Signaling Events Mediating the Additive Effects of Oleic Acid and Angiotensin II on Vascular Smooth Muscle Cell Migration

Eddie L. Greene, Gang Lu, Da Zhang, Brent M. Egan

Abstract—Obese hypertensive patients with cardiovascular risk factor clustering and increased risk for atherosclerotic disease have increased plasma nonesterified fatty acid levels, including oleic acid (OA), and a more active renin-angiotensin-aldosterone system. Vascular smooth muscle cell (VSMC) migration and proliferation participate in the development of atherosclerotic plaque. OA and angiotensin (Ang) II induce synergistic mitogenic responses in VSMCs through sequential signaling pathways dependent on the activation of protein kinase C (PKC), oxidants (reactive oxygen species, ROS), and extracellular signal-regulated kinase (ERK) activation. We tested the hypotheses that (1) OA and Ang II have additive or synergistic effects on VSMC migration and (2) PKC, ROS, and mitogen-activated protein kinase are critical signaling molecules. OA at 100 \( \mu \text{mol/L} \) increases VSMC migration 60\% over control \((P<0.001)\). Ang II \((10^{-9} \text{ mol/L})\) increases VSMC migration by 62\% and 73\% over control, respectively \((P<0.01)\). Coincubation of cells with OA and Ang II produces a nearly additive increase in VSMC cell migration at 107\% over control \((P<0.01)\). Increases in VSMC migration induced by OA alone and combined with Ang II were reduced by PKC inhibition and downregulation. VSMC migration in response to OA alone and with Ang II was also inhibited by \( \text{N-acetyl-cysteine} \), MEK inhibition, and ERK antisense. VSMC migration in response to OA alone or combined with Ang II is dependent on activation of PKC, ROS, and ERK activation, further raising the possibility that increased plasma nonesterified fatty acids and an activated renin-angiotensin-aldosterone system in subjects with the risk factor cluster contribute to accelerated atherosclerosis through a PKC, ROS, and ERK-dependent signaling pathway. \(\text{(Hypertension. 2001;37:308-312.)}\)

Key Words: muscle, smooth, vascular ■ angiotensin II ■ protein kinases ■ signal transduction ■ oxidative stress

Obese hypertensives have insulin resistance and the clustering of cardiovascular risk factors.\(^1\)–\(^4\) Although abdominal obesity and insulin resistance are known independent risk factors for atherosclerosis, the intermediary mechanisms are not well defined. Obese hypertensives have elevated plasma nonesterified fatty acids, including oleic acid,\(^5\) which are highly resistant to suppression by insulin.\(^6\)

The accumulation of vascular smooth muscle cells (VSMCs) in the intima results from both cell proliferation and directed migration of VSMCs from the media into the intima.\(^7\)–\(^8\) Oleic acid induces protein kinase C (PKC), reactive oxygen species (ROS), and extracellular signal-regulated kinase (ERK)-dependent mitogenic response in rat aortic smooth muscle cells.\(^9\) The effects of oleic acid on VSMC migration are unknown.

Subjects with risk factor clustering also have a more active renin-angiotensin system.\(^10\) Angiotensin II (Ang II) is a potent chemotactrant\(^11\) and a moderate mitogen. Oleic acid and Ang II exert a synergistic mitogenic effect on VSMCs that is dependent on activation of PKC, ROS, and ERKs.\(^9\)–\(^13\) This raises the possibility that the increased oleic acid and the enhanced activity of the renin-angiotensin axis in subjects with the risk factor cluster interact to accelerate atherosclerosis by stimulating not only VSMC proliferation but also VSMC migration. In this study, we examined the possibility that oleic acid alone or together with Ang II stimulates VSMC migration and found confirmatory evidence. We then tested whether activation of PKC, ROS, and ERKs participates in VSMC migration mediated by oleic acid alone and combined with Ang II. The role of PI-3 kinase in migration was also examined because evidence implicates a role for this signaling system in the insulin resistance syndrome as well as in VSMC migration.\(^14\)–\(^15\) Observations from these experiments could help define signal transduction mechanism(s) by which the insulin resistance syndrome contributes to the cardiovascular complications associated with atherosclerosis. This information could help foster novel approaches to the prevention and treatment of cardiovascular disease in patients with the cardiovascular risk factor cluster.
Methods

