Original Article

Free Fatty Acids Inhibit Insulin Signaling–Stimulated Endothelial Nitric Oxide Synthase Activation Through Upregulating PTEN or Inhibiting Akt Kinase

Xing Li Wang, Lin Zhang, Keith Youker, Ming-Xiang Zhang, Jian Wang, Scott A. LeMaire, Joseph S. Coselli, and Ying H. Shen

In metabolic syndrome, a systemic deregulation of the insulin pathway leads to a combined deregulation of insulin-regulated metabolism and cardiovascular functions. Free fatty acids (FFAs), which are increased in metabolic syndrome, inhibit insulin signaling and induce metabolic insulin resistance. This study was designed to examine FFAs' effects on vascular insulin signaling and endothelial nitric oxide (NO) synthase (eNOS) activation in endothelial cells. We showed that FFAs inhibited insulin signaling and eNOS activation through different mechanisms. While linoleic acid inhibited Akt-mediated eNOS phosphorylation, palmitic acid appeared to affect the upstream signaling. Upregulation of PTEN (phosphatase and tensin homolog deleted on chromosome 10) activity and transcription by palmitic acid mediated the inhibitory effects on insulin signaling. We further found that activated stress signaling p38, but not Jun NH_2-terminal kinase, was involved in PTEN upregulation. The p38 target transcriptional factor activating transcription factor (ATF)-2 bound to the *PTEN* promoter, which was increased by palmitic acid treatment. In summary, both palmitic acid and linoleic acid exert inhibitory effect on insulin signaling and eNOS activation in endothelial cells. Palmitic acid inhibits insulin signaling by promoting PTEN activity and its transcription through p38 and its downstream transcription factor ATF-2. Our findings suggest that FFA-mediated inhibition of vascular insulin signaling and eNOS activation may contribute to cardiovascular diseases in metabolic syndrome. *Diabetes* 55:2301–2310, 2006

From the Michael E. DeBakey Department of Surgery, Baylor College of Medicine, Houston, Texas.

Address correspondence and reprint requests to Dr. Ying. H. Shen, MS NAB 2010, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. E-mail: hyshen@bcm.edu; or Dr. Xing Li Wang, MS NAB 2010, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. E-mail: xlwang@bcm.edu.

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ATF, activating transcription factor; CREBP, cAMP-responsive element–binding protein; EBM, endothelial cell basic medium; eNOS, endothelial nitric oxide synthase; FFA, free fatty acid; HAEC, human aortic endothelial cell; JNK, Jun NH_2-terminal kinase; PDK, phosphoinositide-dependent kinase; PI, phosphatidylinositol; PIP_3, phosphatidylinositol-3.4,5-triphosphate.

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Metabolic syndrome, a cluster of metabolic and cardiovascular disorders, affects ~25% of adults aged >20 years and 50% of those aged >50 years (1,2). The major consequence of this syndrome is a marked increase of cardiovascular diseases. Understanding the mechanisms of cardiovascular diseases is important for the development of novel therapeutic strategies to reduce the morbidity and mortality of the disease.

The mechanisms for the development of vascular complications are complex. The key feature of the metabolic syndrome is global insulin resistance. Insulin has multiple biological actions not only on metabolism but also on cardiovascular functions. Insulin exerts desirable effects on the vasculature, primarily by enhancing endothelial nitric oxide (NO) synthase (eNOS) activation and expression and thus NO bioavailability (3,4), which in turn engenders a wide array of antiatherogenic actions. In an insulin-resistant state, a systemic deregulation of the insulin pathway leads to a combined deregulation of insulin-regulated metabolism and cardiovascular functions (5) and results in glucose intolerance and cardiovascular diseases. Therefore, vascular insulin resistance may play an important role in the development of cardiovascular diseases in metabolic syndrome.

One of the characteristic abnormalities in metabolic syndrome is high circulating free fatty acid (FFA) concentrations (6). Under insulin-resistant conditions, FFA concentrations are not only high in fasting status but also fail to be suppressed appropriately in response to insulin in the postprandial state (7). Thus, insulin target tissues, such as skeletal muscle and vasculature, are constantly exposed to high levels of FFAs. Clinical studies (8–11) have shown that high FFA levels are associated with impairment of endothelium-dependent vasodilation, high blood pressure, myocardial infarction, stroke, and sudden death. However, mechanisms of the FFA-induced vascular diseases are not completely understood. A number of researchers have shown that high circulating FFA concentrations impair insulin signaling, which leads to resistance to insulin-mediated glucose uptake and utilization (12,13). We hypothesize that high levels of FFAs cause vascular diseases by impairing vascular insulin signaling.

To test this hypothesis, we examined the effects of FFAs on insulin signaling and eNOS activation in cultured endothelial cells. We observed that FFAs inhibited both basal and insulin-stimulated eNOS activation. FFAs also

inhibited the insulin signaling pathway through different mechanisms. Our study suggests that FFA-induced inhibition of insulin signaling and eNOS activation may play a critical role in the development of cardiovascular diseases in metabolic syndrome.

RESEARCH DESIGN AND METHODS

siRNAs and antibodies. PTEN (phosphatase and tensin homolog deleted on chromosome 10) siRNA (Cell Signaling Technology, Beverly, MA), p38 siRNA, Jun NH_2-terminal kinase (JNK) siRNA, and activating transcription factor (ATF)-2 siRNA (Ambion, Austin, TX) and various antibodies (Cell Signaling Technology) were used in this study.

Preparation of fatty acid–albumin complexes. Saturated palmitic acid, polyunsaturated linoleic acid, and monounsaturated oleic acid were used in this study. Lipid-containing media were prepared by conjugation of FFAs with BSA using a modified method described by Svedberg et al. (14). Briefly, FFAs were first dissolved in ethanol at 200 mmol/l and then combined with 10% FFA-free low endotoxin BSA to concentrations of 1–10 mmol/l. The pH of all solution was adjusted to ~7.5, and the stock solutions were filter-sterilized and stored at −20°C. Control solution containing ethanol and BSA was similarly prepared. Fresh working solutions were prepared by diluting stock solution (1:10) in 2% FCS–endothelial cell basic medium (EBM) or 0.5% FCS-EBM as appropriate. The final 1% BSA was consistent in all FFA medium, while the FFA-to-BSA ratio varied with the FFA concentrations.

FFA treatment and insulin stimulation. Primary human aortic endothelial cells (HAECs) (Cell Applications, San Diego, CA) were cultured in endothelium growth medium-2 (Cambrex, East Rutherford, NJ) containing EBM, hydrocortisone, fibroblast growth factor-B, vascular endothelial growth factor, IGF-1, epidermal growth factor, ascorbic acid, gentamycin amphotericin B-1000, heparin, and 2% fetal bovine serum. Cells cultured up to five passages were first grown to confluence before exposure to FFAs (0.1–1 mmol/l) for 24 h. For insulin stimulation, confluent cells were exposed to FFAs (0.2–0.8 mmol/l) for 24 h and then starved in 0.5% FCS EBM containing FFAs (0.2–0.8 mmol/l) for 6 h before being stimulated with 50–100 nmol/l insulin for 30 min.

siRNA-induced gene silencing. Silencing PTEN, JNK, p38, and ATF-2 expression was achieved using the siRNA technique. Transfection of HAECs was carried out using LipofectAMINETM 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Transfected cells were then treated with FFAs and/or insulin for designed concentrations and time periods.

eNOS activity assay. The catalytic activity of eNOS was determined by the conversion of L-[3H] arginine to L-[3H] citrulline using the Nitric Oxide Synthase Assay kit from Calbiochem. Briefly, confluent cells were exposed to FFAs and then starved for 6 h before being stimulated with 100 nmol/l insulin for 30 min. The treated cells were washed, harvested in PBS/EDTA, and lysed in homogenization buffer. A total of 25 μg protein lysate was incubated with 1 μCi L-[3H] arginine in the presence of 75 μmol/l calcium at room temperature for 30 min. Reaction was stopped, and the converted L-[3H] citrulline was separated from the unreacted L-[3H] arginine by iron exchange resin. The radioactivity of L-[3H] citrulline was determined by liquid scintillation counting. Data are expressed as generation of L-[3H] citrulline (percent of control).

