Reactive Oxygen Species Are Critical in the Oleic Acid–Mediated Mitogenic Signaling Pathway in Vascular Smooth Muscle Cells

Gang Lu, Eddie L. Greene, Toshi Nagai, Brent M. Egan

Abstract—Obese hypertensive patients with cardiovascular risk factor clustering have increased plasma nonesterified fatty acid levels and are at high risk for atherosclerotic events. Our previous studies demonstrated that oleic acid induces a mitogenic response in rat aortic smooth muscle cells (RASMCs) through protein kinase C (PKC)– and extracellular signal–regulated kinase (ERK)–dependent pathways. In the present study we investigated the possibility that the generation of reactive oxygen species (ROS) constitutes a critical component of the oleic acid–induced mitogenic signaling pathway in RASMCs. We studied the effect(s) of oleic acid on the generation of ROS using the oxidant-sensitive fluoroprobe 2′,7′-dichlorofluorescin diacetate. Relative fluorescence intensity and fluorescent images were obtained with laser confocal scanning microscopy from 1 to 5 minutes, since preliminary studies demonstrated that the peak fluorescence intensity occurred within 5 minutes. Oleic acid (100 μmol/L) induced a time-dependent increase of cell fluorescence that was 8-fold of that seen in control cells at 5 minutes. This was blocked by catalase, which suggests that H₂O₂ was the principal ROS. The oleic acid–induced increases in H₂O₂ were blocked when PKC was inhibited with the use of bisindolylmaleimide and when PKC activity was downregulated by exposing RASMCs to phorbol 12–myristate 13–acetate for 24 hours. Stearic and elaidic acids, which are weak PKC activators, did not significantly increase H₂O₂ production. The increase of H₂O₂ in response to oleic acid was inhibited by the antioxidant N-acetylcysteine. N-Acetylcysteine also completely blocked ERK activation and the increase of thymidine incorporation in response to oleic acid. The data suggest that generation of H₂O₂ in RASMCs exposed to oleic acid is PKC dependent. Moreover, H₂O₂ production emerges as a critical intermediary event in the oleic acid–mediated mitogenic signaling pathway between the activation of PKC and ERK. These observations raise the possibility that the elevated plasma nonesterified fatty acids, including oleic acid, in obese hypertensive patients contribute to vascular growth and remodeling by a PKC-dependent mechanism to generate ROS that subsequently activate ERK. (Hypertension. 1998;32:1003-1010.)

Key Words: muscle, smooth, vascular ■ oleic acid ■ kinase ■ reactive oxygen species ■ hydrogen peroxide

Obese hypertensive patients have insulin resistance and the associated cardiovascular risk factor cluster. Obese hypertensive patients have insulin resistance and the associated cardiovascular risk factor cluster.1–4 While abdominal obesity and insulin resistance emerge as independent risk factors for atherosclerotic disease, the intermediary mechanisms are not well defined. Evidence suggests that resistance to the fatty acid–lowering actions of insulin among insulin-resistant individuals may contribute to structural and functional vascular changes.5–8 We previously reported that obese hypertensive patients have elevated plasma nonesterified fatty acids (NEFAs), including oleic acid, which reflect resistance to the antilipolytic action of insulin.9 We demonstrated that oleic acid induced a protein kinase C (PKC)– and extracellular signal–regulated kinase (ERK)–dependent mitogenic response in rat aortic smooth muscle cells (RASMCs). Activation of PKC and ERK has been linked to vascular smooth muscle cell (VSMC) proliferation and vascular contraction.9–12 In addition, activation of ERK has been linked to VSMC migration.13 Thus, the activation of PKC and ERK is linked to events associated with hypertension and vascular remodeling.