Materials
Oleic acid and N-acetyl-cysteine (NAC) were purchased from Sigma Chemical Co. Sodium salts of oleic acid were prepared as previously described. Cell culture materials were purchased from Gibco BRL. Fatty acid–free albumin was obtained from ICN Biomed. Inc. Transwell chambers (6.5 mm) were purchased from Corning.

Cell Culture
Rat aortic VSMCs were cultured by procedures modified from Chamley-Campbell et al. Using a protocol approved by the Medical University of South Carolina Animal Research Committee, Sprague-Dawley rats (weight, 150 to 200 g) were euthanized instantly by decapitation. A 10-cm section of aorta was removed and placed in 1× Dulbecco’s Modified Eagle’s Medium (DMEM). Adherent fat and connective tissue were gently removed with fine sterile forceps. The aorta was minced into small cube-shaped specimens and incubated with 1× DMEM/1 mg/mL collagenase for 1 hour. The individual pieces of vessel segments were seeded in a T-25 culture flask for at least 15 minutes to ensure adherence to the bottom surface. They were then incubated with 3 mL of 1× DMEM supplemented with 20% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/mL streptomycin at 37°C in 95% air/5% CO₂. Approximately 7 to 10 days later, the segments were removed and cells were placed into a 150-cm² flask, and the medium was changed to DMEM containing 10% FBS. Cells were characterized morphologically as smooth muscle by phase contrast microscopy and by immunostaining with α-actin.

VSMC Migration
Migration of VSMC was assayed by a modification of the Boyden’s chamber method. VSMC migration assay was performed in Transwell cell-culture chambers with a type I collagen (5 pg/mL) polycarbonate membrane with 8-µm pores. Preconfluent VSMCs were suspended in 0.1% BSA DMEM to a concentration of 5×10⁵ cells/mL. In some experiments, cells were pretreated with bisindolylmaleimide, PDMA, PDGF-BB, oleic acid, and/or Ang II. Cells were then incubated with 3 mL of 1× DMEM supplemented with 20% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/mL streptomycin at 37°C in 95% air/5% CO₂. Approximately 7 to 10 days later, the segments were removed and cells were placed into a 150-cm² flask, and the medium was changed to DMEM containing 10% FBS. Cells were characterized morphologically as smooth muscle by phase contrast microscopy and by immunostaining with α-actin.

VSMC Migration
Migration of VSMC was assayed by a modification of the Boyden’s chamber method. VSMC migration assay was performed in Transwell cell-culture chambers with a type I collagen (5 pg/mL) and fibronectin-coated (100 pg/mL) polycarbonate membrane with 8-µm pores. Preconfluent VSMCs were suspended in 0.1% BSA DMEM to a concentration of 5×10⁵ cells/mL. In some experiments, cells were pretreated with bisindolylmaleimide, PDMA, PDGF-BB, oleic acid, and/or Ang II. Cells were then incubated with 3 mL of 1× DMEM supplemented with 20% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/mL streptomycin at 37°C in 95% air/5% CO₂. Approximately 7 to 10 days later, the segments were removed and cells were placed into a 150-cm² flask, and the medium was changed to DMEM containing 10% FBS. Cells were characterized morphologically as smooth muscle by phase contrast microscopy and by immunostaining with α-actin.

