The key feature of metabolic syndrome, a cluster of metabolic and cardiovascular disorders, is systemic insulin resistance, which is associated with dysregulated endothelial nitric-oxide synthase (eNOS). Stress signaling induced by inflammation can inhibit insulin signaling. However, molecular mechanisms for the cross-talk between stress signaling and insulin resistance are only partially understood. Resistin, an adipokine/cytokine, is involved in inflammatory processes that could lead to insulin resistance status and vascular diseases. In the current study, we observed that resistin inhibited insulin signaling and eNOS activation in endothelial cells. Up-regulation of PTEN (phosphatase and tensin homolog deleted on chromosome ten) expression by resistin may mediate the inhibitory effects. Activated stress signaling p38 MAPK, but not JNK, is involved in PTEN up-regulation. We further found that p38 target transcriptional factor activating transcription factor-2 (ATF-2) bound to ATF sites in the PTEN promoter. The phosphorylation/activation of ATF-2 and its binding to PTEN promoter were increased by resistin treatment. In summary, up-regulation of PTEN is involved in the inhibitory effects of resistin on insulin signaling and eNOS activation in endothelial cells. Resistin induces PTEN expression by activating stress signaling p38 pathway, which may activate target transcription factor ATF-2, which in turn induces PTEN expression. Our findings suggest that resistin-mediated inhibition of insulin signaling and eNOS activation may contribute to cardiovascular diseases.
dantly expressed in mononuclear leukocytes, macrophages, spleen, and bone marrow cells in human subjects (15, 16, 24). Resistin expression can be increased by proinflammatory cytokines (interleukin-1, interleukin-6, tumor necrosis factor-α and C-reactive protein) (25–27). Indeed, resistin also up-regulates interleukin-6 and tumor necrosis factor-α and induces arthritis (27).

In the present study, we examined the hypothesis that resistin, as an inflammatory mediator, may impair insulin signaling in endothelium that leads to the inhibition of eNOS activity and endothelial dysfunction. We further investigated the signaling pathways mediating the effects and the molecular mechanisms for the stress-signaling induced impairment of insulin functions.

**MATERIALS AND METHODS**

**siRNAs and Antibodies**—Human recombinant resistin was purchased from BioVision Research Product (Mountain View, CA). Endotoxin level in the culture medium containing resistin was measured using Pyrogen® Plus Gel Clot Assay Kit (Cambrex, East Rutherford, NJ). PTEN siRNA was purchased from Cell Signaling (Beverly, MA). p38α siRNA and JNK1 siRNA were purchased from Ambion (Austin, TX). For Western blot analysis and immunofluorescence staining, monoclonal and polyclonal antibodies from Cell Signaling and Santa Cruz Biotechnology (Santa Cruz, CA) were used.

**Cell Culture and Treatment**—Primary human aortic endothelial cells (HAECs) from Cell Applications (San Diego, CA) were cultured in EGM-2 medium (Cambrex, containing endothelial cell basic medium (EBM), hydrocortisone, fibroblast growth factor-B, vascular epithelial growth factor, insulin-like growth factor-1, epidermal growth factor, ascorbic acid, GA-1000, heparin, and 2% fetal bovine serum). Cells, cultured up to five passages, were first grown to confluence in 2% FCS-EGM, before they were exposed to resistin (10–100 ng/ml) for the designated time periods. For insulin stimulation, confluent cells were exposed to resistin (50 ng/ml) for 24 h and then starved in 0.5% FCS EGM with 10–100 ng/ml resistin for 6 h before stimulated with 100 nM insulin for 5–60 min.

**siRNA-induced Gene Silencing**—Silencing of gene expression in primary HAECs was achieved using the siRNA technique. Transfection of HAECs was carried out using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s instruction. Transfected cells were then treated with resistin for 24–48 h.

