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ORIGINAL INVESTIGATION

Fatty Acids Differentially Modulate Insulin-Stimulated Endothelial Nitric Oxide Production by an Akt-Independent Pathway

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ABSTRACT

Background: Insulin increases endothelial nitric oxide (NO) production by activating endothelial nitric oxide synthase (eNOS) through protein kinase B (Akt)-mediated phosphorylation of serine residue 1179 (p-eNOS serine 1179). Because fatty acids modulate insulin-stimulated Akt signaling cascades in smooth muscle cells, we hypothesized that fatty acids would differentially regulate endothelial Akt signaling, eNOS phosphorylation, and NO production.

Methods: Porcine pulmonary artery endothelial cells (PAECs) were treated for 3 hours with 100 •M oleic (18:1) or eicosapentaenoic (20:5) acids or with an equivalent volume of ethanol vehicle (0.1%). PAECs were then treated with graded concentrations (10^{-29} -10^{-25} M) of insulin or incubated overnight (24 hours) in culture medium without fatty acids before insulin treatment. Activation and phosphorylation of Akt and eNOS were determined by immunoblotting. NO production was measured with a chemiluminescence NO analyzer or with a NO-selective carbon fiber microelectrode.

Results: Insulin-stimulated Akt phosphorylation, eNOS phosphorylation, and NO production. The phosphatidylinositol-3 kinase inhibitor wortmannin attenuated insulin-stimulated Akt activation and NO production. Treatment with the ω-3 fatty acid 20:5, but not 18:1, enhanced insulin-stimulated NO production but failed to alter insulin-stimulated Akt activation or eNOS serine 1179 phosphorylation.

Conclusion: Individual fatty acyl species have distinct effects on insulin-stimulated endothelial NO production. Although fatty acids alter Akt signaling in muscle cells, the current results indicate that fatty acids do not modulate endothelial NO production through alterations in insulin-stimulated, Akt-mediated eNOS phosphorylation.

KEYWORDS

fatty acid, endothelial cell, nitric oxide

Endothelial cells play a critical role in the regulation of vascular tone. Nitric oxide (NO) released from the vascular endothelium diffuses into underlying smooth muscle layers, where it activates soluble guanylate

cyclase, increases the cyclic guanosine monophosphate content of vascular smooth muscle cells, and thereby promotes vascular smooth muscle relaxation and reduced vascular tone. Endothelium-derived NO also regulates platelet activation, endothelial adhesion molecule expression, and vascular smooth muscle cell proliferation. Endothelial dysfunction, characterized by impaired endothelial NO production, participates in the pathogenesis of atherosclerotic vascular disease and is associated with risk factors for vascular disease, including hypercholesterolemia, diabetes mellitus, insulin resistance, and obesity. These observations indicate that impaired endothelial NO production plays a critical role in the pathogenesis of macrovascular disease. [1]

Patients who suffer from obesity, insulin resistance syndrome, or type 2 diabetes have characteristic metabolic derangements that may include elevated levels of circulating free fatty acids. Compared with lean control subjects, patients with obesity and insulin resistance have two- to threefold increases in serum free fatty acid levels [2] and suffer from an increased incidence and severity of macrovascular disease. Elevated circulating levels of fatty acids have been shown to impair endothelium- and NO-dependent vasorelaxation in humans in vivo. [3,4] In addition to quantitative increases in serum fatty acids, patients with type 2 diabetes also manifest qualitative alterations in the profile of serum and tissue fatty acids. [5-7] Additional studies have also shown that saturated and predominantly ω-6 unsaturated fatty acid emulsions impair endothelial NO production [8] and vascular relaxation, [3] whereas ω-3 fatty acids enhance endothelial [9] and vascular NO production. [10] Taken together, these studies suggest that fatty acids can modulate endothelial NO production and may contribute to altered vascular responsiveness in patients with obesity, insulin resistance, or type 2 diabetes.

The mechanisms by which fatty acids alter endothelial NO production remain to be defined. Because insulin stimulates endothelial NO production [11] and vascular relaxation, [3] we questioned if fatty acids disrupt insulin-stimulated endothelial signaling pathways to impair endothelial NO production. Akt-dependent phosphorylation of endothelial nitric oxide synthase (eNOS) at serine residue 1179 (p-eNOS) activates eNOS. [12-14] Furthermore, insulin-stimulated endothelial NO production is blocked by the phosphatidylinositol-3 (PI-3) kinase inhibitor wortmannin [11] and requires Akt-mediated eNOS phosphorylation on serine 1179. [15] In skeletal muscle, several fatty acids have been shown to impair Akt signaling by decreasing Akt phosphorylation and activation, thereby inhibiting downstream glucose uptake and transport. [16] Taken together, these reports suggest that fatty acids could potentially disrupt insulin-stimulated, Akt-mediated signaling pathways in vascular endothelium to modulate NO production.

MATERIALS AND METHODS

Materials

Minimum essential medium alpha (MEMα), Dulbecco's modification of Eagle's medium (DMEM), penicillin-streptomycin, trypsin, and Hanks balanced salts solution (HBSS) were obtained from Mediatech Cellgro (Herndon, VA). Collagenase was purchased from Worthington Biochemicals (Freehold, NJ). Bovine serum albumin (BSA), fetal bovine serum (FBS), Tris (Tris [hydroxymethyl] aminomethane), insulin, oleic and eicosapentaenoic acids, and amphotericin B were purchased from Sigma Chemical Co (St. Louis, MO). Gentamicin was purchased from SoloPak Laboratories Inc (Elk Grove Village, IL). LumiGlo reagent, antibodies for Akt, phosphorylated Akt (p-Akt), p-eNOS serine 1179, and horseradish peroxidase-linked antirabbit secondary antibodies were purchased from Cell Signaling (Beverly, MA). Precast 4 to 12% bis

tris polyacrylamide gel electrophoresis (PAGE) minigels, NuPage morpholinepropanesulfonic acid (MOPS) sodium dodecyl sulfate (SDS) running buffer, NuPage transfer buffer, NuPage antioxidant, and SDS sample loading buffer were purchased from Novex-Invitrogen (San Diego, CA). Tween-20 was purchased from BioRad (Hercules, CA). Complete protease inhibitor cocktail was obtained from Roche (Basel, Switzerland).