Western blot analysis. Treated cells were collected and lysed as previously described (15). Fifteen micrograms of protein per lane were separated by SDS–polyacrylamide gels and transferred to polyvinylidine fluoride membranes. The membrane was blocked with the primary antibody, washed, and then incubated with the secondary horseradish peroxidase–labeled antibody. Bands were visualized with enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ). For densitometry graphs, phosphoprotein bands and total protein bands were quantified by densitometry using the Quantity One imaging program (Bio-Rad, Hercules, CA). The relative levels of phosphoprotein were quantified after being normalized to the total protein and expressed as the percentage of the control.

Real-time quantitative RT-PCR. Total RNA from treated cells was extracted with Trizol (Invitrogen) according to the manufacturer's protocol. The mRNAs were reverse transcribed into cDNAs using iScript cDNA synthesis kit (Bio-Rad). Real-time PCR was performed using iCycler iQ real-time PCR detection system (Bio-Rad). Primers were designed through Beacon Designer 2.0 software. The primers for human *PTEN* were forward: 5′-CAA GAT GAT GTT TGA AAC TAT TCC AAT G-3′ and reverse: 5′-CCT TTA GCT GGC AGA CCA CAA-3′. The mRNA levels were acquired from the value of threshold cycle (C_t) of *PTEN* and normalized against C_t of β-actin. The data shown was representative of three separate experiments.

Cellular phospholipids analysis. Treated HAECs were lysed in 0.5 mol/l trichloroacetic acid. Phospholipids were first extracted with one for one (vol/vol) methanol:chloroform. The organic phase was collected and washed twice with one for one (vol/vol) methanol:HCl. The organic phase was then dried to a small volume. Phospholipids were spotted onto hybond nitrocellulose. Phosphatidylinositol-3,4,5-triphosphate (PIP_3) was detected using anti-PIP_3 monoclonal antibody (Echelon Research Laboratories, Salt Lake City, UT). The specificity of the antibody was tested with PIP_3 and PIP_3 standards (Echelon Research Laboratories).

Chromatin immunoprecipitation assay. The chromatin immunoprecipitation (ChIP) assay was performed using the histone H3 ChIP assay kit according to the manufacturer's protocol (Upstate). In brief, treated HAECs were first incubated with 1% formaldehyde at 37°C for 15 min to cross-link DNA-protein complexes. Cells were then rinsed, harvested, and lysed. Cell lysates were sonicated and centrifuged to produce chromatin fragments of 300–1000 bp in length. The supernatants were precleared with a mixture of salmon sperm DNA/protein A/protein G, followed by immunoprecipitation with anti-ATF2 antibody–protein A-agarose slurry (with IgG serving as the negative control). The immunocomplex beads were then washed sequentially with low-salt wash buffer, high-salt wash buffer, LiCl wash buffer, and Tris-EDTA buffer. The immunocomplex was eluted with elution buffer (100 mmol/l NaHCO3, 1% SDS). The eluted immunocomplex and the inputs were incubated with 200 mmol/l NaCl at 65°C overnight to reverse the cross-link, followed by incubation with proteinase K to digest the remaining proteins. The DNA was recovered by the phenol/chloroform/isoamyl alcohol extraction. The immunoprecipitated DNA was used as a template for PCR. The PCR products were separated by 1.2% agarose gel. The primers used for the AFT-2 binding site in the 5′-flanking region of the human *PTEN* were forward primer 5′-TCG ACT ACT TGC TTT GTA GA-3′ and reverse primer 5′-TTT ACA GCC CCG ATT GGG CT-3′.

Statistical analysis

All quantitative variables are presented as means ± SE from three separate experiments. We compared the differences of three groups or more using one-way ANOVA. To evaluate the interactive effects of two factors, e.g., the siRNA-specific inhibition together with the treatment of FFAs, two-way ANOVA was used for the analyses. Two-tailed $P < 0.05$ was considered statistically significant.

RESULTS

FFAs inhibited basal and insulin-stimulated eNOS activation. To determine whether FFAs have any effect on eNOS activity, we examined the eNOS activation by measuring its phosphorylation at Ser^{1177}. The HAECs cultured in the endothelium growth medium (with serum and growth factors that maintain the basal activation of eNOS) were treated with palmitic, linoleic, and oleic acids. As shown in Fig. 1*A*, inhibition of eNOS phosphorylation was observed with palmitic and linoleic acids but not with oleic acid treatment (data not shown) in a dose-dependent manner. We next examined the FFAs' effects on insulin-stimulated eNOS phosphorylation. HAECs, preincubated with palmitic and linoleic acids, were starved for 6 h before being stimulated with insulin. As shown in Fig. 1*B*, insulin stimulated eNOS phosphorylation and this upregulation of eNOS phosphorylation was inhibited by palmitic or linoleic acids treatment. We further assessed the FFAs' effects on eNOS enzyme activity by measuring the conversion of L-[^{3}H] arginine to L-[^{3}H] citrulline in the treated cells. Consistent with eNOS phosphorylation/activation, eNOS activity induced by insulin was also repressed by palmitic acid treatment (Fig. 1*C*). Similar findings were observed with the linoleic acid treatment (data not shown). Thus, both palmitic and linoleic acids can inhibit basal as well as insulin-stimulated eNOS phosphorylation and hence inactivate eNOS in endothelial cells.

FFAs inhibited insulin signaling pathway. Since insulin activates eNOS through Akt, we then investigated whether FFAs have any effects on basal and insulin-stimulated Akt activation, which was monitored by phosphorylation at Ser^{473} and Ser^{308} sites. We observed that while linoleic acid had no effects on Akt phosphorylation at Ser^{473}, palmitic acid inhibited Akt phosphorylation at both Ser^{473} and Ser^{308} sites in a dose-dependent fashion (Fig. 2*A*). Palmitic acid also inhibited insulin-stimulated