**eNOS Activity Assay**—The catalytic activity of eNOS was determined by the conversion of L-[3H]arginine to L-[3H]citrulline using a nitric-oxide synthase assay kit from Calbiochem. Briefly, confluent cells in 6-well plates were treated with 10–100 ng/ml resistin for 24 h. The treated cells were washed with phosphate-buffered saline and then harvested in phosphate-buffered saline plus 1 mM EDTA. The collected cells were lysed in homogenization buffer. 25 μg of protein lysate was incubated with 1 μCi of L-[3H]arginine in the presence of 75 μM calcium at room temperature for 30 min. The 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 (% of control).

**Western Blot Analysis**—Treated HAECs were collected, washed with ice-cold phosphate-buffered saline, and lysed in protein lysis buffer (20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na3VO4, 10 μg/ml each protease inhibitors (aprotinin, leupeptin, and pepstatin), and 1 mM phenylmethylsulfonyl fluoride) for 1 h on ice. Protein concentration was measured by the Bradford method (Bio-Rad). 15 μg of protein per lane was separated by 10% or 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membrane was blocked by 5% nonfat powdered milk in TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20). The membrane was incubated with the primary antibody in 2% powdered milk in TBST, washed extensively with TBST, and then incubated with the secondary anti-rabbit or anti-mouse horseradish peroxidase-labeled antibody. Bands were visualized with ECL (Amersham Biosciences).

**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 with an iScript cDNA synthesis kit (Bio-Rad). Real-time PCR was performed by 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’-CAAGATGATGTTGAAACTATTCAAATG-3’; backward, 5’-CCTTATGCGTCGACACCA-3’. The PCR efficiency and specificity were examined as described before (28). The mRNA levels were acquired from the value of threshold cycle (Ct) of the real-time PCR and normalized against the housekeeping gene β-actin. The data shown comprise a representative graph of three separate experiments.

**The ChIP Assay**—The ChIP assay was performed using the histone H3 ChIP assay kit according to the manufacturer’s protocol (Upstate). In brief, ~5 × 106 cells were used per ChIP assay. Cells were cross-linked with 1% formaldehyde at 37 °C for 15 min and rinsed twice with ice-cold phosphate-buffered saline. Cells were then harvested and lysed in SDS lysis buffer (50 mM Tris-HCl, pH 8.1, 1% SDS, 10 mM EDTA, protease inhibitors). Cell lysates were sonicated to produce chromatin fragments of 300–1000 bp in length. Cellular debris was removed by centrifugation, and supernatants were diluted to 10-fold in ChIP dilution buffer (16.7 mM Tris-HCl, pH 8.1, 16.7 mM NaCl, 1.2 mM EDTA, 0.01% SDS, 1.1% Triton X-100, protease inhibitors) (20 μl aliquot was used as the input sample). Following pre-immunoprecipitation clearing with a mixture of salmon sperm DNA/protein A/protein G, immunoprecipitation was carried out by incubating cell lysate with anti-ATF antibody (with IgG served as the negative control in the assay) with rotation at 4 °C overnight, followed by incubating with the salmon sperm DNA/protein A-agarose slurry at 4 °C with rotation for 2 h. The immunocomplex beads were pelleted by centrifugation and washed sequentially with the following buffers: low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris-HCl, pH 8.1), and LiCl wash buffer (0.25 mM LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1). Finally, the beads were washed twice with the TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0). The immunocomplex was eluted with elution buffer (100 mM NaHCO3, 1% SDS), and then eluates were collected by centrifugation. The eluted immunocomplex and the inputs were incubated with 200 μM 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/isooamyl alcohol (25:24:1) extraction and precipitated with ethanol using glycogen as a carrier. The recovered input and immunoprecipitated DNA were resuspended in 30 μl of H2O. Four microliters of the immunoprecipitated DNA was used as a template for PCR with the following protocol: 2-min preincubation at 94 °C, 40 cycles of 30 s denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 45 s with a final incubation at 72 °C for 5 min. The PCR products were separated by electrophoresis through 1.2% agarose. Two pairs of primers were used for flanking AFT-2 binding sites in the 5'-flanking region.
of the human PTEN. Site 1: forward primer, 5'-TCGACTACTTGCT-TTGAGA-3' and backward primer, 5'-TTTACAGCCCCGATTGG-GCT-3'; Site 2: forward primer, 5'-CAGACTTGACAGGTTTG-CC-3' and backward primer, 5'-TCCAGTCACCTACCCCTGAGC-3'.