Pulmonary Artery Endothelial Cell Isolation and Culture

Endothelial cells were isolated from the main pulmonary artery of pigs as previously reported. [17] In brief, pulmonary arteries were isolated immediately following sacrifice and transported on ice from the slaughterhouse to the laboratory. The lumen of each vessel was treated with 0.3% collagenase for 15 minutes at 37°C. Endothelial cells were then gently scraped from the vessel lumen with a sterile scalpel and dispersed in growth medium consisting of MEMα containing 10% FBS, 100 U/mL penicillin-streptomycin, 20 •g/mL gentamicin, and 2 •g/mL amphotericin B. Pulmonary artery endothelial cells (PAECs) were then transferred to 60 mm plastic culture dishes precoated with 0.2% gelatin and maintained in a humidified 95% air-5% CO_2 atmosphere at 37°C until reaching primary confluence. Once confluent, PAECs were passaged by treatment with 0.05% trypsin for 1 to 2 minutes. After reaching confluence, the concentration of FBS was decreased to 4% (maintenance medium). In all experiments, PAECs were studied within 1 to 3 days after confluence. Control and experimental dishes were matched according to the cell line, number of passages, and number of days postconfluence. Monolayers were identified as endothelial cells by phase-contrast microscopy and factor VIII staining. [17]

Fatty Acid and Insulin Treatment Protocols

PAECs were washed with HBSS and then exposed to maintenance medium containing 100 •M oleic (18:1) or eicosapentaenoic (20:5) acids or to an equivalent volume of ethanol vehicle (0.1%), as we have previously reported. [17] This method of enriching PAECs with exogenous fatty acids caused significant and persistent alterations in PAEC fatty acid composition without causing PAEC cytotoxicity or impaired barrier function. [17-21] After 3 hours, the cells were washed again with HBSS and then either incubated in serum-free DMEM containing insulin or incubated overnight in maintenance medium without exogenous fatty acids prior to washing and treatment with insulin. At intervals following insulin treatment, media were collected for NO detection, and cell monolayers were harvested for Western blot analyses and protein determinations.

PAEC NO Release Assays

PAEC NO release was determined by measuring NO and its oxidation products, NO_2^- [2] and NO_3^- [2] (collectively referred to as NO_x), in the culture medium above confluent PAEC monolayers as previously described. [22] Following experimental manipulations, aliquots of PAEC culture medium were aspirated and transferred to centrifuge tubes. All samples were centrifuged at 1,500 3 *g* for 1 minute before transferring 40 •L of the supernatant to a purge vessel containing 0.8% vanadium chloride in 1 N HCl at 95°C. These conditions convert NO_x to $\cdot$NO, which is carried with N_2 into a Sievers chemiluminescence NO analyzer (Model 280, Boulder, CO). Standard curves were generated daily using 0.1 to 10 •M $NaNO_3$.

In separate experiments, acute insulin-induced alterations in PAEC NO release were measured with an

NO-selective electrode as previously reported. [23,24] Carbon fiber microelectrodes 30 •m in diameter and 100 •m in length (World Precision Instruments, Sarasota, FL) were coated with nafion (Sigma-Aldrich, St. Louis, MO). These electrodes were subsequently coated with o-phenylenediamine (Sigma) as previously reported. [23,25] The electrodes were calibrated the day of the experiment using serial dilutions (50, 100, 200, and 400 nM) of saturated 2 mM NO dissolved in phosphate buffered saline (PBS). Prior to use, the electrode was hydrated in PBS. Preliminary experiments demonstrated that the coated electrode did not respond significantly to insulin, nitrite, ascorbic acid, or dopamine (data not shown). Following experimental manipulations, PAECs in 35 mm culture dishes were treated for 2 hours prior to the experiment in serum-free medium to lower background noise detected by the electrode. PAECs were then placed in a temperature-controlled holder (37°C) on a microscope stage, and the electrode was positioned with a micromanipulator 5 •m above the monolayer surface. The electrode was conditioned for 45 to 60 minutes above PAEC monolayers to achieve a stable baseline. After the conditioning period, data were recorded prior to and for 10 minutes following the addition of insulin. Amperometric currents were recorded at 900 mV relative to an Ag/AgCl ground electrode at a sampling rate of 500 •s [21] using software and hardware from Axon Instruments (Union City, CA). The resulting data sets were initially low pass filtered at 1 kHz (8 pole Bessel) and further filtered at 0.2 Hz using software before final analysis.

Western Blot Analysis

Immediately following insulin treatment, PAEC monolayers were washed with cold HBSS and then solubilized for 10 minutes with cell lysis buffer at 4°C (20 mM Tris pH 7.4, 2.5 mM ethylenediaminetetraacetic acid [EDTA], 100 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4, 1% Triton X-100, 0.1% SDS, 1% Na deoxycholate, 1 tablet/10 mL EDTA-free complete protease inhibitor cocktail, 1 mM β-glycerolphosphate, and 2.5 mM Na pyrophosphate). Cells were then scraped into microfuge tubes, sonicated on ice (5 watt-seconds 3 10), and spun at 16,000 3 *g* for 5 minutes. The supernatants were then transferred to new tubes, and protein concentrations were determined using a Pierce (Rockford, IL) bicinchoninic acid (BCA) system. Equal amounts of protein were added to SDS sample buffer solution and heated to 70°C for 10 minutes. Proteins (10-20 •g) were loaded into 4 to 12% bis tris PAGE precast minigels. Proteins were separated by electrophoresis and blotted to polyvinylidene difluoride (PVDF) membranes. Membranes were incubated overnight at 4°C in 1:1,000 dilution of primary antibody. Proteins were visualized using a peroxidase-coupled antirabbit immunoglobulin G in the presence of LumiGlo reagent while being exposed to blue-sensitive autoradiographic film. Densitometric analysis was accomplished with a laser densitometer.

Statistical Analysis

In each experiment, PAECs were matched according to cell line, passage, and number of days postconfluence. Comparisons between multiple groups were made with analysis of variance followed by post hoc Tukey test. The level of statistical significance was taken as $p < .05$.