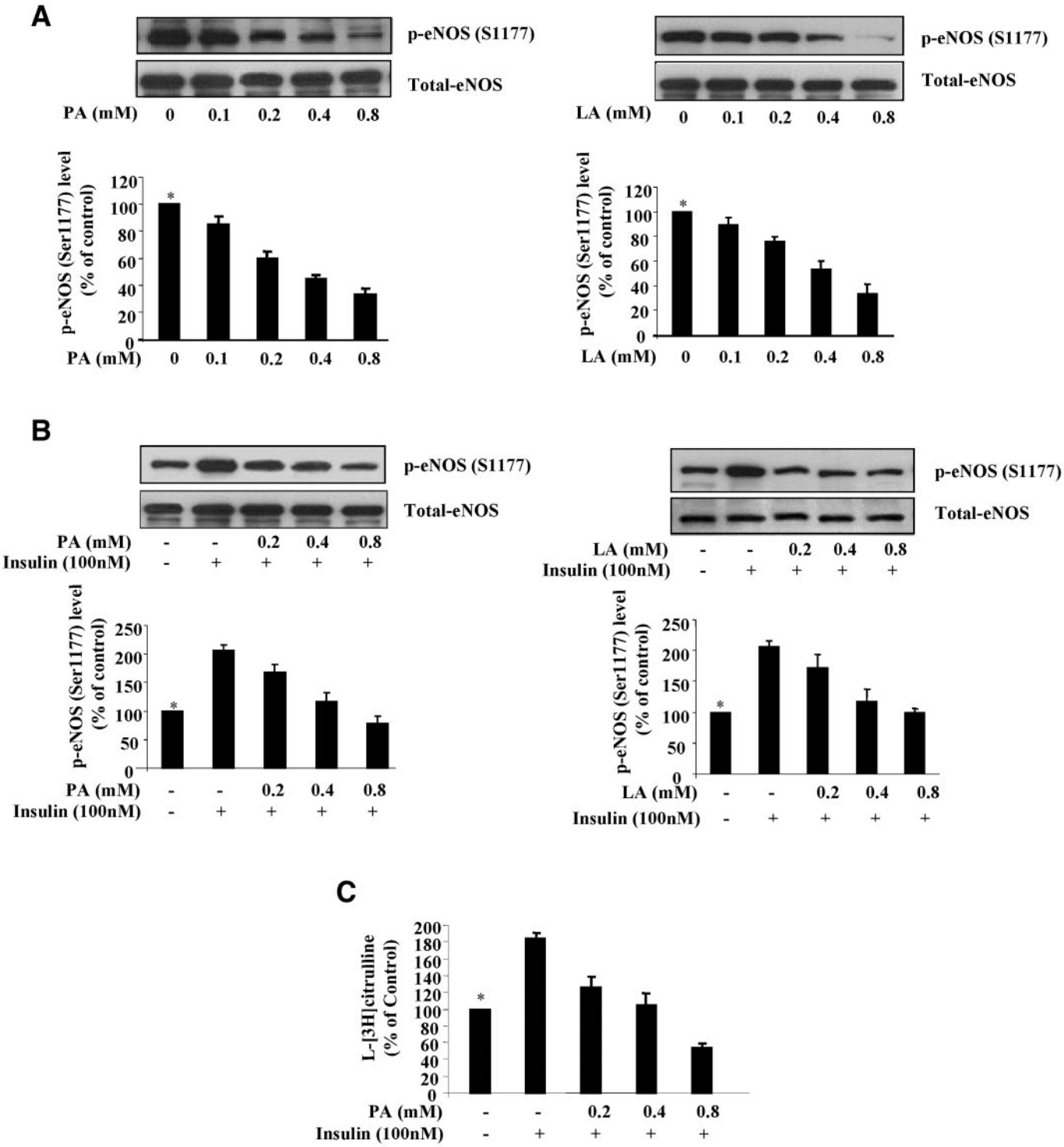

FIG. 1. FFAs inhibited basal and insulin-stimulated eNOS phosphorylation in HAECs. *A*: FFAs inhibited basal eNOS phosphorylation. Phosphorylated and total eNOS were detected using specific antibodies. *B*: FFAs inhibited insulin-stimulated eNOS phosphorylation. HAECs were preincubated with palmitic acid and starved before insulin stimulation. *C*: Palmitic acid inhibited insulin-stimulated eNOS activity. The eNOS activity in the cell lysate was measured. Data represent the means ± SE ($n = 3$, $P < 0.001$).

Akt phosphorylation at Ser^{473} (Fig. 2*B*), suggesting that palmitic acid may block upstream signaling. For the rest of this study, we focused on palmitic acid's effects. Phosphoinositide-dependent kinases (PDK1 and PDK2) are direct Akt upstream kinases, which phosphorylate Akt on Thr^{308} and Ser^{473}, respectively. Therefore, the phosphorylation of PDK1 in palmitic acid–treated cells was examined. As shown in Fig. 2*C*, insulin-stimulated PDK1 phosphorylation at Ser^{241} was also inhibited by palmitic acid treatment. Collectively, palmitic acid inhibited insulin-stimulated phosphorylation of PDK1 and Akt, indicating possible impairment of upstream insulin signaling, whereas linoleic acid had no effects on Akt phosphorylation.

Palmitic acid upregulated PTEN activity and transcription. Akt phosphorylation/activation is regulated by PIP_3, which recruits PDKs and Akt to the plasma mem-

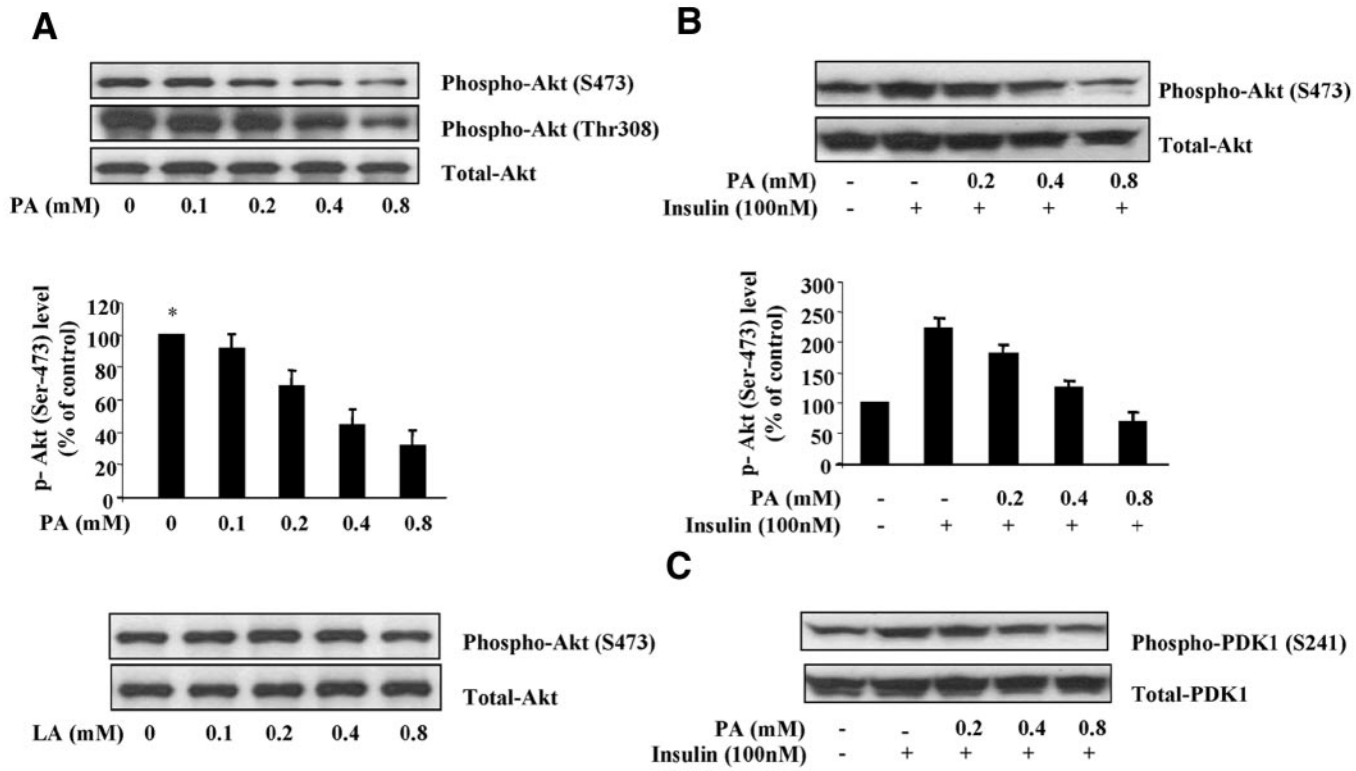

FIG. 2. FFAs inhibited the Akt pathway in HAECs. *A*: Palmitic acid inhibited basal Akt phosphorylation. Phosphorylated and total Akt were measured. *B*: Palmitic acid inhibited insulin-stimulated Akt phosphorylation. HAECs were preincubated with palmitic acid and starved before stimulated with insulin. *C*: Palmitic acid inhibited insulin-stimulated PDK1 phosphorylation. PDK1 phosphorylation and total PDK1 were measured. Data represent the means ± SE ($n = 3$, $P < 0.001$).

brane, allowing Akt regulatory residues to be more accessible to PDKs and to be phosphorylated (16). PIP_3 levels are tightly regulated by phosphatidylinositol (PI) 3K and phosphatases, such as PTEN, which has been shown to antagonize PI3K/Akt signaling by dephosphorylating PIP_3 (17). To investigate the upstream molecules that were targeted by palmitic acid, we examined changes in PTEN. Since PTEN can be activated by dephosphorylation at the PTEN COOH-terminal tail (18), the phosphorylation of PTEN at $Ser^{380}/Thr^{382}/Thr^{383}$ was measured from the palmitic acid–treated cell lysate. As demonstrated in Fig. 3*A*, palmitic acid induced PTEN dephosphorylation, indicating the activation of PTEN, in a dose-dependent manner. We further examined palmitic acid's effects on PTEN expression. Recent studies have shown that dephosphorylation at the PTEN COOH-terminal tail not only activates PTEN but also promotes PTEN degradation (19). We expected that the total PTEN level might be decreased by its activation in palmitic acid–treated cells. However, our data showed that the total PTEN protein level was unchanged by the palmitic acid treatment (Fig. 3*A*), suggesting that increased PTEN production may have occurred to compensate for the protein degradation. We measured the PTEN mRNA level in palmitic acid–treated cells. As shown in Fig. 3*B*, PTEN mRNA levels increased with the palmitic acid treatment, indicating that palmitic acid can induce PTEN expression at mRNA level, either by increasing transcription or decreasing mRNA turnover. In addition, consistent with the increased PTEN activation, both basal and insulin-induced PIP_3 levels were decreased by the palmitic acid treatment (Fig. 3*C*). Thus, palmitic acid induces PTEN transcription and promotes its activation, which leads to decreased PIP_3 levels.