Statistical Analysis—All quantitative variables are presented as means ± S.E. We compared the differences of three groups or more using one-way ANOVA, and \( p < 0.05 \) was considered statistically significant.

RESULTS

Resistin Inhibited eNOS Activation—To determine whether resistin has any effect on eNOS activation, we first examined the effects of resistin on eNOS activity by measuring the conversion of L-[3H]arginine to L-[3H]citrulline in resistin-treated cells. As shown in Fig. 1A, resistin treatment resulted in a dose-dependent reduction of L-[3H]citrulline generation indicating the reduced eNOS activity by resistin treatment. The endotoxin level in the resistin-containing culture medium was tested with levels <0.03 endotoxin unit/ml, which ruled out the possibility of the endotoxic effects of the bacterially expressed recombinant resistin. We also conducted cellular viability assay to rule out general toxic effects of resistin.

We then examined whether this reduced eNOS activity was due to reduced eNOS activity by resistin treatment. eNOS can be activated by phosphorylation at Ser-1177 and dephosphorylation at Thr-495 by multiple protein kinases and phosphatases (29). We therefore examined the effect of resistin on eNOS phosphorylation. The HAECs, which were cultured in the EGM medium (with serum and growth factors that maintain the basal activation of eNOS), were treated with resistin before eNOS phosphorylation at Ser-1177 and Thr-495 were measured. As shown in Fig. 1B, although phosphorylation at Thr-495 site was unchanged, eNOS phosphorylation at Ser-1177 was inhibited by the resistin treatment in a dose-dependent manner, indicating that resistin may inhibit eNOS activity by inhibiting eNOS phosphorylation at Ser-1177.

Because insulin can induce eNOS phosphorylation at Ser-1177 and activation, we next examined whether resistin could inhibit insulin-
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FIG. 2. Resistin-inhibited Akt pathway. A, dose-dependent inhibition of Akt by resistin treatment. HAECS were incubated with resistin (5–50 ng/ml) for 24 h. A cell lysate was prepared. Phosphorylated Akt was detected by Western blots using anti-phospho (Ser-473) Akt antibody. Membranes were stripped and reprobed for total Akt using anti-Akt antibody. Relative levels of the phospho Akt were quantified after normalized to total Akt and expressed as the percentage of the control. B, resistin-inhibited insulin-stimulated Akt phosphorylation. HAECS were preincubated with resistin (10–100 ng/ml) for 24 h and then starved in 0.5% FCS-EBM containing resistin for 6 h before stimulated with insulin (50 nm) for 30 min. A cell lysate was prepared, and the phosphorylation of Akt and total Akt were detected. Relative levels of the phospho Akt were quantified after normalized to total Akt and expressed as the percentage of the control. C, time course of Akt inhibition by resistin treatment. HAECS were treated with the recombinant resistin (50 ng/ml) for different time (0, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 24 h, and 48 h). Phosphorylation of Akt at Ser-473 was detected. Total Akt was measured using anti-Akt antibody. D, dose-dependent inhibition of PDK1 by resistin treatment. PDK1 phosphorylation was detected from the resistin-treated HAECS using anti-phospho (Ser-241) PDK1 antibody. Total PDK1 was measured using anti-PDK1 antibody. Representative blots of three separate experiments are shown in each panel. Data represent the mean ± S.E. from three separate experiments, and the group difference was tested by ANOVA (p < 0.001 for all comparisons in A and B).

stimulated eNOS phosphorylation. HAECS were preincubated with resistin, starved, and followed by stimulation with insulin. As shown in Fig. 1C, insulin stimulated eNOS phosphorylation at Ser-1177. However, this insulin-induced up-regulation of eNOS phosphorylation was inhibited by resistin in a dose-dependent manner. Consistent with eNOS phosphorylation/activation, eNOS activity induced by insulin was also repressed by resistin treatment (Fig. 1D). Thus, resistin can inhibit both growth factor and insulin-stimulated eNOS phosphorylation at Ser-1177 and, hence, inactivates eNOS activity in endothelial cells.