A better understanding of early events in the oleic acid–induced signaling pathway in VSMCs could shed light on intermediary mechanisms by which insulin resistance leads to functional and structural vascular changes. Of note, NEFAs, including oleic acid, induce a PKC-dependent activation of the respiratory burst in white blood cells.14 Recently, reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂) and superoxide radical (O₂−) generated in response to hormone or ligand binding have been implicated as participants in signaling pathways stimulating VSMC growth and DNA synthesis.15–18 Moreover, ROS have been associated with the activation of ERKs,19 transcription factors,20 phospholipase A₂,21...
collagenases, and protooncogene expression. Collectively, the extant literature supports the hypothesis that oleic acid induces a PKC-dependent generation of oxygen radicals that, in turn, activate ERK in VSMC. The present study was undertaken to determine whether ROS are major participants in critical intermediary signaling events between the activation of PKC and ERK in RASMCs. We used the following criteria to establish that oleic acid induces the generation of ROS and that activation of PKC is a critical upstream event and activation of ERK is a downstream event: (1) Stimulation of VSMCs by oleic acid should lead to the generation of measurable quantities of ROS. (2) PKC inhibition and down-regulation should reduce the generation of ROS in VSMCs stimulated with oleic acid. (3) Controlling for nonspecific effects of fatty acids and elaidic and stearic acids, which are poor activators of PKC compared with oleic acid, should induce a lesser amount of ROS than oleic acid. (4) Antioxidants should attenuate ERK activation and thymidine incorporation induced by oleic acid. Observations from these experiments could help to define signaling mechanism(s) by which insulin resistance and abnormal fatty acid metabolism could accelerate vascular remodeling. This information could help to foster novel approaches to the prevention and treatment of cardiovascular disease in patients with the risk factor cluster.

**Methods**

**Materials**

Individual fatty acids, catalase, and N-acetylcysteine (NAC) were purchased from Sigma Chemical Co. Sodium salts were prepared as previously described. Cell culture materials were purchased from GIBCO BRL. Fatty acid–free albumin was obtained from ICN Biomedical Inc.

**Cell Culture**

RASMCs were cultured by procedures previously described. Assay for Intracellular Oxidant Production

RASMCs were plated in 35-mm cell culture plates and grown until confluent. Cells were placed in DMEM containing 0.1% fetal bovine serum for 48 hours to induce growth arrest. Quiescent cells were incubated with the $\text{H}_2\text{O}_2$-sensitive fluorescent probe $\text{DCF}-\text{DA}$ (10 nmol/L) for 30 minutes. Oleic acid (100 $\mu$mol/L) was added to the cells for 10 minutes. Relative fluorescence intensity and fluorescent images were obtained from 1 to 5 minutes after addition of 100 $\mu$mol/L oleic acid with laser confocal microscopy. A, Fluorescent images of control cells and cells treated with 100 $\mu$mol/L oleic acid. B, The intensity of the fluorescent images was quantified with the use of Adobe Photoshop 4.01. Values shown represent the mean ± SEM from 3 experiments. $^*P<0.05$ vs baseline by 1-way ANOVA followed by Duncan’s multiple range test. Differences between the 2 curves were assessed by 2-factor ANOVA.
Proteins in the whole cell lysate were resolved by SDS-PAGE with 10% Laemmli gels. Proteins were electrophoretically transferred to polyvinylidene membranes (Millipore) and then immunoblotted with antibodies against anti-ACTIVE mitogen-activated protein kinase polyclonal antibody (Promega Co) and anti-ERK monoclonal antibody (Transduction Laboratories). IgG horseradish peroxidase–conjugated antibody (Amersham) was used as the secondary antibody. Visualization of the blot was performed with the ECL Western blotting system.

**Statistical Analysis**

Data are presented as mean±SD. Data were analyzed with SPSS 6.0 (SPSS Inc). The time-dependent changes in fluorescence intensity within and across the various treatment conditions were analyzed with 1-way ANOVA followed by Duncan’s multiple range test or 2-factor ANOVA, respectively. *P* values <0.05 were considered statistically significant.

