Resistin decreases expression of endothelial nitric oxide synthase through oxidative stress in human coronary artery endothelial cells

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Chen C, Jiang J, Lui J, Chai H, Wang X, Lin PH, Yao Q. Resistin decreases expression of endothelial nitric oxide synthase through oxidative stress in human coronary artery endothelial cells. Am J Physiol Heart Circ Physiol 299: H193–H201, 2010. First published April 30, 2010; doi:10.1152/ajpheart.00431.2009.—Resistin is a newly discovered adipocyte-derived cytokine that may play an important role in insulin resistance, diabetes, adipogenesis, inflammation, and cardiovascular disease. However, it is largely unknown whether resistin impairs endothelial functions by affecting the endothelial nitric oxide synthase (eNOS) system. In this study, we determined the effect of human recombinant resistin protein on eNOS expression and regulation in human coronary artery endothelial cells (HCAECs). When cells were treated with clinically relevant concentrations of resistin (40 or 80 ng/ml) for 24 h, the levels of eNOS mRNA, protein, and activity and eNOS mRNA stability were significantly reduced. Cellular nitric oxide levels were also decreased. In addition, the cellular levels of reactive oxygen species (ROS), including superoxide anion, were significantly increased in resistin-treated HCAECs. Mitochondrial membrane potential and the activities of catalase and superoxide dismutase were reduced. Three antioxidants, seleno-L-methionine, ginsenoside Rb1, and MnTBAP (superoxide dismutase mimetic), effectively blocked resistin-induced eNOS downregulation. Meanwhile, resistin activated the mitogen-activated protein kinases p38 and c-Jun NH2-terminal kinase (JNK), and the specific p38 inhibitor SB-239063 effectively blocked resistin-induced ROS production and eNOS downregulation. Furthermore, immunoreactivity of resistin was increased in atherosclerotic regions of human aorta and carotid arteries. Thus resistin directly induces eNOS downregulation through overproduction of ROS and activation of p38 and JNK in HCAECs. Resistin-induced mitochondrial dysfunction and imbalance in cellular redox enzymes may be the underlying mechanisms of oxidative stress.

NUMEROUS FACTORS CONTRIBUTE to the risk for atherosclerotic vascular disease and subsequent cardiovascular events. Among them, obesity has become a major health concern bearing important social and economical impacts (5). It is associated with increases in all-cause mortality, including death from cardiovascular disease and heart failure (13, 51). Recent studies indicate that adipose tissue is not only an organ of energy storage but also an endocrine organ producing a number of bioactive molecules such as leptin, adiponectin, tumor necrosis factor-α (TNF-α), plasminogen activator inhibitor type 1, and resistin (21). Over the past several years, much effort has been made to explore the mechanisms linking these adipokines to insulin resistance, endothelial dysfunction, and vascular disease (21, 37).

Resistin is a newly described adipokine that has been suggested to play a role in the development of insulin resistance and obesity (31). In patients with type 1 and 2 diabetes, obesity, and inflammatory conditions, plasma levels of resistin can be elevated to >40 ng/ml (14, 16, 30). Chronic inflammation contributes to the formation of atherosclerosis (32). Clinical studies have indicated that high plasma levels of resistin are associated with the severity of coronary disease and hypertension (7, 8, 26, 33, 41, 50). Several types of cells can express resistin. In mice, adipocytes appear to be the major source of resistin (47). In humans, resistin may also come from macrophages and monocytes (42, 44). Macrophages in inflammatory sites such as obese adipose tissues and atherosclerotic lesions may secrete resistin (24). High levels of resistin can be detected at the atherosclerotic tissues (4). Although human vascular endothelial cells did not express resistin (44), high systemic and/or local concentrations of resistin could induce endothelial dysfunction. Indeed, resistin directly induces endothelin-1 production, upregulates adhesion molecules and chemokines, and downregulates TNF receptor-associated-factor-3 (49). Our previous reports and other studies also showed that resistin can impair endothelium-dependent vasorelaxation (18, 29) and induce angiogenesis (38).