RESULTS

PAECs were treated with graded concentrations of insulin (1 3 10 [25] -10 [29] M) for 1 hour followed by analysis of Akt activation and NO production. Compared with control cells, treatment with ≥ 0.05 •M insulin for 1 hour activated the serine kinase Akt, as evidenced by increased levels of p-Akt (Figure 1

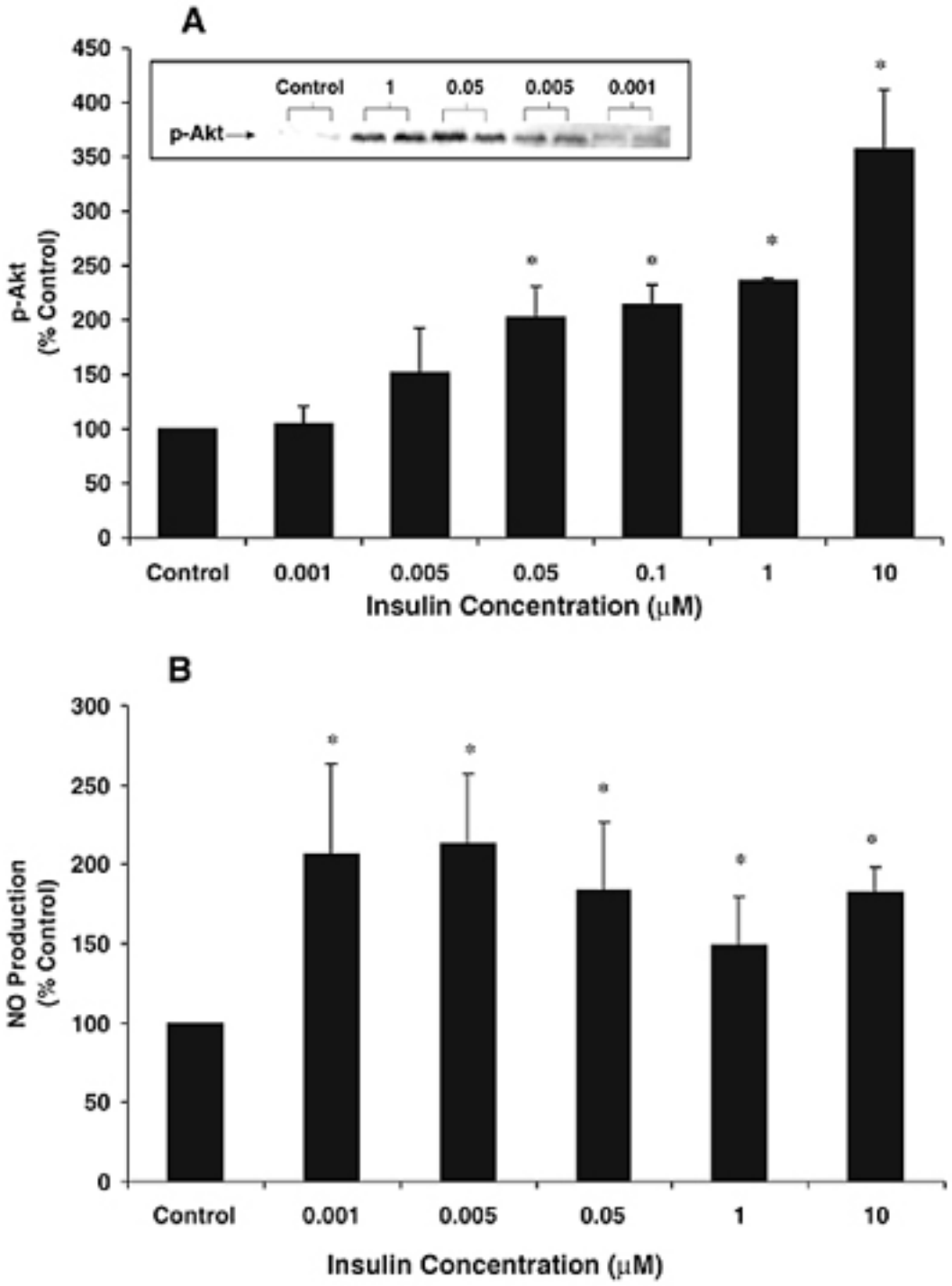


FIGURE 1 Insulin-stimulated Akt activation and nitric oxide (NO) production in pulmonary artery endothelial cells (PAECs). PAECs were exposed to serum-free Dulbecco's modification of Eagle's medium alone (control) or medium containing insulin (1 3 10[25]-10[29] M) for 1 hour. Cell lysates were prepared and examined by immunoblotting for phosphorylated Akt (p-Akt). The densitometric intensity of p-Akt was measured and expressed as % control ± SEM, *n* = 3. *Inset*, Representative Western blot of p-Akt in human umbilical vein endothelial cells treated with 0.001 to 1 •M insulin (*A*). After treatment with insulin for 1 hour, the culture media above PAEC monolayers were collected and analyzed by chemiluminescence for NO concentration,

and monolayers were collected for protein determination. Each bar represents the mean NO concentration/mg protein expressed as % control ± SEM, *n* = 4 (*B*). *$p < .05$ versus control.

A). Insulin also increased PAEC NO production at all concentrations examined, including 1 and 5 nM, where enhanced NO production was not associated with detectable increases in Akt activation compared with control cells. In contrast to previous reports using human umbilical vein endothelial cells (HUVECs), [11] insulin-stimulated PAEC NO production was not dependent on insulin concentration in the range from 1 3 10 [25] to 10 [29] M. These increases in NO production were attributable to insulin because the buffer vehicle used for insulin delivery had no effect on PAEC NO production (Figure 2),