**Results**

**Effect of Oleic Acid on Production of H$_2$O$_2$**

To fulfill the first criterion, quiescent RASMCs were pretreated with DCF-DA for 30 minutes and then treated with 100 μmol/L oleic acid for up to 20 minutes. We postulated that oleic acid would increase the generation of ROS. Relative fluorescence intensity and microscopic fluorescent images of the cells were obtained from 1 to 20 minutes after exposure to oleic acid with laser confocal scanning microscopy. Initial studies indicated that the increase in fluorescence intensity peaked after 5 minutes and persisted at the same levels over the next 15 minutes. Therefore, subsequent imaging studies were limited to measuring change in fluorescence intensity from 1 to 5 minutes. As shown in Figure 1A, oleic acid induced a time-dependent increase in fluorescence in RASMCs from 1 minute to 5 minutes, which presumably reflects an increase of intracellular H$_2$O$_2$ production. In the control RASMCs, fluorescence intensity increased minimally over 5 minutes. As shown in Figure 2, 1000 IU/mL catalase inhibited H$_2$O$_2$ production in response to oleic acid. Quiescent RASMCs were incubated with 100 μmol/L oleic acid in the presence or absence of a 30-minute pretreatment with either 1000 or 2000 IU/mL catalase. Fluorescent images were obtained at 5 minutes with laser confocal microscopy. A, DCF control; B, 100 μmol/L oleic acid; C, 1000 IU/mL catalase; D, 1000 IU/mL catalase + 100 μmol/L oleic acid; E, 2000 IU/mL catalase; F, 2000 IU/mL catalase + 100 μmol/L oleic acid.

**Immunoblot Assay for Phosphorylation of Mitogen-Activated Protein Kinase**

Confluent RASMC in 100-mm cell culture plates were incubated in media containing 0.1% fetal bovine serum and DMEM for 48 hours. Oleic acid (100 μmol/L) was added to control cells and also cells pretreated for 10 to 20 minutes with NAC (30 mmol/L). The incubation was stopped by adding ice-cold PBS. Cells were scraped into PBS and then pelleted by centrifugation for 20 seconds at 2000g. The cell pellet was suspended in cold lysis buffer containing 10 mmol/L Tris, 10 mmol/L NaCl, 3 mmol/L MgCl$_2$, 500 μmol/L sodium vanadate, 2 mmol/L phenylmethylsulfonyl fluoride, and 10 000 U/mL aprotinin. The suspension was maintained at 4°C for 10 minutes. The supernatant was collected as the whole cell lysate.
to deplete PKC. As shown in Figure 3, in cells pretreated with 4 μmol/L bisindolylmaleimide for 30 minutes to block PKC or 200 nmol/L PMA for 24 hours to deplete PKC, the increase in H2O2 after treatment with oleic acid was completely blocked. Basal H2O2 production in cells treated with bisindolylmaleimide or PMA pretreatment for 24 hours was not significantly altered (data not shown).

**Effect of Elaidic and Stearic Acids on H2O2 Production**

To ensure that the effect(s) of oleic acid did not represent nonspecific effects of fatty acids (criterion 3), RASMCs were treated with 100 μmol/L concentrations of elaidic acid, the trans isomer of oleic acid, and stearic acid, an 18-carbon saturated fatty acid. RASMCs treated with stearic and elaidic acids did not show significant increases in fluorescence intensity, which raises the possibility of a selective effect of oleic acid on cell function (Figure 4).