However, the precise mechanisms of resistin-mediated effects on vascular cells are largely unknown. In the present study, we hypothesized that resistin may affect endothelial nitric oxide synthase (eNOS) expression and regulation through oxidative stress and mitogen-activated protein kinase (MAPK) activation. Specifically, the effects of resistin on eNOS mRNA, protein levels, and activity were determined in human coronary artery endothelial cells (HCAECs). Reactive oxygen species (ROS) production, mitochondrial membrane potential, and internal antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD) activities, as well as MAPK phosphorylation were investigated. Expression levels of resistin in human atherosclerotic tissues were also determined. This study provides new insights into the mechanisms by which resistin interacts with endothelial cells, which may contribute to cardiovascular disease.

MATERIALS AND METHODS

Cell culture. HCAECs and endothelial growth medium-2 were purchased from Cambrex BioWhittaker (Walkersville, MD). Cells were used at passage 4 to 6. Human recombinant resistin was obtained from Phoenix Pharmaceuticals (Belmont, CA). In the culture condition, we confirmed that almost all HCAECs had positive immunostaining for eNOS, one of the most important functional markers for oxidative stress; mitogen-activated protein kinase

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mature endothelial cells [Supplemental Fig. S1 (Supplemental data for this article may be found on the American Journal of Physiology: Heart and Circulatory Physiology website.)].

Real-time PCR. Total RNA from HCAECs was isolated. cDNA was generated by reverse transcription from mRNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control to account for variations in mRNA loading. Real-time PCR was performed in an iCycler iQ real-time PCR detection system (Bio-Rad). To assess the mRNA stability or half-life of eNOS mRNA, HCAECs were treated with 5 μg/ml actinomycin D [a direct inhibitor of RNA polymerase II (RNAPII)] in the presence or absence of resistin (40 ng/ml). Total cellular RNA was isolated at multiple time points (0, 0.5, 1, 3, 6, and 12 h) and analyzed for eNOS mRNA levels by real-time PCR. In separate experiments, HCAECs, human aorta smooth muscle cells (AoSMCs), and human THP-1-derived macrophages were treated with or without TNF-α (10 ng/ml) for 24 h, and resistin mRNA levels were determined by real-time PCR.

Western blot. Equal amounts of endothelial proteins (6 μg) were resolved electrophoretically by one-dimensional SDS-PAGE (10% polyacrylamide). Subsequently, the proteins were electrophoretically transferred to nitrocellulose. eNOS was detected using a mouse anti-human eNOS monoclonal antibody (BD Biosciences, San Jose, CA), and β-actin was detected using a mouse anti-human β-actin monoclonal antibody (Sigma). The eNOS and β-actin primary antibodies were detected with horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody.

NOS activity assay. A fluorometric cell-associated NOS detection system (Sigma) was used to measure intracellular production of nitric oxide (NO) from supplemented L-arginine by a nonradioactive method.

Nitrite detection. NO levels released from HCAECs were determined by measuring the accumulation of its stable degradation products, nitrite and nitrate (Griess reaction NO assay kit; Calbiochem). Total nitrite levels in cell culture supernatants were measured and normalized to total proteins of HCAECs (pmol/mg protein).