Liposomal Transfection With Antisense ODNs
The antisense ODN, a 17-mer (5’GCCGCCGCCGCCGCAT-3’) directed against a consensus sequence that initiates the translation of rat ERK-1 and ERK-2, sense ODN (5’-ATTCGCGCGCGCGCGGCGGC-3’), and scrambled controls (5’-CTCGCGCACCC-3’) were synthesized at the Nuclear Synthesis Facility of the Medical University of South Carolina by an automated DNA synthesizer (EXPEDETE PerSeptive Biosystems). All bases were phosphorothioate protected. The ODNs were desalted by N-butanol precipitation, dried, and resuspended in sterile water. Primary rat aortic VSMCs were grown in 100-mm Petri dishes to 70% confluence. Transfection with 0.8-µm ODNs including antisense and scrambled ODNs was performed in Opti-Media (GIBCO BRL) with 10 pg/mL lipofectin. Cells were incubated for 5 hours at 37°C in a 5% CO₂ incubator for 4 hours. The transmembrane was then fixed with methanol for 10 minutes at room temperature, followed by counterstaining with hematoxylin. The number of VSMCs per high-power field that had migrated to the lower surface of the membrane was determined microscopically. Cells from 4 randomly chosen high-power fields (at ×400) were counted. Experiments were performed in triplicate and repeated on 4 separate occasions.

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Effects of Oleic Acid on VSMC Migration
As shown in Figure 1, the addition of 50 and 100 µmol/L oleic acid to primary cultures of rat aortic smooth muscle cells induced a concentration-dependent increase of VSMCs that migrated from the upper to the lower chamber. At concentrations of 50 and 100 µmol/L, oleic acid increased VSMC migration to values roughly 60±10% over control, whereas 10 ng/mL PDGF-BB, which served as the positive control, increased VSMC migration 3 to 4 times that of control. Ang II at 10⁻⁹ and 10⁻⁷ mol/L raised VSMC migration to 61% and 73% above control, respectively. When VSMC cells were incubated with both 50 µmol/L oleic acid and 10⁻⁷ or 10⁻⁵ mol/L Ang II for 4 hours, VSMC migration rose to 207% and 234% of control, which demonstrated that the combined effect of these two agents was approximately additive.

Effects of PKC Inhibition and PKC Downregulation on Oleic Acid and/or Ang II–Induced VSMC Migration
Studies from other laboratories on human platelets and Swiss 3T3 fibroblasts demonstrated that bisindolylmaleimide at concentrations of 5 pmol/L provides highly selective inhibition of PKC activity. We reported that 4 pmol/L bisindolylmaleimide completely blocks oleic acid–stimulated VSMC proliferation as measured by thymidine incorporation and cell number. In the present study, 4 pmol/L bisindolylmaleimide significantly inhibited the increase in VSMC migration induced by oleic acid (50 and 100 µmol/L) and 10⁻⁷ mol/L. Ang II alone or combined (Figure 2A). When VSMCs were treated with 200 nmol/L PMA for 24 hours,
PKC isoforms were no longer detected in whole-cell lysates, PKC downregulation after prolonged incubation with PMA also significantly inhibits VSMC migration in response to oleic acid and Ang II singly and combined (Figure 2A). Both PKC inhibition with bisindolylmaleimide and downregulation with PMA did not significantly alter basal VSMC migration.

Effects of NAC on Oleic Acid and/or Ang II–Induced VSMC Migration

Pretreatment of cells with 30 mmol/L NAC (Figure 2B) did not change basal VSMC migration. NAC significantly reduced VSMC migratory response to both oleic acid and Ang II singly and together.

Effects of MEK Inhibition and ERK Antisense on VSMC Migration

When cells were preincubated for 1 hour with 20 pmol/L PD 98059, a specific MEK inhibitor, VSMC migration was unchanged from basal values. However, VSMC migration in response to 50 pmol/L oleic acid and 10^{-7} mol/L Ang II both singly and in combination was reduced by 75% to 95% by PD 98059 (Figure 2C). The antisense ODN (5′-GCCGCCG-CGGCCGCGCAT-3′) has been used successfully to downregulate ERK-1 and ERK-2 in 3T3 cells, rat cardiac myocytes, and rat aortic VSMCs. Our previous study demonstrated that this antisense ODN reduced ERK expression by 83±3% and prevented the activation of ERK in response to oleic acid. Antisense ODNs did not reduce basal migration but did inhibit the rise of migration in response to 50 pmol/L oleic acid and 10^{-7} mol/L Ang II alone or together (Figure 2C). The scrambled ODNs did not significantly affect either basal migration or the increase in response to oleic acid and Ang II.