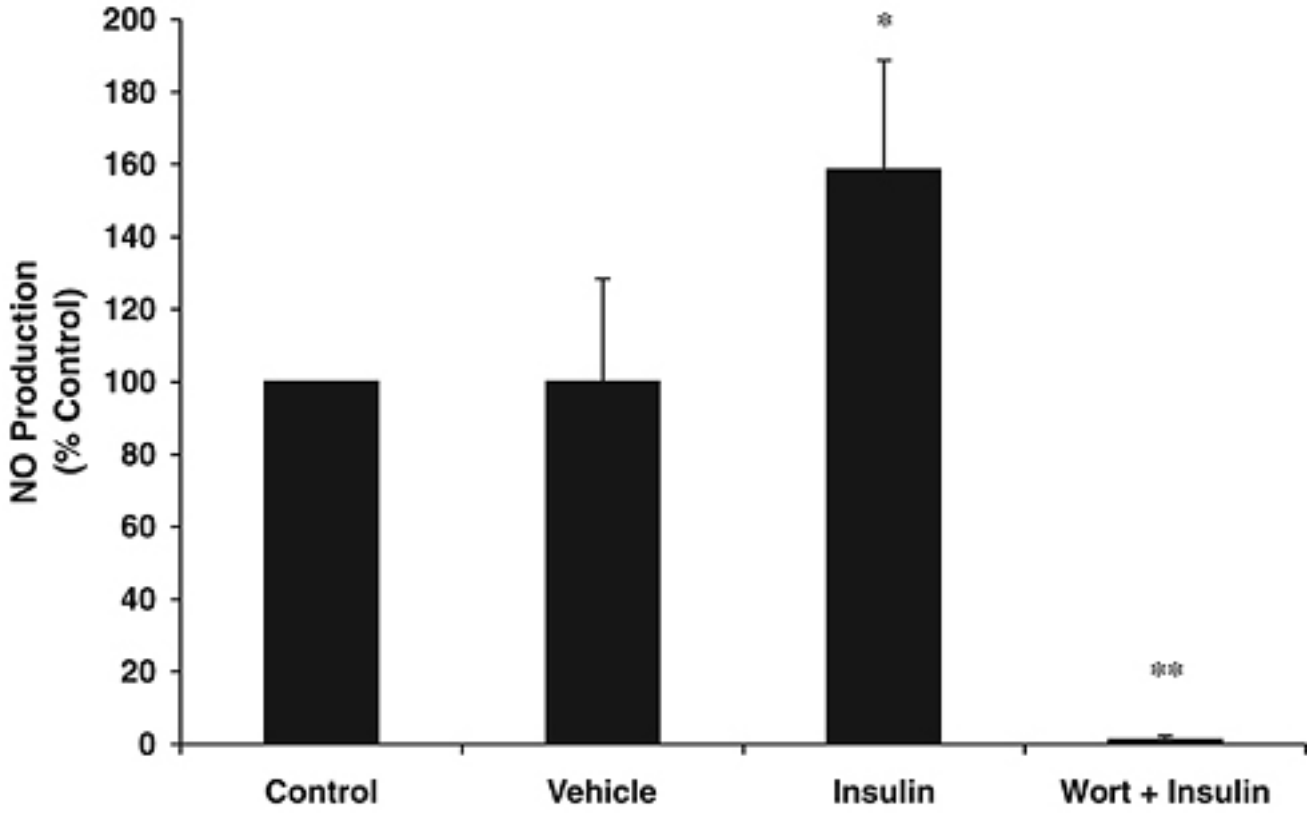


FIGURE 2 Wortmannin blocks insulin-stimulated nitric oxide (NO) production. Pulmonary artery endothelial cells (PAECs) were exposed to serum-free Dulbecco's modification of Eagle's medium alone (control), medium containing 25 •M HEPES, pH 8.0 (vehicle), or medium containing 1 •M insulin in vehicle. Selected PAECs were pretreated with wortmannin (100 nM) for 30 minutes and then incubated with medium containing 1 •M insulin and 100 nM wortmannin for 1 hour (wort + insulin). After 1 hour, media were collected for analysis of NO release, and monolayers were collected for protein determinations. Each bar represents NO concentration/mg protein expressed as % control ± SEM, *n* = 4. *$p < .05$ versus control; **$p < .05$ versus insulin.

and wortmannin, the PI-3 kinase inhibitor that links insulin receptor ligation to Akt activation, abolished insulin-stimulated NO production (see Figure 2).

Because eNOS represents a downstream target of Akt signaling in vascular endothelial cells, we examined the time course of Akt activation as well as eNOS phosphorylation relative to NO production in PAECs following treatment with 1 •M insulin. Insulin stimulated the rapid phosphorylation and activation of Akt that was temporally associated with increased p-eNOS serine 1179 (Figure 3