Flow cytometry analyses. Cells were harvested with 0.025% Trypsin/EDTA and adjusted to 1 × 106 cells per each FACS tube. For ROS and NO staining, respectively, dihydroethidium (DHE, 3 μM; Molecular Probes) and 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF, 10 μM; Molecular Probes) were added and incubated at 37°C for 30 min. DHE is often used for the detection of ROS production because it can be oxidized by ROS such as superoxide anion (O2−) into two red fluorescent molecules [2-hydroxyethidium (2-EOH, excitation wavelength 490 and emission wavelength 580 nm) and ethidium (excitation wavelength 480 and emission wavelength 580 nm)] (35). Samples were analyzed using FACSscan and Cell Quest software (Becton Dickinson, Franklin Lakes, NJ). Loss of mitochondrial membrane potential was assessed using flow cytometry analysis of cells stained with 5,5′,6,6′-tetrachloro-1,1′,3′,3′-tetraethylbenzimidazolylcarbo cyanide iodine (JC-1, MitoScreen kit; BD Biosciences). HPLC analysis of O2−. We confirmed the increased production of O2− in the resistin-treated HCAECs by DHE staining and HPLC analysis as previously described, which is able to separate 2-EOH and ethidium (15, 53, 55). DHE was added in the cell culture medium with a final concentration of 25 μM and incubated for 30 min. The cells were harvested in 200 μl methanol and sonicated for 10 s, and the cell lysates were subjected to HPLC analysis.

Cellular glutathione assay. GSH-Glo Glutathione assay (Promega, Madison, WI) measures a change in the redox state of the cell due to oxidants, which are downstream metabolites of O2−.

Measurement of CAT and SOD activity. CAT and SOD enzyme activities were measured with commercial enzyme assay kits (Cayman Chemical, Ann Arbor, MI) following the manufacturer’s protocols. The CAT and SOD activity was represented as nanomoles per minute per milliliter and units per milliliter, respectively.

BioPlex immunoassay. HCAECs were cultured with 40 ng/ml of resistin for 0, 5, 10, 20, 30, 45, 60, or 90 min. Cell lysate was prepared. Detection of phospho- and total extracellular signal-regulated kinase (ERK) 1/2, c-Jun NH2-terminal kinase (JNK), and p38 was performed by the BioPlex LumineX system 2200 (Bio-Rad).

Immunohistochemical analysis. Full-thickness arterial wall specimens of aorta and carotid arteries were obtained from five patients with or without atherosclerosis undergoing autopsy [National Disease Research Interchange (NDRI), Philadelphia, PA]. Immunohistochemistry was done using anti-resistin antibody (1:200) (Phoenix Pharmaceuticals, Burlingame, CA), biotinylated secondary antibody, and avidin-biotin reaction using peroxidase enzyme (ABC kit; Vector Laboratories, Burlingame, CA). The protocol of use of human tissues obtained from NDRI was approved by the Institutional Review Board at the Baylor College of Medicine. The investigation conformed to the principles outlined in the Declaration of Helsinki.

Statistical analysis. Data from the different treatment groups and control groups were compared with a paired Student’s t-test (two tails). Significance was considered if P < 0.05. Data are reported as means ± SE.

Details and methods are provided in the online supplementary materials.

RESULTS

Resistin decreases eNOS expression and NO levels in HCAECs. HCAECs were treated with resistin in a concentration- and time-dependent manner. eNOS mRNA and protein levels were detected using real-time PCR (n = 3) and Western blot (n = 3), respectively. When cells were treated with resistin (40 or 80 ng/ml) for 24 h, eNOS mRNA levels were decreased by 32 and 44%, respectively, compared with controls (P < 0.05, Fig. 1A). When cells were cultured with resistin (40 ng/ml) for different times, only the 24-h treatment group showed a significant reduction of eNOS mRNA levels compared with controls (P < 0.01, Fig. 1B). To determine the specific effect of resistin on eNOS expression, HCAECs were treated with resistin (40 ng/ml) and anti-resistin antibody (1 or 4 μg/ml), isotype IgG (4 μg/ml), or anti-bromodeoxyuridine antibody (4 μg/ml) for 24 h. Anti-resistin antibody at a concentration of 4 μg/ml significantly blocked the decrease in eNOS induced by resistin (P < 0.05, Fig. 1C). Isotype and anti-bromodeoxyuridine antibodies as negative controls at the same concentration showed no effect on the resistin-induced eNOS mRNA decrease (Fig. 1C). In addition, by using actinomycin D, a direct inhibitor of RNA polymerase II, we showed that eNOS mRNA stability was significantly decreased in cells treated with resistin (40 ng/ml) compared with control cells (P < 0.05, Fig. 1D). The half-life of eNOS mRNA decreased from >12 h in control cells to <6 h in resistin-treated HCAECs.