PTEN was involved in palmitic acid–induced impairment of the Akt pathway. We recently showed that PTEN was involved in the inactivation of Akt and eNOS induced by resistin (15). We therefore investigated whether upregulated PTEN was also responsible for the palmitic acid–induced inhibition of Akt and eNOS. As shown in Fig. 4, insulin stimulated Akt and eNOS phosphorylation, which were suppressed by the palmitic acid treatment. PTEN siRNA specifically suppressed PTEN expression and reversed the palmitic acid–induced inhibition of Akt and eNOS phosphorylation. These findings suggest that PTEN activation is directly involved in palmitic acid–induced inhibition of the insulin pathway and eNOS activation in endothelial cells.

Involvement of activated JNK and p38 stress pathways in palmitic acid–induced inhibition of Akt and eNOS. We next investigated the mechanisms by which palmitic acid induced the expression of PTEN and inhibition of Akt and eNOS. Mitogen-activated protein kinase kinases JNK and p38 can be activated in response to a wide variety of stimuli and are involved in stress responses by regulating many cellular functions. Activated JNK and p38 are also implicated in the development of insulin resistance and cardiovascular diseases (20,21). Since FFAs increase ceramide content, which has been shown to activate MLK3, the upstream kinase of JNK and p38 pathways, we examined the activation of these pathways by palmitic acid treatment. JNK and p38 can be activated by phosphorylation at Thr^{183}/Tyr^{185} and Thr^{180}/Tyr^{182}, respectively. As shown in Fig. 5*A*, palmitic acid induced both JNK and p38 phosphorylation, which were paralleled by a dose-dependent decrease in eNOS phosphorylation. We next explored the involvement of JNK and p38 in palmitic

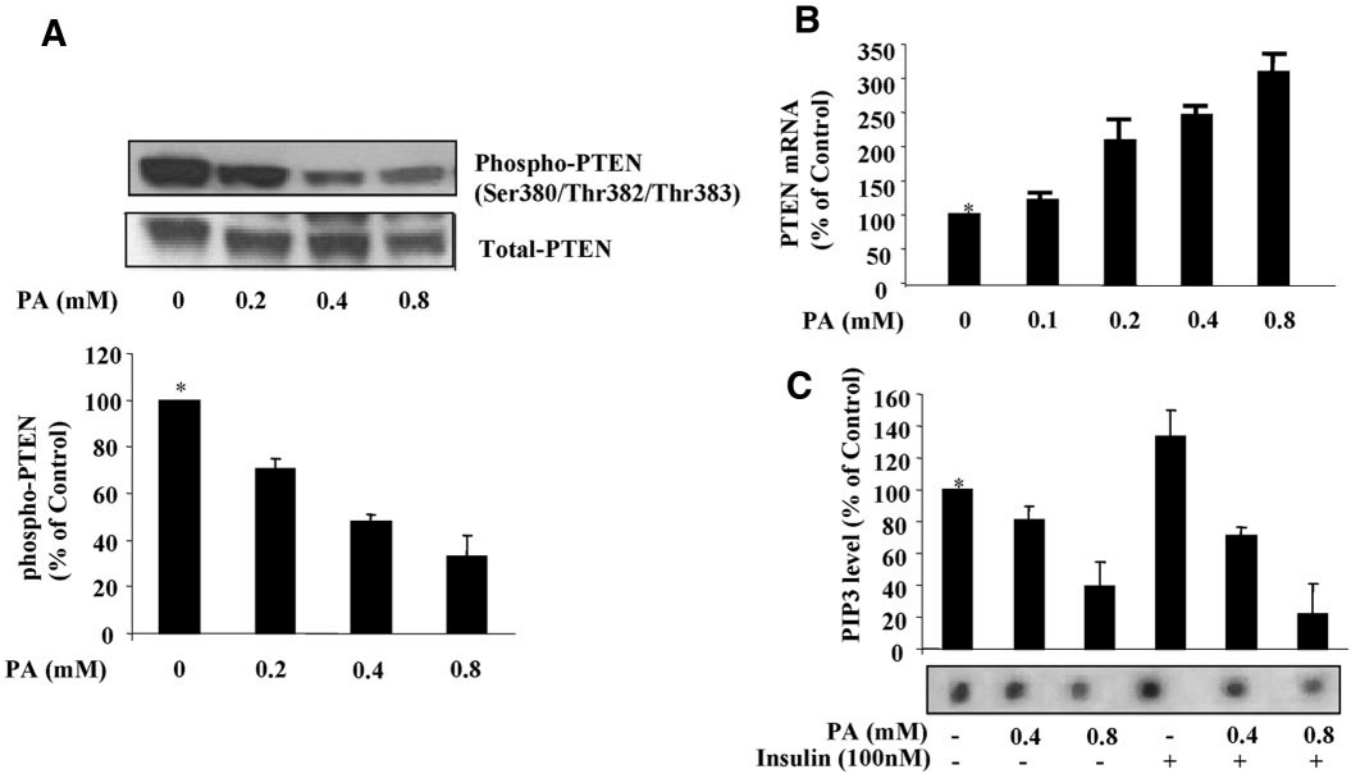

FIG. 3. Upregulation of PTEN activity and expression by palmitic acid in HAECs. *A*: Palmitic acid induced dephosphorylation of PTEN. Phosphorylated PTEN and total PTEN were measured. *B*: Palmitic acid increased PTEN mRNA. Results are expressed as percentage of control. *C*: Palmitic acid decreased PIP_3 levels. HAECs were treated with palmitic acid and starved before insulin treatment. Phospholipids were extracted, and PIP_3 was detected with anti-PIP_3 antibody. Data represent the means ± SE ($n = 3$, $P < 0.001$).

acid–induced inhibition of insulin signaling by blocking their expressions with specific siRNAs, which have previously been shown to specifically inhibit JNK or p38 expression (15). As shown in Fig. 5*B*, palmitic acid–induced inhibition of Akt and eNOS phosphorylation was completely reversed by the JNK siRNA and p38 siRNA, indicating that both pathways are involved in palmitic acid–induced inhibition of Akt and eNOS. Taken together, these data suggest that both JNK and p38 pathways are activated by palmitic acid and are involved in mediating the inhibitory effects on insulin signaling and eNOS activation in endothelial cells.

Activated p38 stress pathway mediated palmitic acid–induced PTEN transcription. As demonstrated above (Figs. 3 and 4), palmitic acid inhibited insulin signaling by upregulating PTEN activity. Palmitic acid also appeared to counterbalance the activation-induced PTEN degradation by promoting PTEN transcription (Fig. 3*B*). To investigate whether JNK and p38 are involved in these processes, we examined the effects of JNK and p38 on PTEN expression by suppressing JNK and p38 expression. As shown in Fig. 6*A*, in p38 siRNA-treated cells, PTEN protein levels were decreased along with the decreased p38 expression. In contrast, JNK siRNA had minimal effects on basal PTEN expression, even though it efficiently suppressed JNK expression. These results suggest that p38, rather than JNK, may be involved in upregulating PTEN expression. Additionally, as shown in Fig. 6*B*, while basal PTEN mRNA was slightly decreased with the increased amount of p38 siRNA, palmitic acid–induced PTEN transcription was significantly repressed by the p38 siRNA, indicating that p38 is involved in palmitic acid–induced upregulation of PTEN mRNA. Taken together, these

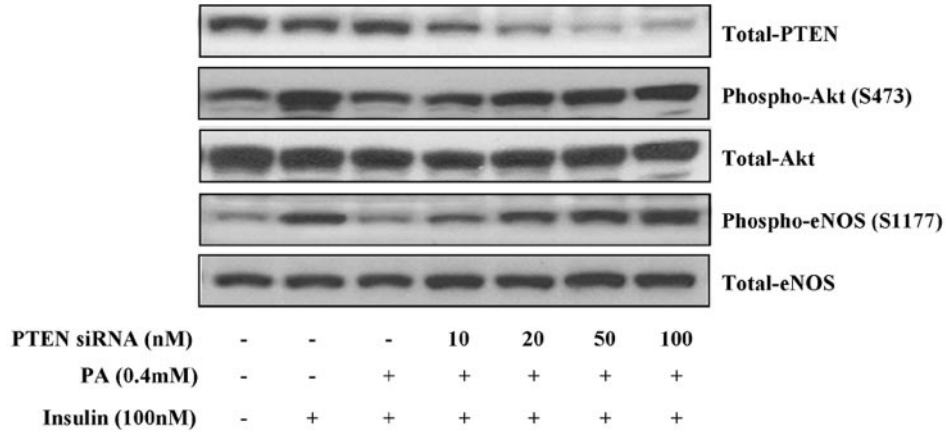

FIG. 4. Involvement of PTEN in palmitic acid–induced impairment of insulin signaling. HAECs were transfected with PTEN siRNA followed by palmitic acid treatment. The cells were then starved before insulin stimulation. Representative blots from three experiments are shown.