**Effect of NAC on ERK Activation and 3H-Thymidine Incorporation in Response to Oleic Acid**

To fulfill the fourth criterion, ie, that antioxidants should inhibit ERK activation and thymidine incorporation, we treated cells with NAC. Treatment of quiescent RASMCs with 100 μmol/L oleic acid for 10 minutes induced a marked increase in the phosphorylation of ERK-1 and ERK-2 (Figure 5A, top panel). Pretreatment of cells with 30 mmol/L NAC completely blocked phosphorylation of ERKs. In the bottom panel, an immunoblot with anti-ERK antibody confirms that equal amounts of proteins were loaded in each lane. Treatment of cells with NAC alone did not change either ERK protein expression or the phosphorylation of ERK-1 and ERK-2. NAC (30 mmol/L) also completely blocked the increase of thymidine incorporation in response to oleic acid (Figure 5B). To support the notion that NAC blocked ERK activation and thymidine incorporation by inhibiting ROS, we...
examined whether NAC would limit the increase of DCF fluorescence intensity in cells stimulated with oleic acid. When RASMCs were pretreated with 30 mmol/L NAC for 30 minutes, the increase in fluorescence after stimulation with 100 μmol/L oleic acid was blocked (Figure 6).

**Discussion**

Oxidative stress and the generation of ROS in vascular cells have been postulated as contributors to atherosclerosis and related cardiovascular complications. Oxidants could conceivably participate in vascular injury through several mechanisms, which include (1) effects on cellular function through lipid peroxidation of plasma membrane and subcellular membrane lipids, (2) oxidation of critical cellular proteins and enzymes, and (3) direct cytotoxicity. However, oxidants are not always generated in quantities that are immediately cytotoxic or injurious. Moreover, limited quantities of ROS can be generated in response to hormone or ligand binding to cell surface receptors and function as second messengers involved in cell growth and proliferation.15-18 ROS fulfill several criteria as possible signaling molecules. For example, ROS are small diffusible molecules that are ubiquitously present and can be rapidly synthesized and destroyed.26

The findings of the present study suggest that oleic acid induces a PKC-dependent increase in the generation of ROS leading to ERK activation in cultured VSMCs. Fluorescence intensity in DCF-loaded RASMCs treated with oleic acid increased rapidly as a function of time up to 5 minutes (Figure 1). Furthermore, the increase in fluorescence was inhibited by catalase (Figure 2). These data suggested that the oleic acid–induced increases in intracellular fluorescence intensity principally reflect a rise in H2O2.24,25 Of note, the 100 μmol/L concentration of oleic acid used in our experiments falls within the range of plasma levels measured in healthy normotensives, which are roughly two thirds the values obtained in obese hypertensives. Interstitial fluid concentra-

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**Figure 4.** Effect of elaidic and stearic acids on H2O2 production. Quiescent RASMCs were incubated with 100 μmol/L elaidic or stearic acid for 10 minutes. Relative fluorescence intensity and fluorescent images were obtained from 1 to 5 minutes with laser confocal microscopy. A, Fluorescent images of cells treated with 100 μmol/L oleic, elaidic, or stearic acid. B, The intensity of the fluorescent images was quantified with the use of Adobe Photoshop 4.01. Values shown represent the mean±SEM from 3 experiments. *P<0.05 vs baseline by 1-way ANOVA followed by Duncan’s multiple range test. Differences between the 2 curves were assessed by 2-factor ANOVA.
OE8 Reactive Oxygen and Oleic Acid Signaling

Figure 5. Effect of NAC on ERK activation and thymidine incorporation in response to oleic acid (OA). A, Quiescent RASMC were stimulated with 100 μmol/L oleic acid for 10 minutes. Some cells were pretreated with 30 mmol/L NAC for 30 minutes. Cells were washed, harvested, and subjected to SDS-PAGE and immunoblotted for phospho–mitogen-activated protein kinase (MAPK). Top lane, Representative immunoblot of phospho–mitogen-activated protein kinase protein of 3 independent experiments. Bottom lane, Representative immunoblot of ERKs. C indicates control. B, Oleic acid (100 μmol/L) was added to quiescent RASMCs for 24 hours at 37°C, some of which were pretreated with 30 mmol/L NAC. 3H-dT was measured 6 hours after addition of 3H-thymidine. Data are presented as mean±SEM for 3 independent experiments in triplicate.