Western blot showed significant decreases in eNOS protein levels after treatment with resistin (40 or 80 ng/ml) for 24 h, compared with controls (P < 0.05, Fig. 2A). NOS activities were also studied using a NOS detection system (n = 3). Consistent with the real-time PCR and Western blot data, resistin at 40 and 80 ng/ml significantly decreased NOS activity by 25 and 36%, respectively, compared with controls (P < 0.05, Fig. 2B).

We also measured cellular NO levels in HCAECs after resistin treatment using the fluorescent dye DAF. DAF staining is a unique method measuring NO production in living cells or solutions (28). Treated HCAECs were incubated with 10 μM
of DAF for 30 min and then washed. The stained cells were studied using flow cytometry assay. Resistin at 40 and 80 ng/ml decreased the NO positive cell number by 10 and 31%, respectively, compared with controls (Fig. 2C). Furthermore, NO-derived nitrite levels in the supernatants of HCAECs were determined by Griess assay. Resistin treatment at 40 ng/ml for 24 h led to significantly decreased GSH levels compared with controls (Fig. 2D). HCAECs were also treated with resistin for different time points (0, 10, 20, 40, and 120 min), and reduced GSH levels were seen after treatment for 120 min (Fig. 3D), indicating oxidative stress.

Resistin decreases mitochondrial membrane potential and ATP production in HCAECs. Mitochondria are known to be a major source of ROS, and they are also particularly susceptible to oxidative damage produced by the action of ROS on lipids, proteins, and DNA (17). For this reason, the membrane potential can serve as an indicator of mitochondrial respiratory chain function. HCAECs were seeded on six-well plates and cultured to oxidative damage produced by the action of ROS on lipids, proteins, and DNA (17). For this reason, the membrane potential can serve as an indicator of mitochondrial respiratory chain function. HCAECs were seeded on six-well plates and cultured with or without resistin (40 ng/ml) for 24 h. Treatment with resistin substantially reduced mitochondrial membrane potential by 68% compared with controls (Fig. 3E).

Energy produced by mitochondrial respiration is used for ATP synthesis by a complex mechanism referred to as “oxidative phosphorylation.” Impairment in mitochondrial respiration can lead to a decrease in ATP production. We determined ATP levels in HCAECs by using the ATPLite kit. Treatment with resistin (40 ng/ml) significantly reduced ATP levels by 29% compared with controls (P < 0.05, n = 3, Supplemental Fig. S2).

To further confirm the mitochondrial source of ROS, HCAECs were treated with resistin, the mitochondrial inhibitor thenoyltrifluoroacetone (TFFA), or a combination of both for 24 h, and ROS production was detected by GSH assay. Resistin reduced GSH levels, whereas TFFA effectively blocked the effect of
Resistin in HCAECs (Fig. 3C). This indicates that mitochondria contribute to resistin-induced ROS production in HCAECs.

To determine whether resistin causes cell death in the current experimental conditions, HCAECs were treated with resistin (80 ng/ml) for 24 and 48 h, and cell viability was studied with a MTS assay. There was no significant difference of cell viability between control and resistin-treated cells at either the 24- or 48-h time points (Supplemental Fig. S3). Thus resistin used in the current study did not affect cell viability or apoptosis in HCAECs.

Resistin decreases CAT and SOD activities in HCAECs. A variety of intrinsic antioxidants, including CAT and SOD, are present in organisms and protect them from oxidative stress (39). The enzyme activities of CAT and SOD were studied with commercial assay kits (n = 3 for each). Treatment with resistin at 40 or 80 ng/ml significantly reduced CAT activities by 19 and 26%, respectively, compared with controls (P < 0.05, Fig. 3F). Similarly, SOD activities were also reduced by 30 and 53%, respectively (P < 0.05, Fig. 3G).