Resistin Inhibited Insulin-stimulated Akt Phosphorylation—Because insulin activates eNOS through the IRS/PI3K/PDK/Akt pathway, we investigated whether resistin can inhibit this pathway. Akt is activated by phosphorylation at Ser-473 and Thr-308. The phosphorylation of Akt from resistin-treated cells was detected. As shown in Fig. 2A, Akt phosphorylation at Ser-473 was inhibited with resistin treatment in a dose-dependent fashion. In addition, resistin also inhibited insulin-stimulated Akt phosphorylation at Ser-473 (Fig. 2B) suggesting that resistin may block insulin signaling. Time-course study showed that resistin exhibited dual effects on Akt phosphorylation (Fig. 2C). Resistin induced a transient Akt phosphorylation that peaked at 30 min and returned to the basal level within 60 min. However, prolonged incubation with resistin decreased Akt phosphorylation, which started as early as 2 h and progressed with a maximal inhibition at 48 h, indicating that prolonged resistin treatment inhibited Akt activation. Because phosphoinositide-dependent kinases (PDKs) PDK1 and PDK2 are direct upstream kinases, which phosphorylate Akt and play an important role in regulating the insulin pathway (30), PDK1 activation was monitored by its phosphorylation at Ser-241. As shown in Fig. 2D, the PDK phosphorylation at Ser-241 was also inhibited by the resistin treatment. Collectively, these data suggest that resistin may inhibit Akt signaling axis in endothelial cells.

Up-regulation of PTEN Expression by Resistin—Akt phosphorylation/activation is regulated by PIP3. PIP3 recruits PDK and Akt to the plasma membrane where Akt undergoes conformational changes that allow its regulatory residues more accessible to PDKs and to be phosphorylated (31). PIP3 levels are tightly regulated by PI3K and phosphatases, such as PTEN, which has been shown to antagonize PI3K/Akt signaling by dephosphorylating PIP3 (32, 33). To further investigate the upstream insulin signaling molecules that were regulated by the resistin, we examined the expression of PTEN from resistin-treated cell lysate. As shown in Fig. 3A, PTEN protein levels were increased by the resistin treatment in a dose-dependent manner. Additionally, PTEN mRNA was also significantly increased (Fig. 3B) indicating that resistin can induce PTEN expression at mRNA level, either by increasing transcription or by decreasing mRNA turnover.

Involvement of PTEN in Inhibitory Effects of Resistin on Akt and eNOS—We then investigated whether up-regulated PTEN was responsible for resistin-induced inhibition of Akt and eNOS. We first examined the effects of PTEN on Akt and eNOS phosphorylation by blocking the PTEN expression using PTEN-specific siRNA. As shown in Fig. 4A, PTEN expression was specifically suppressed by the PTEN siRNA, whereas Akt and eNOS phosphorylation was increased by the PTEN siRNA treatment indicating the involvement
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**Activation of JNK and p38 Pathways by Resistin Treatment**—We next examined the mechanisms for resistin-induced expression of PTEN and inhibition of Akt and eNOS. While the receptor of resistin has not yet been identified, little is known about the downstream signaling pathways triggered by resistin. MAPKs, JNK, and p38 can be activated in response to a wide variety of stimuli such as inflammatory cytokines and are involved in stress responses by regulating many cellular functions. Activated p38 and JNK are implicated in the development of insulin resistance and cardiovascular diseases (34–38). We therefore examined the effects of resistin on the activation of stress signaling molecules p38 and JNK. As shown in Fig. 5A, resistin treatment increased both p38 and JNK phosphorylation indicating the activation of these kinases. The activation of p38 and JNK was paralleled by a dose-dependent decrease in eNOS activity. The phosphorylation of upstream kinases MKK6 and MLK3 was also increased (Fig. 5B) indicating the activation of the p38 stress signaling pathways.