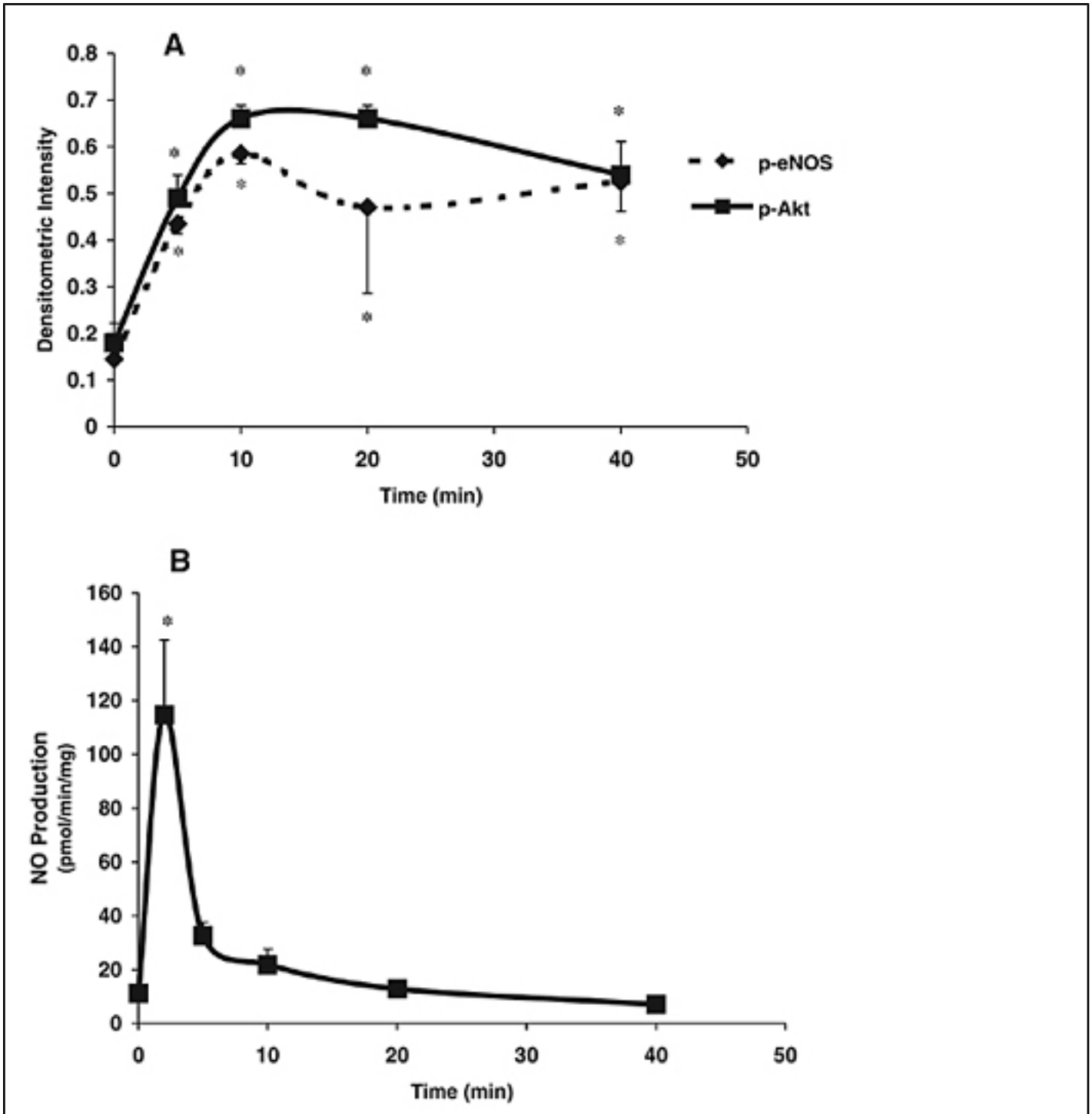


FIGURE 3 Time course of insulin-stimulated Akt activation, endothelial nitric oxide synthase (eNOS) phosphorylation, and nitric oxide (NO) production. Pulmonary artery endothelial cells (PAECs) were incubated in culture medium alone (0 minutes) or

culture medium containing 1 •M insulin. PAECs were collected at the indicated time points, and lysates were subjected to immunoblotting for phosphorylated Akt (p-Akt) and phosphorylated e-NOS (p-eNOS). Each point represents average densitometric intensity of p-Akt or p-eNOS ± SEM, *n* = 4 (*A*). At the indicated times, media were collected, and NO concentration was determined by chemiluminescence analysis. Each point represents average pmol NO/min/mg protein ± SEM, *n* = 4 (*B*). **p* < .05 versus 0 minute time point.

A). Associated with the rapid insulin-induced phosphorylation of Akt and eNOS, PAEC NO release increased after insulin treatment but followed a different time course (Figure 3 B). NO production dramatically increased within 2 minutes following insulin stimulation and thereafter rapidly decreased by 5 minutes with continued reductions in NO release 10 to 40 minutes following insulin stimulation.

The chemiluminescence technique for measuring endothelial NO release provides a cumulative index of NO release over the 1-hour period following insulin treatment. To confirm and more accurately assess the rapid changes in endothelial NO production following insulin stimulation as well as the impact of fatty acids on insulin-stimulated PAEC NO production, we employed a NO-specific electrode. PAECs were treated with 100 •M concentrations of 18:1 or 20:5 for 3 hours and then incubated in maintenance medium for 24 hours, as previously reported. [17] These conditions cause persistent alterations in PAEC fatty acid composition. [17,19,21] Insulin (1 •M) caused a rapid increase in NO-induced current detected by the electrode that was converted to NO concentrations using a standard calibration curve (Figure 4