**P**<0.05 vs control by 1-way ANOVA followed by Duncan’s multiple range test.


tions of fatty acids are likely approximately one fourth those in plasma. Thus, the 100 μmol/L concentration of oleic acid probably lies at the upper end of interstitial fluid values observed in insulin-resistant patients, which includes obese hypertensives.

Both PKC inhibition with bisindolylmaleimide and downregulation by 24-hour pretreatment with PMA blocked the increase in cellular fluorescence in response to oleic acid (Figure 3). We have shown that PKC inhibition with bisindolylmaleimide and downregulation with PMA prevent agonist-induced increases of PKC activity. However, neither of these treatments adversely affected cell morphology or the increase of 3H-thymidine uptake in response to 10% fetal bovine serum. These findings suggest that oleic acid induces a PKC-dependent increase in the generation of H2O2 in VSMCs.

To control for possible nonspecific effects of fatty acids, additional experiments were performed with elaidic and stearic acids. In contrast to 100 μmol/L oleic acid, an 18-carbon, cis-monounsaturated fatty acid, identical concentrations of elaidic acid, the trans isomer of oleic acid, and stearic acid, an 18-carbon saturated fatty acid, caused minimal changes in cell fluorescence (Figure 4). In comparison to oleic acid, stearic and elaidic acids are weak activators of PKC. Thus, the generation of H2O2 in response to these three 18-carbon NEFAs corresponds to their known capacity to activate PKC. These data are consistent with the notion that oleic acid induces a specific PKC-dependent increase of ROS in VSMCs.

We reported that oleic acid induced a PKC-dependent activation of ERK in RASMCs and subsequently demonstrated that the mitogenic response to oleic acid was also ERK dependent. As noted above, other studies have shown that ROS activate ERK. Activation of ERK has emerged as an important signaling event in VSMC migration, growth, and vascular tone. Therefore, our studies of ROS and oleic acid–mediated activation of ERK complement the existing literature. NAC, an antioxidant, blocked the generation of ROS in oleic acid–treated cells (Figure 6). NAC also blocked the ability of oleic acid to induce phosphorylation of ERK-1 and ERK-2 and DNA synthesis in cells stimulated with oleic acid (Figure 5). These observations suggest that the generation of ROS is a critical event in the activation of ERK and cell proliferation by oleic acid. Collectively, our data suggest that the generation of ROS represents a critical intermediary event between the activation of PKC and ERK and cell growth in VSMCs stimulated with oleic acid.

A large body of literature has established activation of both PKC and ERK as signaling events that may play an important role in vascular remodeling. A more recent and rapidly growing number of studies implicates oxygen radicals in vascular pathophysiology. ROS trigger several events linked with vascular remodeling and cardiovascular complications, including activation of ERKs, transcription factors, phospholipase A2, insulin-like growth factor 1, DNA synthesis, and metalloproteinases. ROS have also been associated with apoptosis and impairment of endothelium-dependent vasodilation. Thus, oleic acid is now associated with 3 steps of the signaling cascade in VSMCs (ie, PKC, ROS, and the ERKs) that are implicated in vascular pathophysiology.

In summary, the cardiovascular risk factor cluster associated with insulin resistance includes resistance to the fatty acid–lowering action of insulin. Obese hypertensives, in particular, are highly resistant to the antilipolytic effect of insulin and have elevated plasma oleic acid concentrations. Oleic acid induces a PKC-dependent increase in the generation of ROS, which, in turn, activate ERK in VSMCs. Therefore, the study data suggest that ROS represent a critical event in the oleic acid–mediated mitogenic signaling pathway between PKC and ERK. These findings raise the possibility that oleic acid participates in the vascular remodeling and complications associated with the risk factor cluster and may shed further light on intermediary events by which insulin resistance accelerates vascular remodeling.

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References


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