**Involvement of JNK and p38 Pathways in Inhibitory Effects of Resistin on Akt and eNOS**—We further studied whether p38 and JNK play any role in the inhibitory effects of resistin on Akt and eNOS activation. We first examined the effects of JNK and p38 on Akt and eNOS phosphorylation by blocking their expressions with specific siRNAs. Both JNK and p38 have different isoforms. We selectively silenced JNK1 expression, because it has been reported to be involved in insulin resistance (75). We chose to suppress p38α expression, because it was the most thoroughly studied isoform. As shown in Fig. 6A, Akt and eNOS phosphorylation was increased by both p38α siRNA and JNK1 siRNA treatments suggesting that both pathways have inhibitory effects on insulin pathway. We next tested whether activated p38 and JNK pathways are involved in the effects of resistin on insulin pathway. As shown in Fig. 6B, resistin inhibited Akt and eNOS phosphorylation, which was completely reversed by p38α siRNA and JNK1 siRNA indicating that p38 and JNK are involved in resistin-induced inhibition of Akt and eNOS. We further examined whether the JNK and p38 activations were responsible for the resistin-induced inhibition of insulin signaling. As shown in Fig. 6C, insulin stimulated Akt and eNOS phosphorylation, which were suppressed by resistin. p38α siRNA and JNK1 siRNA specifically suppressed p38 and JNK expression and reversed resistin-induced inhibition of Akt and eNOS phosphorylation. Taken together, these data suggest that both pathways are involved in mediating resistin-induced inhibition of insulin signaling and eNOS activation.
p38 MAPK Inhibits Insulin Pathway by Promoting PTEN Expression—We explored the possibility that p38 and JNK could inhibit insulin pathway by up-regulating PTEN expression. We first examined the effects of p38 and JNK on basal PTEN expression by measuring the PTEN expression in cells treated with either JNK1-specific siRNA or p38-specific siRNA. As shown in Fig. 7A, basal JNK expression was specifically suppressed by the JNK1 siRNA, and the p38 expression was suppressed by the p38a siRNA. In p38a siRNA-treated cells, PTEN protein levels were decreased along with the decreased p38 expression. However, although JNK1 siRNA efficiently suppressed JNK expression, it had minimal effects on PTEN expression. These results suggest that p38, rather than JNK, may be involved in regulating PTEN expression. We then examined whether JNK and p38 mediate resistin-induced PTEN expression. As shown in Fig. 7B, JNK and p38 expression was specifically suppressed by the specific siRNAs. Resistin-induced PTEN expression was reduced along with the decreased p38 but not JNK expression indicating that p38 pathway, but not JNK pathway, may be involved in mediating resistin-induced PTEN expression. Furthermore, as shown in Fig. 7C, basal PTEN mRNA was decreased with increased amount of p38a siRNA, and resistin-induced PTEN transcription was also repressed by the p38a siRNA in a dose-dependent manner (Fig. 7D). Taken together, these data suggest that, although both JNK and p38 pathways are involved in mediating resistin-induced inhibition of insulin signaling and eNOS activation, mechanisms may differ. Although JNK may inhibit the insulin pathway through PTEN-independent mechanisms, p38 may impair insulin signaling by up-regulating PTEN, possibly at the transcriptional level.