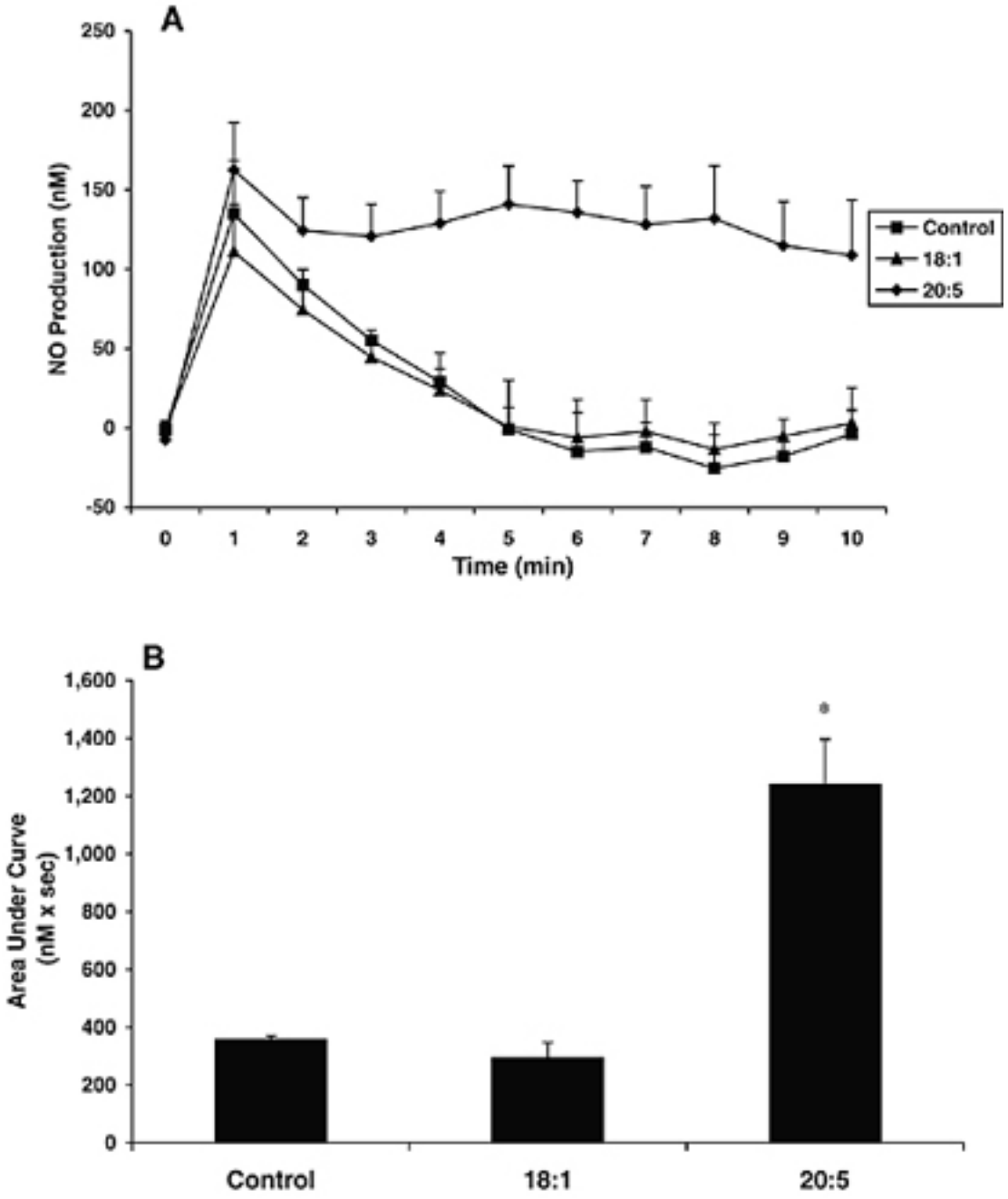
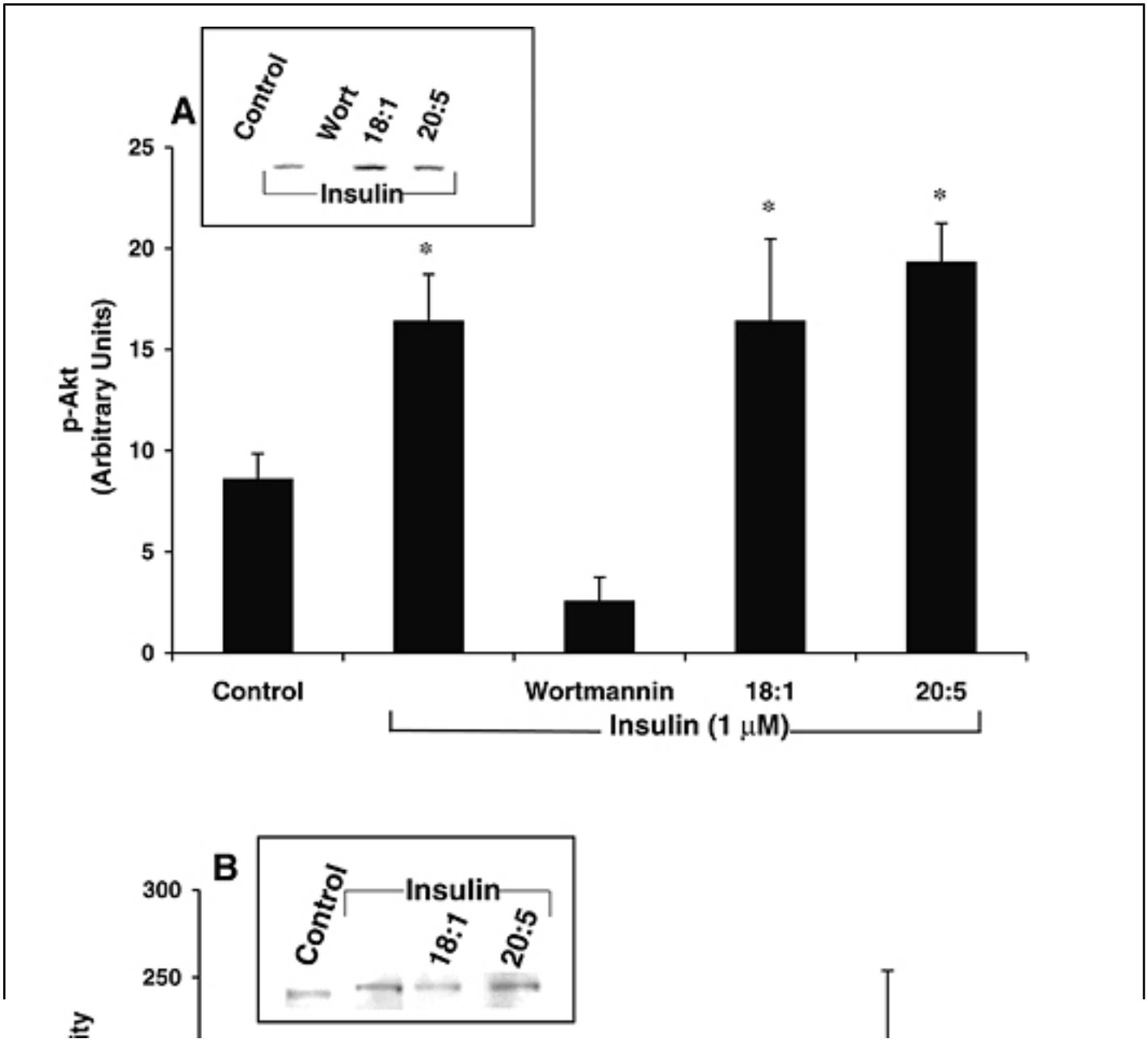


FIGURE 4 The effect of fatty acids on insulin-stimulated pulmonary artery endothelial cell (PAEC) nitric oxide (NO) production detected by an NO-specific electrode. PAECs were treated with 100 •M 18:1, 20:5 or an equivalent volume of ethanol vehicle (control) for 3 hours. Monolayers were then rinsed and incubated in standard culture medium overnight. PAECs were then placed in serum-free medium for 2 hours, and after obtaining a stable baseline, NO release was measured with a NO-specific electrode positioned 5 •M above the monolayer before and after stimulation with insulin (1 •M). Insulin was added at t = 0, and NO release was recorded for 10 minutes (*A*). Each point represents the average NO concentration (nM) ± SEM from three separate experiments. The data presented in *A* are expressed as average area under the curve ± SEM following insulin stimulation (*B*). **p* < .05 versus control and 18:1.