LY-83583 induces ROS overproduction and eNOS mRNA reduction. To demonstrate the direct link between increased ROS and decreased eNOS mRNA expression levels, we included LY-83583, a known superoxide generator (22), in our experiments as a positive control. HCAECs were treated with LY-83583 (3 μM) for 24 h. DHE staining showed an increase in ROS production by 61% compared with controls (P < 0.05, n = 3, Fig. 4A and B). Meanwhile, in HCAECs treated with LY-83583, eNOS mRNA levels showed a decrease (33%), which was similar to that seen in resistin-treated cells. Coculture with the antioxidant selenomethionine (SeMet) reduced ROS production and increased eNOS mRNA expression to the level of untreated controls (Fig. 4C). Furthermore, NO released from HCAECs was detected by Griess assay. LY-83583 significantly reduced both basal and bradykinin-stimulated levels of NO (Fig. 2D).

Antioxidants SeMet, ginsenoside Rb1, and SOD mimetic MnTBAP block resistin-induced eNOS downregulation in HCAECs. Because oxidative stress is involved in the action of resistin, we further tested whether antioxidants could block the reduction in eNOS mRNA induced by resistin. HCAECs were cocultured with resistin (40 ng/ml) and antioxidant SeMet (20 μM) for 24 h. The eNOS mRNA decrease seen in cells treated with resistin alone was significantly blocked by the addition of SeMet (P < 0.05, n = 3, Fig. 4D). SeMet alone showed no effect on eNOS expression. In addition, resistin-induced decreases in cellular CAT and SOD activities were also blocked by SeMet (P < 0.05, n = 3, Fig. 3, F and G). To determine the effect of the specific O2− scavenger MnTBAP on resistin-induced eNOS downregulation in HCAECs, the cells were treated with resistin (40 ng/ml) and/or MnTBAP (2 μM) for 24 h. The eNOS mRNA and protein levels were determined by real-time PCR and Western blot analysis, respectively. As shown in Fig. 4, E and F, MnTBAP effectively blocked resistin-induced eNOS downregulation at both mRNA and protein levels. In addition, the action of ginsenoside Rb1 on these resistin-mediated events
was examined in HCAECs. Adding ginsenoside Rb1 effectively blocked resistin-induced eNOS downregulation ($P < 0.05$, $n = 3$, Fig. 5A). Also, ginsenoside Rb1 was able to reduce resistin-induced ROS production (Fig. 3).

**Resistin induces MAPK p38 and JNK activation.** Phosphorylation of MAPKs was investigated with a BioPlex immunoassay. After resistin treatment (40 ng/ml), there were an early p38 phosphorylation peak at 5–10 min and a second peak at 30–45 min. JNK activation only showed a single peak at 45 min after the treatment. However, ERK1/2 did not show any significant changes at any time points (Fig. 5B). To confirm the functional role of p38 activation in the action of resistin, a p38 inhibitor (SB-239036, 1 μM) was used to pretreat HCAECs for 1 h. Next, the cells were cultured with resistin (40 ng/ml) for 24 h, and eNOS mRNA levels were detected using real-time PCR. Pretreatment with SB-239036 effectively blocked the resistin-induced eNOS decrease ($P < 0.05$, $n = 3$, Fig. 5A).