p38-Target Transcriptional Factor ATF-2 Mediates Resistin-induced PTEN Up-regulation—p38 MAPK involves in gene expression regulation by activating an array of transcriptional factors, including p53, Max, Myc, ELK1, MEF2, cAMP response element-binding proteins (CREBs), and activating transcription factors (ATFs) (39–46). To further investigate the mechanisms by which p38 mediates resistin-induced PTEN mRNA up-regulation, we first identified p38-target transcription factors that can be activated by resistin treatment. Compare with other transcriptional factors tested such as c-Myc, ELK1, and MEF2 (data not shown), the phosphorylation of ATF-2 was strongly induced by the resistin treatment in a dose-dependent manner (Fig. 8A) indicating that ATF-2 may be a target of activated p38 MAPK in resistin-treated cells. ATF-2 belongs to ATF/CREB family of transcription factors. It binds to target DNA either as homodimers or heterodimers with ATF/CREB proteins or AP-1 transcription factors. These dimers bind to ATF/CREB site 5′-TGACGTCA-3′ and AP-1 site 5′-TGACTCA-3′ (47, 48). Because the promoter region in the PTEN gene contains two putative ATF half binding sites: site 1 (cctTGACGggtggg) and site 2 (ggcT-GACGgccatt) (Fig. 8B), chromatin immunoprecipitation assays were conducted to determine whether ATF-2 can bind to these sites in the PTEN promoter in vivo. Cross-linked DNA-protein complex from intact cells was immunoprecipitated with ATF-2 antibody. The ATF binding sites present in the immunoprecipitates were amplified. As demonstrated in Fig. 8C, ATF-2 directly bound to both ATF binding sites in the PTEN promoter detected specifically by the anti-ATF-2 antibody immunoprecipitation but not by the control IgG. Interestingly, although ATF-2 bound to both sites in the PTEN promoter, the basal binding of ATF-2 to the site 2 was higher than to the site 1 suggesting that these two ATF sites may contribute differently to the basal PTEN expression. These results clearly indicate that p38 target transcription factor ATF-2 can bind to PTEN promoter and may be involved in the regulation of PTEN gene.

We next tested whether the binding of ATF-2 to PTEN promoter is regulated by the resistin. As shown in Fig. 8D, although the binding of ATF-2 to the site 2 was unchanged, its binding to the site 1 was significantly increased by the resistin treatment in a dose-dependent manner. The findings suggest that ATF-2 binding to site 1 may be involved in resistin-induced PTEN expression. We suggest that ATF-2 is an important transcription factor in PTEN gene expression and may be involved in mediating p38-induced PTEN expression in response to resistin treatment.

DISCUSSION

In the present study, we have shown that resistin inhibits basal and insulin-stimulated phosphorylation/activation of Akt and its downstream target eNOS. Additionally, using resistin as a tool, we have explored the mechanisms for stress signaling-induced insulin resistance in cultured endothelial cells. We show that resistin-induced PTEN expression appears to mediate the inhibitory effects of resistin on Akt and eNOS. Moreover, activation of p38 by resistin up-regulates PTEN expression through activating transcriptional factor ATF-2 and promoting its binding to the PTEN promoter. Taken these findings...
together, we propose a pathway of the resistin-induced inhibition of eNOS activation (Fig. 8E).

Increasing evidence suggest that inflammation causes insulin resistance, impairs endothelium function, and initiates or promotes pathogenesis of vascular diseases (49–51). Resistin as a potent inflammatory regulator (27) may affect insulin signaling and endothelial functions. Endothelial dysfunction and eNOS deregulation are the prominent features in metabolic syndrome and an early event in the atherogenesis. Little data is available, however, about whether resistin could interfere with endothelial functions and insulin signaling in endothelial cells. Our study shows that human recombinant resistin inhibits basal and insulin-stimulated eNOS phosphorylation and activation. As far as we are aware, our study is the first to report the direct eNOS inhibition by resistin. Because NO is a key regulator in endothelial functions, eNOS inhibition by resistin could provide a mechanism for endothelial dysfunction, which is consistent with recent reports of the elevated resistin levels in patients with coronary artery disease. Together with other findings that resistin up-regulates the expression of adhesion molecules (52) and promotes smooth muscle cell proliferation (53), we suggest that resistin may provide a causal link for accelerated cardiovascular diseases in metabolic syndrome.

Although human and murine resistin shares only 53% homology, our study shows that human resistin has similar functions as the murine resistin, which induces insulin resistance in vivo and in vitro (54). It has been reported that resistin forms a complex multimeric structure, which may be an obligatory step toward activation (55). Therefore, whether the recombinant resistin is physiologically relevant has been questioned. However, studies of ours and others have shown that the recombinant resistin can exert pathological effects (27, 53, 56–58).