A). The magnitude of insulin-stimulated NO production was comparable in all treatment groups 1 minute following insulin addition (control = 6.03 ± 1.50, 18:1 = 4.97 ± 1.32, and 20:5 = 7.27 ± 1.33 picoamperes). However, NO released by control cells and 18:1-treated PAECs returned to baseline values 5 minutes after insulin treatment, whereas NO released from 20:5-treated PAECs remained elevated at 10 minutes following insulin addition. To assess total NO production over the 10- minute time course, we analyzed the area under the curve of the NO concentration tracings. As shown in Figure 4 B, the total area under the curve, reflecting the magnitude of NO release over time, was increased significantly in PAECs treated with 20:5 compared with control cells or 18:1-treated PAECs. The effect of insulin stimulation on PAEC NO release was inhibited by pretreatment with the nitric oxide synthase inhibitor N G-nitro-L-arginine methyl ester (L-NAME) (data not shown).

To further examine the mechanisms of fatty acid- induced alterations in insulin-stimulated PAEC NO production, Akt activation and eNOS phosphorylation were examined in fatty acid- and insulin-treated PAECs. PAECs were treated with fatty acids for 3 hours as described above and then stimulated with insulin for 1 hour either immediately after treatment with fatty acids or after incubation in maintenance medium without exogenous fatty acids for 24 hours. As illustrated in Figure 5,



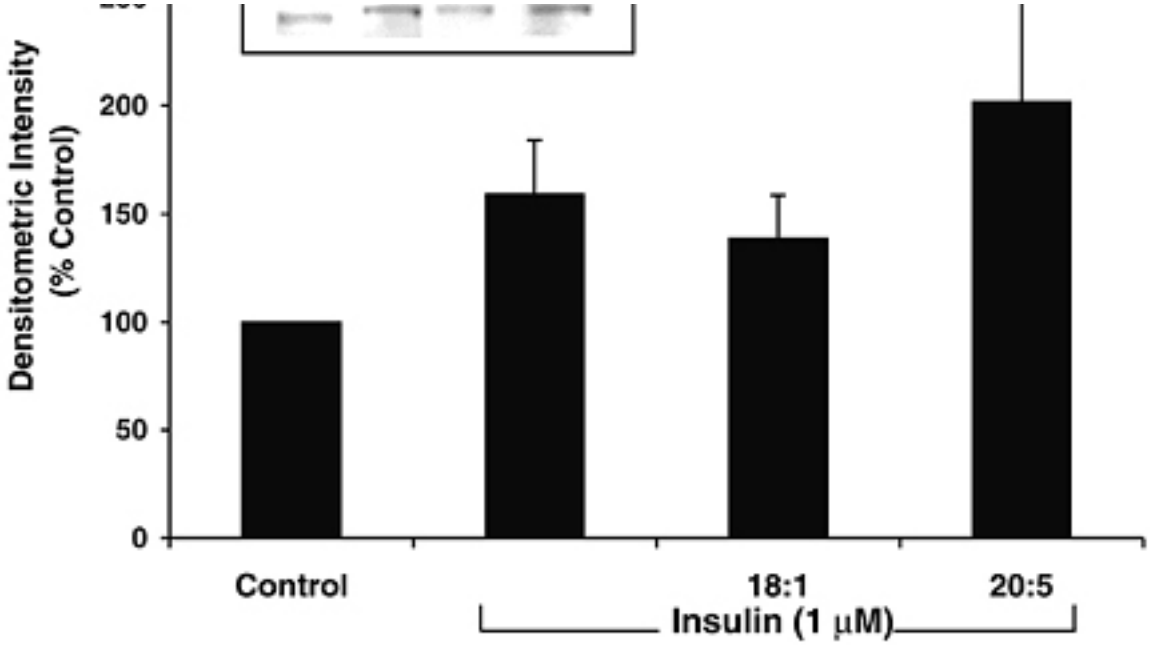


FIGURE 5 Fatty acid effects on insulin-stimulated Akt activation and endothelial nitric oxide synthase (eNOS) phosphorylation. Pulmonary artery endothelial cells (PAECs) were exposed to vehicle-containing culture medium (control) or culture medium containing 100 •M 18:1 or 20:5. After 3 hours, PAECs were washed with Hanks balanced salts solution and treated with fresh medium containing 1 •M insulin for 1 hour. PAECs were then harvested, and Akt activation was measured by immunoblotting for phosphorylated Akt (p-Akt) (*A*), and eNOS activation was measured by immunoblotting for phosphorylated e-NOS (p-eNOS) (*B*). Each bar represents the relative densitometric intensity of p-Akt ± SEM or p-eNOS as % control ± SEM, *n* = 4. **p* < .05 versus control. *Inset,* Representative Western blots of p-Akt (*A*) or p-eNOS (*B*).

PAEC Akt remained activated 60 minutes following treatment with 1 •M insulin consistent with the persistent but declining levels of p-Akt reported 40 minutes following insulin stimulation in Figure 3. Furthermore, consistent with Figure 2, insulin-stimulated Akt activation was blocked by treatment with wortmannin. Whether examined immediately after treatment with fatty acids (see Figure 5 A) or after incubation in maintenance medium without fatty acids for 24 hours (not shown), fatty acids did not alter insulin-stimulated Akt activation. Similarly, fatty acids failed to alter insulin-stimulated, Akt-mediated, phosphorylation of eNOS at serine 1179 either immediately after treatment with fatty acids (see Figure 5 B) or after incubation in maintenance medium without fatty acids for 24 hours (not shown). Fatty acid treatments did not alter overall eNOS or Akt protein expression immediately or 24 hours after treatment with fatty acids for 3 hours (data not shown).

DISCUSSION

Patients with diabetes, insulin resistance, and obesity have complex metabolic derangements that include increased levels of circulating free fatty acids. [2,5-7] Increased levels of circulating free fatty acids are associated with impaired insulin-induced, NO-mediated vasodilation and occur prior to the development of

overt hyperglycemia. [26,27] Experimentally, acute elevations of circulating free fatty acids caused by systemic infusion of exogenous fatty acids for 2 hours or by induction of endogenous lipolysis with somatostatin-induced insulinopenia also impaired endothelium-dependent vasodilation in normal subjects. [3] The role of fatty acids in altered vascular function is further supported by evidence that oleic acid infusions increased systemic blood pressure in rats. [28] These findings suggest that quantitative increases in serum fatty acids may represent an important metabolic variable that contributes to dysregulation of vascular endothelial function and NO production.