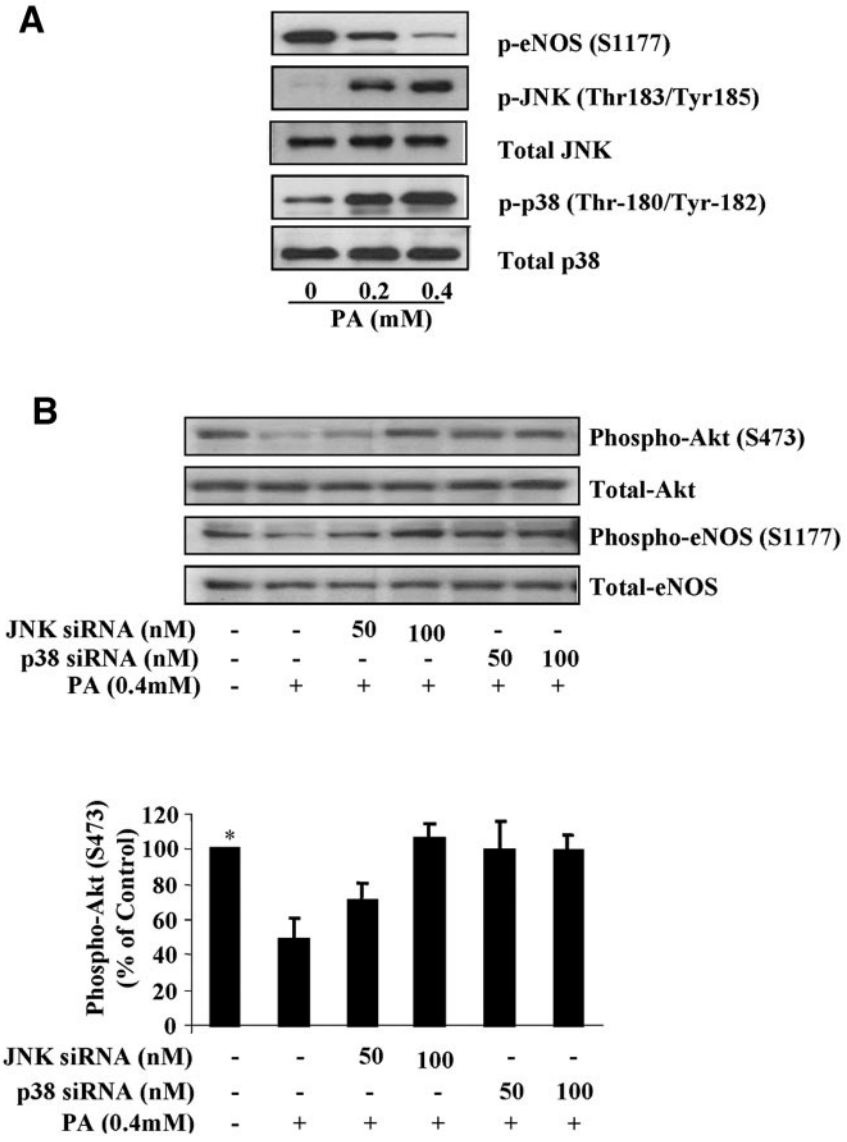

FIG. 5. Involvement of JNK and p38 in palmitic acid–induced impairment of Akt pathway. *A*: Activation of JNK and p38 was paralleled with decreased eNOS activation. Phosphorylated p38, JNK, and eNOS were detected. *B*: Involvement of JNK and p38 in palmitic acid–induced impairment of Akt and eNOS activation. HAECs were transfected with JNK siRNA or p38 siRNA, followed by palmitic acid treatment. The data shown are the means ± SE ($n = 3$) and compared by ANOVA; the effects of JNK siRNA and p38 siRNA on palmitic acid–induced inhibition of phospho-Akt and phospho-eNOS were significant ($P < 0.05$).

data suggest that although both JNK and p38 pathways are involved in mediating palmitic acid–induced inhibition of insulin signaling and eNOS activation, mechanisms may differ. While JNK may inhibit the insulin pathway through PTEN-independent mechanisms, p38 impairs insulin signaling by upregulating PTEN activity and transcription.

p38-target transcriptional factor ATF-2 mediated palmitic acid–induced PTEN upregulation. p38 is involved in gene regulation by activating an array of transcriptional factors such as p53, Max, Myc, ELK1, monocyte-specific enhancer factor 2, cAMP-responsive element–binding proteins (CREBPs), and ATFs (22). To further investigate the mechanisms by which p38 mediates palmitic acid–induced PTEN mRNA upregulation, attempts were made to identify p38 target transcriptional factors that could be activated by the palmitic acid treatment. Compared with other transcriptional factors tested, such as c-Myc, ELK1, and monocyte-specific enhancer factor 2 (data not shown), the phosphorylation of ATF-2 was strongly induced by the palmitic acid treatment in a dose-dependent manner (Fig. 7*A*), indicating that ATF-2 might be a target of the activated p38. ATF-2 belongs to the ATF/CREBP family of transcription factors. It binds to the target DNA either as homodimers or as heterodimers with CREBP or AP-1 transcription factors. These dimers bind to the ATF/CREBP site 5′-TGACGTCA-3′ and the AP-1 site 5′-TGACTCA-3′ (23). The promoter region in the *PTEN* gene contains two putative ATF half-binding sites: site 1 cct*TGACG*ggtggg and site 2 ggc*TGACG*gccatt (Fig. 7*B*). Using chromatin immunoprecipitation assays, we previously showed that ATF-2 bound to these sites in the *PTEN* promoter in vivo (15). Here, we showed that ATF-2 binding to site 1 of the *PTEN* promoter was significantly increased by the palmitic acid treatment (Fig. 7*B*), indicating that p38 target transcription factor ATF-2 may be involved in the palmitic acid–induced PTEN transcription. Indeed, we observed that basal PTEN mRNA was slightly decreased with the increased amount of ATF-2 siRNA, and palmitic acid–induced PTEN mRNA was significantly repressed by the ATF-2 siRNA in a dose-dependent manner (Fig. 7*C*), further suggesting that ATF-2 is involved in the palmitic acid–induced upregulation of PTEN transcription.

DISCUSSION

In the present study, we have shown that palmitic and linoleic acids can inhibit insulin-stimulated eNOS activation. However, different FFAs inhibit eNOS activation

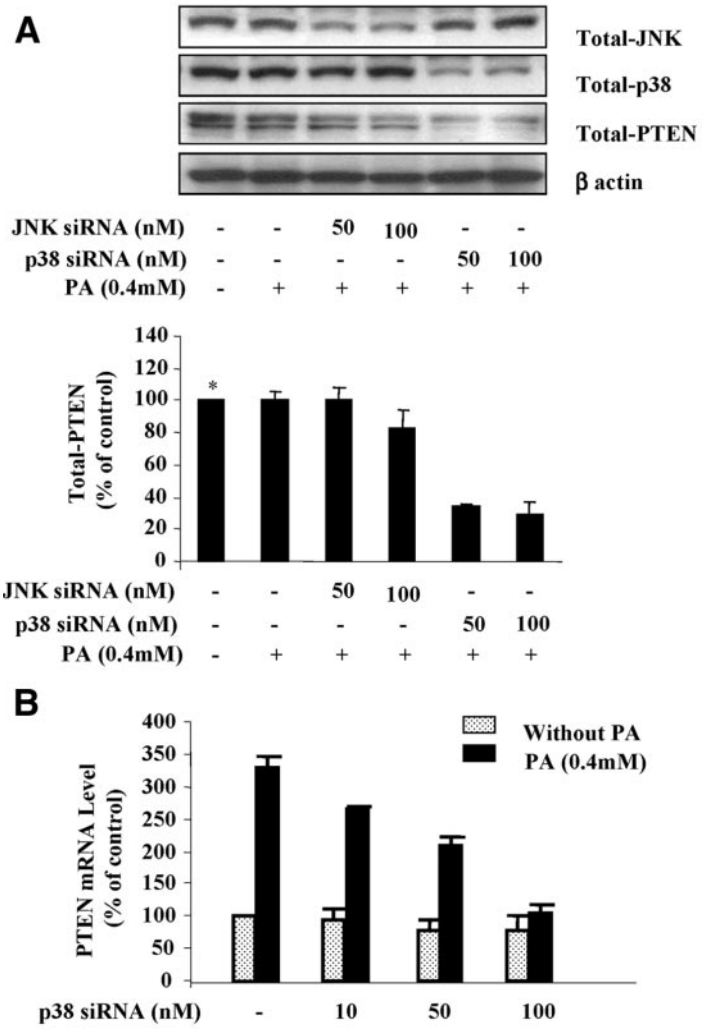

FIG. 6. p38 was involved in palmitic acid–induced PTEN expression. *A*: Role of p38 in PTEN expression in palmitic acid–treated cells. HAECs were transfected with scrambled JNK or p38 siRNAs followed by the palmitic acid treatment. *B*: Involvement of p38 in palmitic acid–induced PTEN mRNA upregulation. HAECs were transfected with p38 siRNA followed by the treatment with palmitic acid. The data shown are means ± SE ($n = 3$) and compared by ANOVA, with a significant effect of p38 siRNA on PTEN protein and mRNA ($P < 0.05$).