Meanwhile, treatment with SB-239036 alone did not show any effect on eNOS expression. Also, in the assay for ROS production, SB-239036 effectively blocked resistin-induced ROS production in HCAECs (Fig. 3)

**Level of human resistin is increased in human atherosclerotic vessels.** Because resistin can be expressed by human macrophages (42), we performed immunostaining to determine

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**Fig. 3.** Effect of resistin on oxidative stress in HCAECs. A: reactive oxygen species (ROS) production [dihydroethidium (DHE) staining and flow cytometry analysis]. HCAECs were treated with 40 or 80 ng/ml of resistin for 24 h. * $P < 0.05$, $n = 3$. B: superoxide anion (O$_2^-$) levels (DHE staining and HPLC analysis). HCAECs were treated with resistin (40 ng/ml) for 3 h, and then DHE (25 μM) was added to the cells for 30 min. Cellular levels of 2-hydroxyethidium (2-EOH) and ethidium were determined by HPLC analysis. Positive control of 2-EOH was prepared by using nitrosodisulfonate radical dianion (NDS) with DHE in the aqueous phosphate buffer, pH 7.4, containing diethylene triamine pentaacetic acid (DTPA). Specific O$_2^-$ scavenger manganese [III] tetrakis(4-benzoic acid)porphyrin [MnTBAP, superoxide dismutase (SOD) mimetic] was used. C: ROS production (glutathione assay). HCAECs were treated with resistin and/or other molecules for 24 h. FL1-H, relative light units; TFFA, thenoyltrifluoroacetonate. * $P < 0.05$, $n = 3$ [compared with dimethyl sulfoxide (DMSO) controls]. # $P < 0.05$, $n = 3$ (compared with resistin treatment alone). D: time course of ROS production (glutathione assay). HCAECs were treated with resistin (40 μg/ml) for different time points. * $P < 0.05$, $n = 3$ [compared with dimethyl sulfoxide (DMSO) controls]. # $P < 0.05$, $n = 3$ (compared with resistin treatment alone). E: mitochondrial membrane potential (JC-1 staining and flow cytometry). F: catalase activity assay. HCAECs were treated with resistin (40 or 80 ng/ml) and/or selenomethionine (SeMet, 20 μM) for 24 h. * $P < 0.05$, $n = 3$ (compared with DMSO controls). # $P < 0.05$, $n = 3$ (compared with resistin treatment alone). G: SOD activity assay. HCAECs were treated with resistin (40 or 80 ng/ml) and/or SeMet (20 μM) for 24 h. * $P < 0.05$, $n = 3$ (compared with DMSO controls). # $P < 0.05$, $n = 3$ (compared with resistin treatment alone).
resistin levels in human arteries. Nonatherosclerotic vessels, including human aorta and carotid arteries, showed no or very limited resistin immunoreactivity. In contrast, atherosclerotic regions of these arteries displayed strong resistin immunoreactivity. The increased signal of resistin was mainly located in the regions of the intima and media of atherosclerotic plaques (Fig. 6). To confirm the capability of three cell types of vascular tissues (macrophages, smooth muscle cells, and endothelial cells) to express resistin in response to TNF-α, the cells were treated with or without TNF-α (10 ng/ml) for 24 h, and resistin mRNA levels were determined by real-time PCR. As shown in Supplemental Fig. S4, macrophages expressed much higher levels of resistin mRNA than AoSMCs and HCAECs, which had very low levels of resistin mRNA; and TNF-α had no effect on resistin expression in all cell types.

DISCUSSION

In this study, we present evidence that human recombinant resistin is able to induce endothelial dysfunction in HCAECs.
Specifically, resistin significantly reduces eNOS mRNA, protein levels, and NO activity as well as NO bioavailability in HCAECs. In addition, resistin directly induces oxidative stress and activation of p38 and JNK MAPKs in HCAECs. This study provides insight into the biological functions and molecular mechanisms of resistin in the vascular system.

Consistent with previous reports (4), we observed increased immunochemistry reactivity of resistin in human atherosclerotic lesions; however, we did not know which cell types in the vascular wall could express resistin. Our in vitro experiments showed that macrophages expressed much higher levels of resistin mRNA than AoSMCs and HCAECs, which had very low levels of resistin mRNA; and TNF-α had no effect on resistin expression in all cell types. Thus we speculate that resistin in the vascular lesion might be produced by vascular macrophages. It is also possible that plasma resistin could enter the vascular wall. Further studies of the source of resistin in the vascular wall are warranted.