In addition to the quantitative increase in serum fatty acids, patients with type 2 diabetes also manifest qualitative alterations in the profile of serum and tissue fatty acids, [5-7] suggesting that specific types of fatty acids may be particularly deleterious for vascular endothelial function. This concept that specific fatty acids may differentially modulate vascular function is further supported by evidence that prolonged feeding with ω-3 fatty acid-rich diets enhanced eNOS activity in the arterial wall of rats, [10] lowered blood pressure, [29] and improved endothelium-dependent vasodilation responses in patients with type 2 diabetes. [30] These studies suggest that ω-3 fatty acids, in contrast to other fatty acyl families, may enhance vascular endothelial NO production and responsiveness. Thus, we hypothesized that specific fatty acids would differentially modulate vascular endothelial NO production. The mechanisms accounting for these effects of fatty acids on vascular endothelial function remain to be defined. A major pathway linking insulin receptor ligation to endothelial NO production is activation of PI-3 kinase coupled to downstream activation of Akt-mediated phosphorylation of eNOS on serine 1179. [11] Fatty acids disrupted the Akt pathway in smooth muscle cells in vitro, [16] and free fatty acid infusions caused insulin resistance in rat skeletal muscle by inhibiting insulin-stimulated PI-3 kinase activation in vivo. [31] Thus, we investigated if fatty acids altered vascular endothelial NO production through effects on insulin-stimulated activation of Akt and eNOS.

To investigate the effects of fatty acids on insulin-stimulated endothelial NO production, we took advantage of our well-characterized model in which PAECs are treated for 3 hours with exogenous fatty acids. We have previously reported that these conditions (1) produce free fatty acid-to-albumin ratios in the high pathophysiologic range, (2) cause no endothelial cytotoxicity or altered barrier function, [17-21] and (3) significantly alter endothelial fatty acid composition. [19,21] Using this protocol, PAECs were treated with ethanol vehicle (0.1%) or 100 •M oleic (18:1 ω-9) or eicosapentaenoic (20:5 ω-3) acid. These fatty acids were selected for study because they represent the ω-9 and ω-3 (fish oil) families of fatty acids, respectively, and previous studies have suggested that they may have opposite effects on endothelial function. Our model thereby challenges vascular endothelial cells with a "bolus" of a single fatty acid species somewhat analogous to increases in serum fatty acids that occur in the postprandial state. However, it must be emphasized that the high concentrations of 18:1 and 20:5 selected for study exceed the levels of these fatty acids in vivo, limiting the ability to extrapolate our in vitro findings to physiologic or pathophysiologic conditions in vivo.

Similar to previous reports investigating insulin-stimulated NO production in vascular endothelial cells, our results illustrate that insulin stimulates Akt activation and increases NO released from cultured PAECs into the surrounding medium. However, the characteristics of the insulin response in our cells differed from previous reports in several respects. In serum-starved HUVECs, maximal Akt activation occurred 30 to 60 minutes after insulin treatment, [32] whereas we observed maximal stimulation within 20 minutes following insulin treatment. Insulin was also reported to cause dose-dependent increases in NO release in serum-

starved, nonadherent HUVECs. [11] In contrast, in the current study, PAEC NO release was increased to a comparable degree by insulin concentrations between 1 nM and 10 •M. In fact, at the lower range of insulin concentrations studied, increased PAEC NO production was not associated with significant increases in Akt activation compared with PAECs lacking insulin stimulation. We speculate that the presence of serum in our studies activates Akt leading to increased eNOS phosphorylation and NO production in control cells. As previously reported in HUVECs, [11] the PI-3 kinase inhibitor wortmannin attenuated insulin-stimulated PAEC NO production, demonstrating that PI-3 kinase-mediated Akt activation participates in insulin-stimulated endothelial NO production. However, wortmannin lowered NO release in insulin-stimulated cells to levels below those in control cells without insulin treatment consistent with serum-induced basal Akt activation and eNOS phosphorylation. Finally, although longer insulin treatment regimens have been demonstrated to increase eNOS expression in bovine aortic endothelial cells, [33] PAEC eNOS protein expression was not increased after treatment with insulin for 1 hour.

The major observation of this study was that enriching endothelial cells in vitro with the ω-3 fatty acid 20:5 significantly increased NO release in response to insulin, whereas the ω-9 fatty acid 18:1 had no effect. Based on previous reports that fatty acids modulate PI-3 kinase and Akt signaling in vascular smooth muscle, we felt that the endothelial Akt pathway was a plausible target linking fatty acids and altered endothelial NO production. However, our results demonstrate that whereas insulin stimulates PAEC Akt activation, eNOS phosphorylation, and NO production by a wortmannin-sensitive pathway, the fatty acids selected for study do not mediate their effects on endothelial NO production through alterations in this pathway. The role of fatty acids in altered intracellular signaling remains an active area of interest in our laboratory, one that could modulate eNOS activity through several potential mechanisms. The signaling pathways regulating eNOS activity and endothelial NO production are complex and sometimes overlapping. For example, in addition to PI-3 kinase, insulin also activates mitogen-activated protein kinase (MAPK) pathways. [34] MAPK pathways phosphorylate eNOS at residues other than serine 1179 to inhibit eNOS activity. [35] For example eNOS contains multiple phosphorylation sites in addition to serine 1179, and increasing evidence has implicated the participation of a complex variety of kinase and phosphatase activities in the regulation of eNOS activity. [36] These phosphorylation events also participate in controlling the interactions between eNOS and other regulating proteins. [37] The intracellular targeting of eNOS to specific intracellular domains through cotranslational myristoylation and post-translational palmitoylation plays an important role in regulating enzyme activity, colocalizing eNOS with other signaling molecules, and optimizing enzyme activity. Evidence that exogenous fatty acids can modulate protein acylation [38] suggests that fatty acids could also modulate endothelial NO production through alterations in the intracellular localization of eNOS. Furthermore, it is possible that fatty acids, rather than altering the production of NO, may alter its degradation by modulating the intracellular production of superoxide. Superoxide combines with NO at diffusion-limited rates to form peroxynitrite, thereby reducing bioavailable NO. Thus, the experimental model described in the current report provides an initial approach to dissection of the complex mechanisms linking fatty acids to altered vascular endothelial NO production. Clarification of these mechanisms has the potential to contribute to a better understanding of the relationships between metabolic derangements associated with obesity, insulin resistance, and type 2 diabetes and enhanced risk of cardiovascular disease.

FOOTNOTES

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