through different mechanisms. While linoleic acid may directly inhibit Akt-mediated eNOS phosphorylation, palmitic acid appears to affect upstream signaling. Importantly, our data show that palmitic acid can induce activation and transcription of PTEN, which appears to mediate palmitic acid's inhibitory effects on the insulin signaling pathway and eNOS activation. Moreover, activation of p38 by palmitic acid upregulates PTEN transcription through activating transcriptional factor ATF-2 and promoting its binding to the *PTEN* promoter. Taking these findings together, we propose a pathway of the FFA-induced inhibition of eNOS activation (Fig. 7*D*).

FFAs have been shown to have a strong linear correlation with insulin resistance (24). FFAs have also been shown to induce inflammation and impair vascular reactivity (25,26), promote endothelial apoptosis (27), and modulate microvascular function (28). Clinical studies have shown that high FFA concentrations are significantly associated with higher blood pressure (29), endothelial dysfunction (8,9), myocardial infarction, cardiovascular disease (10), and sudden death (11). Our study shows that palmitic and linoleic acids can inhibit insulin-stimulated eNOS phosphorylation/activation and activity, consistent with one recent report (30) that demonstrated that FFA induced inhibition of eNOS through activation of IκB kinase β. Since NO is a key regulator in endothelial functions, eNOS inhibition by FFAs could provide a mechanism for endothelial dysfunction in metabolic syndrome. We suggest that a sustained elevation of certain FFAs, including palmitic and linoleic acids, may be a major risk factor for cardiovascular disease in metabolic syndrome.

FFAs have been shown to inhibit insulin signaling in other insulin target tissues such as liver, adipose, and skeleton muscles. We show that FFAs also inhibit insulin signaling in endothelial cells, supporting a notion that in metabolic syndrome, there is a systemic deregulation of the PI3K pathway leading to a combined deregulation of insulin-regulated metabolism and cardiovascular functions (31). However, palmitic and linoleic acids inhibit insulin-stimulated eNOS activation with different effects on Akt activation. Akt inducing eNOS phosphorylation at Ser^{1177} requires Akt phosphorylation at Ser^{473} and Thr^{308} and the formation of the Hsp90/CaM/Akt/eNOS complex. In quiescent endothelial cells, eNOS can be found in a complex with caveolin-1, a negative regulator. Upon stimulation, eNOS is released from the caveolin inhibitory clamp and binds to Hsp90, which facilitates the docking of Akt and phosphorylation of eNOS. Thus, the Hsp90/CaM/Akt/eNOS complex is necessary for eNOS phosphorylation at Ser^{1177} and for NO release (32,33). Linoleic acid may inhibit Akt-mediated eNOS phosphorylation by changing Akt and/or eNOS location, preventing or disturbing the functional Hsp90/CaM/Akt/eNOS complex formation. In addition, eNOS activity is also regulated by other signaling pathways, including the extracellular signal–related kinase–mitogen-activated protein kinase pathway (34), the AMP-activated protein kinase pathway (35), and the Akt pathway, upon which palmitic and linoleic acids may also exert the inhibition.

Reactive oxygen species production and inflammatory pathways (36–39) have been implicated in the development of insulin resistance. In addition to these important mechanisms, our present study shows that p38-activated PTEN is an additional mechanism for the development of insulin resistance. PTEN is a member of serine/threonine/tyrosine phosphatase subfamily of protein phosphatases. It dephosphorylates PtdIns(3,4,5)P3 into PtdIns(4,5)P2, thus antagonizing PI3K-dependent signaling pathways (17,40). Our results show that palmitic acid can induce PTEN activation, and silencing PTEN can reverse the inhibitory effects on Akt and eNOS activation, suggesting that the upregulated PTEN may mediate palmitic acid's inhibitory effects on insulin signaling in the endothelial cells. PTEN overexpression has been shown to inhibit insulin signaling (17,40). Recently, it has been shown in liver (41), muscle (42), and adipose tissue (43) that tissue-specific deletion of PTEN results in insulin hypersensitivity with improved systemic glucose tolerance. Thus, PTEN may play a critical role in the development of insulin resistance in metabolic syndrome.

Stress signaling pathways, which are activated by inflammation and metabolic stresses, have been implicated in the induction of insulin resistance. In searching for the mechanisms and pathways for palmitic acid–induced PTEN upregulation and inhibition of insulin signaling, we found that palmitic acid activated stress signaling kinases p38 and JNK, which was paralleled by eNOS inactivation.