It is established that eNOS activity can be regulated by post-translational modifications through different signaling pathways, including the ERK MAPK pathway (59), AMP-activated protein kinase (AMPK) pathway (60) and Akt pathway. Insulin can induce eNOS activation through the IRS/PI3K/PDK/Akt cascade, which plays a key role in insulin signal-
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FIG. 7. Involvement of p38 in PTEN up-regulation. A, p38 but not JNK is involved in basal PTEN expression. HAECs were transfected with scrambled siRNA, JNK1 siRNA, or p38α siRNA (50 and 100 nM) for 24 h. Total JNK, p38, and PTEN from treated cells were measured by specific antibodies. β-Actin was measured as loading control. B, involvement of p38 in resistin-induced PTEN up-regulation. HAECs were transfected with JNK1 siRNA or p38α siRNA (50 and 100 nM) followed by the treatment with resistin (50 ng/ml) for 24 h. Total JNK, p38, and PTEN were measured by specific antibodies. β-Actin was measured as loading control. C, p38 is involved in basal PTEN expression at mRNA level. HAECs were transfected with JNK1 siRNA or p38α siRNA (10–100 nM) for 24 h. Total RNA was extracted, and the mRNAs were reverse-transcribed into cDNAs. PTEN mRNA level was quantified by real-time PCR and normalized to β-actin mRNA. Results are expressed relative to control. The data shown was the mean ± S.E. from three separate experiments and compared by ANOVA (p < 0.001). D, involvement of p38 in resistin-induced PTEN mRNA up-regulation. HAECs were transfected with p38α siRNA (10–100 nM) followed by the treatment with resistin (50 ng/ml) for 24 h. Total RNA was extracted, and the mRNAs were reverse-transcribed into cDNAs. PTEN mRNA level was quantified by real-time PCR and normalized to β-actin mRNA. Results are expressed relative to control. The data shown was the mean ± S.E. from three separate experiments and compared by ANOVA (p < 0.001).

Although there are extensive studies on insulin resistance, the molecular mechanisms responsible for insulin resistance are not completely understood. PTEN overexpression has been shown to inhibit activation of insulin signaling (33, 67). Recently, it has been shown, in liver (68), muscle (69), and adipose tissue (70), that tissue-specific deletion of PTEN results in insulin hypersensitivity with improved systemic glucose tolerance. PTEN is a member of the serine/threonine/tyrosine phosphatase subfamily of protein phosphatases. It dephosphorylates phosphatidylinositol 3,4,5-trisphosphate into phosphatidylinositol 4,5-bisphosphate, thus antagonizing PI3K-dependent signaling pathways (33). PTEN plays a critical role in embryonic development, cell growth, apoptosis, differentiation, and migration (71). Our results show that resistin can induce PTEN expression; silencing PTEN by the specific siRNA can reverse the inhibitory effects of resistin on Akt and eNOS activation. These results suggest that the up-regulated PTEN may mediate the inhibitory effects of resistin on insulin signaling and eNOS activation in endothelial cells. 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 resistin-induced PTEN up-regulation and inhibition of insulin signaling, we found that resistin activated stress signaling kinases p38 and JNK, which was paralleled by Akt and eNOS inactivation. Inhibition of p38 and JNK fully reversed resistin-induced Akt and eNOS inhibition. p38 MAPK has been shown to be over-activated in type 2 diabetic patients (37, 38) and involved in the induction of insulin resistance by tumor necrosis factor α (72) and glucose (73). JNK activity is also abnormally elevated in obesity. Mice lacking JNK display significantly improved insulin sensitivity and enhanced insulin receptor signaling in the high fat and ob/ob models (74, 75). Thus, activated p38 and JNK may be an important mediator for stress signal-induced inhibition of insulin signaling and responsible for the development of diabetes and cardiovascular diseases in metabolic syndrome.