Effects of Phosphoinositide-3′ Kinase Inhibition on Oleic Acid and/or Ang II–Induced VSMC Migration

Activation of phosphoinositide-3′ kinase (PI-3 kinase) reportedly plays a major role in growth factor–stimulated migration of VSMCs. In our study, the importance of the PI-3 kinase pathway was examined by pretreating VSMC with 100 nmol/L wortmannin, a PI-3 kinase inhibitor, for 20 minutes. As shown in Figure 3, Ang II–induced migration was substantially inhibited by 100 nmol/L wortmannin (≈27%). Wortmannin did not significantly inhibit migration induced by oleic acid. When VSMCs were preincubated with both 100 nmol/L wortmannin and 4 pmol/L bisindolylmaleimide to inhibit both PI-3 kinase and PKC, VSMC migration in response to oleic acid and Ang II was not reduced below that seen with bisindolylmaleimide alone.

Discussion

This study demonstrates for the first time that oleic acid increases the migration of VSMCs in vitro. Through the use of selective inhibitors of signaling molecules, the migratory response of VSMCs to oleic acid appears to be mediated by a sequential signaling pathway that includes activation of PKC (inhibition and downregulation), generation of ROS (NAC), and activation of ERK (PD 98059, antisense ODN to ERK1 and ERK2). These results coincide with but do not precisely replicate our prior experiments with oleic acid–induced mitogenesis in cultured VSMCs. More specifically, augmentation of H-thymidine incorporation and VSMC number in response to oleic acid was completely abrogated by identical pretreatment of cells to inhibit the PKC, ROS, and ERK pathway(s). These same inhibitors significantly but incompletely inhibited VSMC migration in response to oleic acid. These findings suggest that another pathway participates in the migration of VSMCs stimulated by oleic acid.

PI-3 kinase represents one alternative signaling pathway by which oleic acid could induce VSMC migration. Although oleic acid has not been shown to directly activate PI-3 kinase, insulin, which is elevated among subjects with the risk factor cluster, is known to activate this pathway. Moreover, both oleic acid and cis-unsaturated fatty acids have been shown to activate atypical isoforms of PKC, for example, PKC-ζ, which leads to important downstream signaling events in VSMCs. PI-3 kinase can participate in VSMC migration. Despite the evidence implicating a role for PI-3 kinase in the migratory response of VSMCs to oleic acid, our experiments did not confirm this premise.

Our observations do confirm that Ang II augments VSMC migration. The combination of oleic acid and Ang II produces an enhancement of VSMC migration that appears to be slightly less than additive. These findings are similar but not identical to our previous studies that examined the effects of oleic acid and Ang II on thymidine incorporation and cell number in cultured VSMCs. We observed that Ang II alone did not significantly augment either measure of mitogenesis in VSMCs. Oleic acid and Ang II together increased thymidine incorporation and VSMC number to values significantly greater than the sum of their independent effects, that is, a synergistic response. In contrast, Ang II alone significantly augmented VSMC migration and together with oleic acid induced an effect that was less than additive. The failure of the oleic acid and Ang II together to produce a more robust increase of VSMC migration was not the result of a “ceiling effect” because the response to PDGF alone was greater. In concert with previous findings, inhibition and downregulation of PKC as well as MEK inhibition with PD 98059 and antisense ODNs to ERK1 and ERK2 significantly reduced the combined effect of oleic acid and Ang II on both VSMC proliferation and migration (Figure 2, A and C).

