscript{8,9,12} PKC can activate ERK by effects at multiple points that converge at MEK. Therefore, we focused on the AT\textsubscript{1} receptor, PKC, MEK, and ERK in an attempt to explain the mitogenic interactions between Ang II and oleic acid. Losartan, an AT\textsubscript{1}-receptor antagonist,\textsuperscript{32} blocked the capacity of Ang II to synergistically enhance thymidine uptake in response to oleic acid, whereas PD 123319, an AT\textsubscript{2}-receptor antagonist, did not (Fig 2). These data suggest that the capacity of Ang II to augment the mitogenic response to oleic acid is mediated via the AT\textsubscript{1} receptor, as noted previously.\textsuperscript{20,33} The findings do not implicate a growth-inhibitory role for AT\textsubscript{2} receptors in these rat aortic VSMCs.\textsuperscript{20}

PKC inhibition and depletion eliminated the synergistic mitogenic effect of the oleic acid–Ang II combination (Fig 1). The effects of PKC inhibition and depletion on thymidine incorporation did not reflect nonspecific toxicity, since basal thymidine incorporation and the increase in response to 10% FBS were unaffected.\textsuperscript{12} These data confirm that the mitogenic response to oleic acid is PKC dependent\textsuperscript{12} and indicate that PKC is also required for the synergistic interaction between oleic acid and Ang II.

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To address this concern, experiments were conducted using antisense ODNs to ERK-1 and ERK-2. The antisense ODNs reduced ERK protein expression by 83% and ERK activation in response to oleic acid by 75% (Fig 4). Antisense also blocked the significant increase of thymidine incorporation in response to oleic acid alone and the synergistic interaction with Ang II. In contrast to PD 98059, antisense more effectively limited the synergistic interaction with Ang II. Nevertheless, the findings do not implicate a growth-inhibitory role for AT\textsubscript{2} receptors in these rat aortic VSMCs.\textsuperscript{20}
suggest that serum can induce significant cell growth despite marked limitation of MEK and ERK activation. This response may be mediated in part via other members of the mitogen-activated protein kinase family, eg, p38 and JNK.\textsuperscript{36,39} Oleic acid can potently stimulate nuclear phospholipase D. However, the nuclear ADP-ribosylation factor-dependent phospholipase D activity is associated with cell proliferation,\textsuperscript{30,41} while olate-dependent phospholipase D activity is not.

### Limitations

While the synergistic mitogenic effect of oleic acid and Ang II is PKC-, MEK-, and ERK-dependent, the data do not establish that activation of ERK alone is sufficient to explain this synergy. Our studies have not defined the important nuclear signaling events. In other studies, the effects of Ang II on VSMC proliferation were heterogeneous,\textsuperscript{31,42,43} and coincided inversely with changes in transforming growth factor-\(\beta 1\).\textsuperscript{32,44} Quantifying the effects of oleic acid on transforming growth factor-\(\beta 1\) responses to Ang II in future studies might prove instructive.

### Summary

Subjects with the risk factor cluster have elevated fatty acids and a more active renin-angiotensin system. Angiotensin, acting on the \(AT_1\) receptor, synergistically augments the mitogenic response to oleic acid. Activations of PKC, MEK, and ERKs are necessary steps in the synergistic mitogenic response to oleic acid and Ang II. A synergistic mitogenic interaction between oleic acid and Ang II may accelerate vascular disease among subjects with the cardiovascular risk factor cluster.

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