Although JNK and p38 have been implicated in the impairment of
insulin signaling, the molecular mechanisms for stress signaling-mediated impairment of insulin signaling remain to be elucidated. Our study has discovered that up-regulated PTEN may be a novel mechanism for stress signaling p38-mediated insulin resistance and the inhibitory effects of resistin on insulin signaling. Interestingly, while inhibition of JNK expression completely reversed eNOS activation and Akt activation, it only marginally reduced PTEN up-regulation suggesting that JNK may mediate the inhibitory effects of resistin on insulin signaling through PTEN-independent mechanisms. JNK has been shown to induce phosphorylation of the Ser-307 in IRS-1 (74, 76), which has been identified as a negative regulatory site (77) for insulin signaling. Similar mechanisms may also be responsible for JNK-mediated the inhibitory effects of resistin on insulin signaling and eNOS activation.

We have further shown that the p38 target transcriptional factor ATF-2 can bind to ATF sites in the PTEN promoter. ATF-2 phosphorylation/activation and its binding to PTEN promoter, which may lead to the increased PTEN transcription, can be increased by the resistin treatment. Our study indicates that activated p38 in resistin-treated cells may promote PTEN gene expression by activating its target transcription factor ATF-2. Furthermore, ATF-2 binding to the target sites in the PTEN promoter may be regulated differently. Although the basal binding to one site is high and unchanged by the resistin treatment, the basal binding to the other site is low, and is increased by the resistin treatment. This finding suggests that these two sites may have different regulatory roles in PTEN transcription.

In summary, we have demonstrated, for the first time, that resistin exerts an inhibitory effect on the insulin signaling and eNOS activation in endothelial cells. Resistin inhibits the insulin pathways by up-regulating PTEN and activating stress signaling p38 and JNK pathways. Activated p38 may promote PTEN gene expression by activating its target transcription factor ATF-2. Resistin-induced insulin resistance and eNOS inhibition in endothelial cells may play an important role in the pathophysiology of cardiovascular disease such as hypertension and atherosclerotic process in metabolic syndrome. Characterization of molecular mechanisms involved in resistin-induced insulin resistance in endothelium provides an important mechanistic link implicating PTEN, p38, and JNK in the inhibitory effects of resistin on insulin vascular actions. These findings may help to develop pharmacological agents to effectively prevent endothelial dysfunction associated with insulin resistance.

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FIG. 8. A, up-regulation of ATF-2 by Resistin. HAECs were incubated with resistin (5–100 ng/ml) for 24 h, and a cell lysate was prepared. Phosphorylated ATF-2 was detected by Western blotting using anti-phospho (Thr69/71)-ATF-2 antibodies. The membrane was stripped and re-blotted with anti-ATF-2 (20F1) antibody to monitor total ATF-2 level. Representative blots of three separate experiments are shown. B, locations of putative ATF-2-binding motifs in the PTEN gene promoter. ATF/CREB sites in human PTEN promoter region were examined in the ChIP assay. C, ATF-2 binds to ATF/CREB sites in PTEN promoter in vivo. HAECs were treated with formaldehyde to cross-link the DNA and associated proteins. Protein-DNA complex was immunoprecipitated using anti-ATF-2 antibody. The specificity of the assay was determined by the presence of PCR products. The details of the assay are described under “Materials and Methods.” Two pairs of primers were used for flanking ATF/CREB sites in the 5′ flanking region of the human PTEN promoter. Site 1: 5′-TTCAGCTTACCTGTTGAGA-3′ and backward primer, 5′-TTTACAGCGCGATTGGGCT-3′; Site 2: forward primer, 5′-CAGACTTTGGGTCT-3′ and backward primer, 5′-TGCACTACTTGGTAGA-3′. D, changes in the binding of ATF-2 to ATF/CREB sites in PTEN promoter by resistin treatment. HAECs were treated with resistin for 24 h. A ChIP assay was conducted. ATF-2 DNA binding was examined by PCR analysis of immunoprecipitated samples from control cells and resistin-treated cells using the same set of primers as described in C, E, proposed pathway of the resistin-induced inhibition of eNOS activation. Resistin induces activation of the stress signaling MLK3/MKK3/6/p38 pathway. The activated p38 can up-regulate PTEN expression by activating target transcription factor ATF-2. The up-regulated PTEN decreases the PIP3 level, which leads to reduced activation of Akt and PDK1, hence inhibited eNOS activation.
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