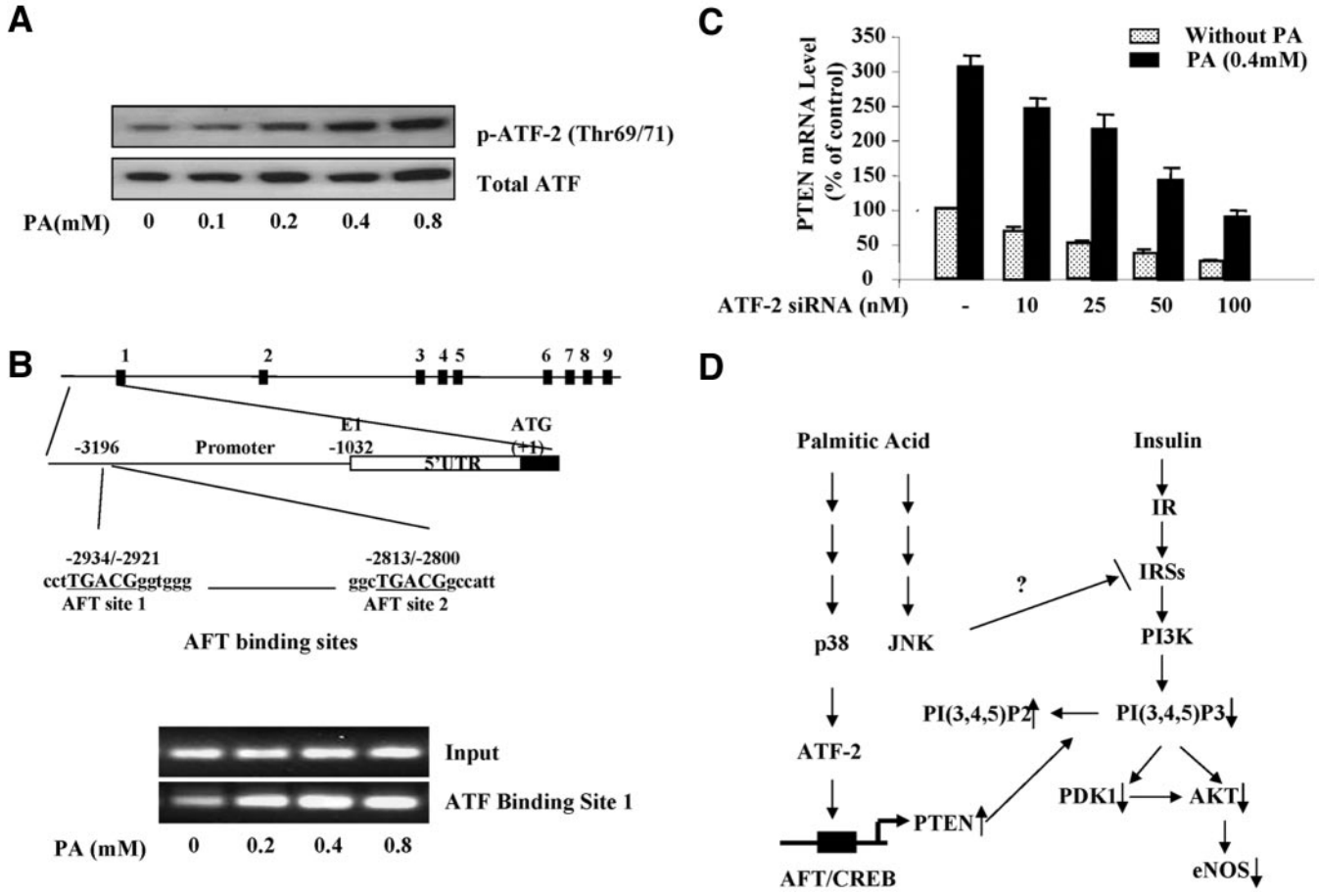

FIG. 7. p38 target ATF-2 was involved in palmitic acid–induced PTEN expression. *A*: Upregulation of ATF-2 in palmitic acid–treated HAECs. Phosphorylated and total ATF-2 were measured. *B*: Palmitic acid increased the binding of ATF-2 to ATF/CREBP site in the *PTEN* promoter. Protein-DNA complex cross-linked by formaldehyde was immunoprecipitated using anti–ATF-2 antibody. The specifically bound DNA was amplified by PCR. ATF/CREBP sites in the *PTEN* promoter are shown. *C*: Involvement of ATF-2 in palmitic acid–induced PTEN mRNA upregulation. HAECs were transfected with ATF-2 siRNA followed by the treatment with palmitic acid. The data were compared by two-way ANOVA and showed a significant effect by palmitic acid on PTEN ($P < 0.001$) in the presence of ATF-2 siRNA. *D*: Proposed pathway of the palmitic acid–induced inhibition of eNOS activation.

Inhibition of p38 and JNK fully reversed palmitic acid–induced Akt and eNOS inhibition, indicating that both pathways are involved in palmitic acid–induced impairment of insulin signaling. p38 mitogen-activated protein kinase is overactivated in type 2 diabetic patients (21,44) and is involved in the induction of insulin resistance by tumor necrosis factor α (45) and glucose (46). JNK activity is also abnormally elevated in obesity, and mice lacking JNK1 displayed significantly improved insulin sensitivity and enhanced insulin signaling in the high-fat dietary and *ob/ob* knockout models (47,48). Thus, activated p38 and JNK may be important mediators for stress signal–induced inhibition of insulin signaling and responsible for the development of diabetes and cardiovascular diseases in metabolic syndrome.

Furthermore, our study has discovered that upregulation of PTEN transcription may be a novel mechanism for p38-mediated, but not JNK-mediated, insulin resistance. While inhibition of JNK expression completely reversed eNOS and Akt inactivation by palmitic acid, it only marginally reduced PTEN transcription, suggesting that JNK may mediate palmitic acid's inhibitory effects on insulin signaling through *PTEN* activation or PTEN-independent mechanisms. JNK has been shown to induce phosphorylation of Ser^{307} in insulin receptor substrate-1 (47,49), which has been identified as a negative regulatory site (50) for insulin signaling. Similar mechanisms may also be responsible for JNK-mediated palmitic acid's inhibitory effects on insulin signaling and eNOS activation.

We have further shown that p38 target transcriptional factor ATF-2 can bind to ATF sites in the *PTEN* promoter. Palmitic acid enhanced ATF-2 phosphorylation/activation and its binding to *PTEN* promoter, which may lead to the increased PTEN transcription. Our study demonstrates that ATF-2 may be a critical link between the p38 stress signaling and *PTEN* gene expression in the development of insulin resistance.

In summary, we have demonstrated that palmitic and linoleic acids exert an inhibitory effect on insulin signaling and eNOS activation in endothelial cells. While linoleic acid inhibits Akt-mediated eNOS phosphorylation, palmitic acid appears to affect upstream signaling by upregulating PTEN transcription and activity. Palmitic acid promotes *PTEN* gene transcription by activating p38 stress signaling and its downstream target transcription factor ATF-2. FFA-induced insulin resistance and eNOS inhibition in endothelial cells may play an important role in the pathogenesis of cardiovascular disease in metabolic syndrome. The characterization of molecular mechanisms involved in the palmitic acid–induced insulin resistance in endothelium provides an important mechanistic link implicating

PTEN, p38, and JNK in the inhibitory effects of FFAs on vascular insulin signaling.