Inhibition of PI-3 kinase reduced VSMC migration in response to Ang II but not to oleic acid, as noted earlier. However, the combination of PKC inhibition with bisindolylmaleimide and PI-3 kinase inhibition with wortmannin did not decrease VSMC migration in response to Ang II more than that observed with bisindolylmaleimide alone (Figure 3). These observations are consistent with reports in the literature indicating that the products of PI-3 kinase can activate PKC.

The findings of the current study may help elucidate mechanisms by which the insulin resistance syndrome accelerates the atherosclerotic process. The insulin resistance syndrome is operative in patients with hypertension, especially that associated with obesity, as well as among individ-
uals with non–insulin-dependent diabetes mellitus and end-stage kidney disease. Previous research has identified several potential components of the cardiovascular risk factor cluster in these patients, which may contribute to the accelerated atherosclerosis including hyperinsulinemia, resistance to insulin-mediated glucose disposal, a complex dyslipidemia, and elevations of plasminogen activator inhibitor-1. We have been intrigued by evidence that many of these patients appear to be resistant to the nonesterified fatty acid–lowering action of insulin and have a more active renin-angiotensin-aldosterone axis. Consequently, we have examined the effects of oleic acid and Ang II on VSMC proliferation and migration, which comprise important events in the development and expansion of the atherosclerotic plaque. Our findings raise the possibility that the elevated oleic acid and Ang II in patients with the risk factor cluster may combine to accelerate atherosclerosis by enhancing VSMC migration and proliferation. The activation of PKC, the generation of ROS, and the activation of ERK emerge as critical signaling events in mediating the effects of oleic acid and Ang II on VSMC proliferation and migration. The experimental observations provide support for further studies to examine the role of these signaling molecules in the accelerated remodeling of the vascular wall and associated

Figure 2. A, Effect of selective inhibition of PKC signaling pathway(s) (by direct inhibition with 4 μmol/L bisindolylmaleimide [Bisi] and PKC depletion with 200 nmol/L PMA) on oleic acid (OA) and Ang II–induced VSMC migration. Quiescent VSMC were incubated with 50 or 100 nmol/mL oleic acid alone, 10^{-7} mol/L Ang II alone, or a combination of oleic acid (50 μmol/L) and Ang II (10^{-7} mol/L) in presence or absence of pretreatment with 4 μmol/L bisindolylmaleimide for 30 minutes. Cells were also pretreated with phorbol ester (200 nmol/L PMA) for 24 hours to deplete PKC. Data are presented as mean±SD from 3 experiments. One-way ANOVA followed by Duncan’s multiple range test was used to compare cell number changes in migration between treatment and control groups. Probability values <0.05 were considered statistically significant. B, Effect of NAC on oleic acid and Ang II–induced VSMC migration. Quiescent VSMC were incubated with 50 or 100 nmol/mL oleic acid alone, 10^{-7} mol/L Ang II alone, or a combination of oleic acid (50 μmol/L) and Ang II (10^{-7} mol/L) in presence or absence of pretreatment with 30 nmol/L NAC for 30 minutes. Data are presented as mean±SD from 3 experiments. One-way ANOVA followed by Duncan’s multiple range test was used to compare cell number changes in migration between treatment and control groups. Probability values <0.05 were considered statistically significant.

Figure 3. Effect of PI-3 kinase inhibition (with 100 nmol/L wortmannin) and PKC inhibition (with 4 μmol/L bisindolylmaleimide [Bisi]) on oleic acid (OA) and Ang II–induced VSMC migration. Cells were also treated with combination of wortmannin (100 nmol/L) and bisindolylmaleimide (4 μmol/L). Data are presented as mean±SD from 3 experiments. One-way ANOVA followed by Duncan’s multiple range test was used to compare cell number changes in migration between treatment and control groups. Probability values <0.05 were considered statistically significant.

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cardiovascular events observed among subjects with the insulin resistance syndrome.

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