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REFERENCES

1. Kereiakes DJ, Willerson JT: Metabolic syndrome epidemic. *Circulation* 108:1552–1553, 2003
2. Haffner S, Taegtmeyer H: Epidemic obesity and the metabolic syndrome. *Circulation* 108:1541–1545, 2003
3. Aljada A, Dandona P: Effect of insulin on human aortic endothelial nitric oxide synthase. *Metabolism* 49:147–150, 2000
4. Zeng G, Nystrom FH, Ravichandran LV, Cong LN, Kirby M, Mostowski H, Quon MJ: Roles for insulin receptor, PI3-kinase, and Akt in insulin-signaling pathways related to production of nitric oxide in human vascular endothelial cells. *Circulation* 101:1539–1545, 2000
5. Bonora E, Kiechl S, Willeit J, Oberhollenzer F, Egger G, Targher G, Alberiche M, Bonadonna RC, Muggeo M: Prevalence of insulin resistance in metabolic disorders: the Bruneck Study. *Diabetes* 47:1643–1649, 1998
6. Laws A, Hoen HM, Selby JV, Saad MF, Haffner SM, Howard BV: Differences in insulin suppression of free fatty acid levels by gender and glucose tolerance status: relation to plasma triglyceride and apolipoprotein B concentrations: Insulin Resistance Atherosclerosis Study (IRAS) Investigators. *Arterioscler Thromb Vasc Biol* 17:64–71, 1997
7. Groop LC, Saloranta C, Shank M, Bonadonna RC, Ferrannini E, DeFronzo RA: The role of free fatty acid metabolism in the pathogenesis of insulin resistance in obesity and noninsulin-dependent diabetes mellitus. *J Clin Endocrinol Metab* 72:96–107, 1991
8. Steinberg HO, Tarshoby M, Monestel R, Hook G, Cronin J, Johnson A, Bayazeed B, Baron AD: Elevated circulating free fatty acid levels impair endothelium-dependent vasodilation. *J Clin Invest* 100:1230–1239, 1997
9. Steinberg HO, Paradisi G, Hook G, Crowder K, Cronin J, Baron AD: Free fatty acid elevation impairs insulin-mediated vasodilation and nitric oxide production. *Diabetes* 49:1231–1238, 2000
10. Carlsson M, Wessman Y, Almgren P, Groop L: High levels of nonesterified fatty acids are associated with increased familial risk of cardiovascular disease. *Arterioscler Thromb Vasc Biol* 20:1588–1594, 2000
11. Jouven X, Charles MA, Desnos M, Ducimetiere P: Circulating nonesterified fatty acid level as a predictive risk factor for sudden death in the population. *Circulation* 104:756–761, 2001
12. Dresner A, Laurent D, Marcucci M, Griffin ME, Dufour S, Cline GW, Slezak LA, Andersen DK, Hundal RS, Rothman DL, Petersen KF, Shulman GI: Effects of free fatty acids on glucose transport and IRS-1-associated phosphatidylinositol 3-kinase activity. *J Clin Invest* 103:253–259, 1999
13. Kuhlmann J, Neumann-Haefelin C, Belz U, Kalisch J, Juretschke HP, Stein M, Kleinschmidt E, Kramer W, Herling AW: Intramyocellular lipid and insulin resistance: a longitudinal in vivo 1H-spectroscopic study in Zucker diabetic fatty rats. *Diabetes* 52:138–144, 2003
14. Svedberg J, Bjorntorp P, Smith U, Lonnroth P: Free-fatty acid inhibition of insulin binding, degradation, and action in isolated rat hepatocytes. *Diabetes* 39:570–574, 1990
15. Shen YH, Zhang L, Gan Y, Wang X, Wang J, Lemaire SA, Coselli JS, Wang XL: Up-regulation of PTEN (phosphatase and tensin homolog deleted on chromosome ten) mediates p38 MAPK stress signal-induced inhibition of insulin signaling: a cross-talk between stress signaling and insulin signaling in resistin-treated human endothelial cells. *J Biol Chem* 281:7727–7736, 2006
16. Nicholson KM, Anderson NG: The protein kinase B/Akt signalling pathway in human malignancy. *Cell Signal* 14:381–395, 2002
17. Leslie NR, Downes CP: PTEN: the down side of PI 3-kinase signalling. *Cell Signal* 14:285–295, 2002
18. Vazquez F, Grossman SR, Takahashi Y, Rokas MV, Nakamura N, Sellers WR: Phosphorylation of the PTEN tail acts as an inhibitory switch by preventing its recruitment into a protein complex. *J Biol Chem* 276:48627–48630, 2001
19. Vazquez F, Ramaswamy S, Nakamura N, Sellers WR: Phosphorylation of the PTEN tail regulates protein stability and function. *Mol Cell Biol* 20:5010–5018, 2000
20. Manning AM, Davis RJ: Targeting JNK for therapeutic benefit: from junk to gold? *Nat Rev Drug Discov* 2:554–565, 2003
21. Koistinen HA, Chibalin AV, Zierath JR: Aberrant p38 mitogen-activated protein kinase signalling in skeletal muscle from type 2 diabetic patients. *Diabetologia* 46:1324–1328, 2003
22. Tan Y, Rouse J, Zhang A, Cariati S, Cohen P, Comb MJ: FGF and stress regulate CREB and ATF-1 via a pathway involving p38 MAP kinase and MAPKAP kinase-2. *EMBO J* 15:4629–4642, 1996
23. Hockings SC, Kahn JD, Crothers DM: Characterization of the ATF/CREB site and its complex with GCN4. *Proc Natl Acad Sci U S A* 95:1410–1415, 1998
24. Boden G: Effects of free fatty acids (FFA) on glucose metabolism: significance for insulin resistance and type 2 diabetes. *Exp Clin Endocrinol Diabetes* 111:121–124, 2003
25. Staiger H, Staiger K, Stefan N, Wahl HG, Machicao F, Kellerer M, Haring HU: Palmitate-induced interleukin-6 expression in human coronary artery endothelial cells. *Diabetes* 53:3209–3216, 2004
26. Tripathy D, Mohanty P, Dhindsa S, Syed T, Ghanim H, Aljada A, Dandona P: Elevation of free fatty acids induces inflammation and impairs vascular reactivity in healthy subjects. *Diabetes* 52:2882–2887, 2003
27. Artwohl M, Roden M, Waldhausl W, Freudenthaler A, Baumgartner-Parzer SM: Free fatty acids trigger apoptosis and inhibit cell cycle progression in human vascular endothelial cells. *FASEB J* 18:146–148, 2004
28. de Jongh RT, Serne EH, Ijzerman RG, de Vries G, Stehouwer CD: Free fatty acid levels modulate microvascular function: relevance for obesity-associated insulin resistance, hypertension, and microangiopathy. *Diabetes* 53:2873–2882, 2004
29. Egan BM, Lu G, Greene EL: Vascular effects of non-esterified fatty acids: implications for the cardiovascular risk factor cluster. *Prostaglandins Leukot Essent Fatty Acids* 60:411–420, 1999
30. Kim F, Tysseling KA, Rice J, Pham M, Haji L, Gallis BM, Baas AS, Paramsothy P, Giachelli CM, Corson MA, Raines EW: Free fatty acid impairment of nitric oxide production in endothelial cells is mediated by IKKbeta. *Arterioscler Thromb Vasc Biol* 25:989–994, 2005
31. Baron AD: Vascular reactivity. *Am J Cardiol* 84:25J–27J, 1999
32. Gratton JP, Fontana J, O'Connor DS, Garcia-Cardena G, McCabe TJ, Sessa WC: Reconstitution of an endothelial nitric-oxide synthase (eNOS), hsp90, and caveolin-1 complex in vitro: evidence that hsp90 facilitates calmodulin stimulated displacement of eNOS from caveolin-1. *J Biol Chem* 275:22268–22272, 2000
33. Takahashi S, Mendelsohn ME: Synergistic activation of endothelial nitric-oxide synthase (eNOS) by HSP90 and Akt: calcium-independent eNOS activation involves formation of an HSP90-Akt-CaM-bound eNOS complex. *J Biol Chem* 278:30821–30827, 2003
34. Wyatt AW, Steinert JR, Wheeler-Jones CP, Morgan AJ, Sugden D, Pearson JD, Sobrevia L, Mann GE: Early activation of the p42/p44MAPK pathway mediates adenosine-induced nitric oxide production in human endothelial cells: a novel calcium-insensitive mechanism. *FASEB J* 16:1584–1594, 2002
35. Morrow VA, Foufelle F, Connell JM, Petrie JR, Gould GW, Salt IP: Direct activation of AMP-activated protein kinase stimulates nitric-oxide synthesis in human aortic endothelial cells. *J Biol Chem* 278:31629–31639, 2003
36. Dandona P, Aljada A, Mohanty P, Ghanim H, Hamouda W, Assian E, Ahmad S: Insulin inhibits intranuclear nuclear factor kappaB and stimulates IkappaB in mononuclear cells in obese subjects: evidence for an anti-inflammatory effect? *J Clin Endocrinol Metab* 86:3257–3265, 2001
37. Aljada A, Ghanim H, Assian E, Dandona P: Tumor necrosis factor-alpha inhibits insulin-induced increase in endothelial nitric oxide synthase and reduces insulin receptor content and phosphorylation in human aortic endothelial cells. *Metabolism* 51:487–491, 2002
38. Arkan MC, Hevener AL, Greten FR, Maeda S, Li ZW, Long JM, Wynshaw-Boris A, Poli G, Olefsky J, Karin M: IKK-beta links inflammation to obesity-induced insulin resistance. *Nat Med* 11:191–198, 2005
39. Cai D, Yuan M, Frantz DF, Melendez PA, Hansen L, Lee J, Shoelson SE: Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. *Nat Med* 11:183–190, 2005
40. Jiang G, Zhang BB: Pi 3-kinase and its up- and down-stream modulators as potential targets for the treatment of type II diabetes. *Front Biosci* 7:d903–d907, 2002
41. Stiles B, Wang Y, Stahl A, Bassilian S, Lee WP, Kim YJ, Sherwin R, Devaskar S, Lesche R, Magnuson MA, Wu H: Live-specific deletion of negative regulator Pten results in fatty liver and insulin hypersensitivity. *Proc Natl Acad Sci U S A* 101:2082–2087, 2004
42. Wijesekara N, Konrad D, Eweida M, Jefferies C, Liadis N, Giacca A, Crackower M, Suzuki A, Mak TW, Kahn CR, Klip A, Woo M: Muscle-specific Pten deletion protects against insulin resistance and diabetes. *Mol Cell Biol* 25:1135–1145, 2005
43. Kurlawalla-Martinez C, Stiles B, Wang Y, Devaskar SU, Kahn BB, Wu H: Insulin hypersensitivity and resistance to streptozotocin-induced diabetes in mice lacking PTEN in adipose tissue. *Mol Cell Biol* 25:2498–2510, 2005
44. Carlson CJ, Koterski S, Sciotti RJ, Poccard GB, Rondinone CM: Enhanced

basal activation of mitogen-activated protein kinases in adipocytes from type 2 diabetes: potential role of p38 in the downregulation of GLUT4 expression. *Diabetes* 52:634–641, 2003
45. De Alvaro C, Teruel T, Hernandez R, Lorenzo M: Tumor necrosis factor-alpha produces insulin resistance in skeletal muscle by activation of inhibitor kB kinase in a p38 mitogen-activated protein kinase-dependent manner. *J Biol Chem* 279:17070–17078, 2004
46. Huang C, Somwar R, Patel N, Niu W, Torok D, Klip A: Sustained exposure of L6 myotubes to high glucose and insulin decreases insulin-stimulated GLUT4 translocation but upregulates GLUT4 activity. *Diabetes* 51:2090–2098, 2002
47. Aguirre V, Uchida T, Yenush L, Davis R, White MF: The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307). *J Biol Chem* 275: 9047–9054, 2000
48. Hirosumi J, Tuncman G, Chang L, Gorgun CZ, Uysal KT, Maeda K, Karin M, Hotamisligil GS: A central role for JNK in obesity and insulin resistance. *Nature* 420:333–336, 2002
49. Lee YH, Giraud J, Davis RJ, White MF: c-Jun N-terminal kinase (JNK) mediates feedback inhibition of the insulin signaling cascade. *J Biol Chem* 278:2896–2902, 2003
50. De Fea K, Roth RA: Modulation of insulin receptor substrate-1 tyrosine phosphorylation and function by mitogen-activated protein kinase. *J Biol Chem* 272:31400–31406, 1997