In the endothelium, NO is constitutively generated from the conversion of L-arginine to L-citrulline by the enzymatic action of eNOS, which is considered to be the most important factor in maintaining normal vascular function. However, under various pathological conditions, eNOS may become dysfunctional or its expression may be decreased (25). Dysfunctional eNOS or low levels of eNOS not only impair endothelium-dependent vasorelaxation but also accelerate atherosclerotic lesion formation (45). In the present study, we demonstrated a direct effect of resistin on cultured HCAECs. Using real-time PCR analysis, we showed that resistin at concentrations relevant to plasma concentrations of obese individuals (14, 16) can decrease eNOS mRNA levels in a time- and concentration-dependent manner. Western blot analysis also confirmed that eNOS protein levels were decreased after resistin treatment. This effect is specific because anti-resistin antibody effectively blocked the effect of resistin on eNOS expression. Indeed, treatment with LY-83583 led to a decrease in eNOS expression. These data are consistent with our previous publication in which we showed, using the lucigenin-enhanced chemiluminescence method, that resistin increased O₂⁻ production in porcine coronary artery rings; this effect was inhibited by the antioxidant seleno-L-methionine (29).

Cardiovascular disease is a multifactorial disease, but ROS have been shown to be key mediators for vascular inflammation and atherogenesis (36). Human investigations support the oxidative stress hypothesis of atherogenesis (2, 36). This is further supported by impaired vascular function and enhanced atherogenesis in animal models that have deficiencies in internal antioxidant enzymes (12). In the present study, we stained cells with a fluorescent DHE dye to detect ROS in HCAECs using flow cytometry assay. We found that resistin significantly induced an increase in ROS levels in HCAECs. DHE is often used for the detection of ROS production because it can be oxidized by ROS such as O₂⁻ into two red fluorescent molecules (2-EOH and ethidium) (35). 2-EOH is generated specifically by O₂⁻ oxidation of DHE, whereas ethidium is associated mainly with pathways involving H₂O₂ and metal-based oxidizing systems, including heme proteins and peroxides. Flow cytometry analysis at the current condition can detect both 2-EOH and ethidium but cannot separate 2-EOH and ethidium. Recent studies showed that HPLC analysis can separate 2-EOH and ethidium after DHE staining (15, 54, 55).

In the current study, we confirmed the increased production of O₂⁻ in the resistin-treated HCAECs by HPLC analysis. Specific O₂⁻ scavenger MnTBAP (SOD mimetic) is also able to effectively block the effect of resistin on 2-EOH increase. The data obtained from DHE staining were confirmed using an assay of cellular GSH levels, which are negatively correlated to oxidative stress. Meanwhile, LY-83583 (O₂⁻ generating molecule) was used in this study to confirm the role of O₂⁻ in eNOS expression. Indeed, treatment with LY-83583 led to a decrease in eNOS expression in HCAECs. These data are consistent with our previous publication in which we showed, using the lucigenin-enhanced chemiluminescence method, that resistin increased O₂⁻ production in porcine coronary artery rings; this effect was inhibited by the antioxidant seleno-L-methionine (29). Thus resistin has a strong impact on ROS production, which is likely one of the mechanisms for eNOS downregulation and dysfunction. Dick et al. (12), however, did not observe ROS production in resistin-treated canine coronary arteries by DHE tissue staining; there were, however, no quantitative data presented. The reason for this discrepancy is not clear. It could be because of experimental conditions, different species, and/or sensitivity of detection methods.

O₂⁻ is produced by a variety of sources, including the mitochondrial respiratory chain (17), NADH/NADPH oxidase (20), xanthine oxidase (46), and lipooxygenases (9). In addition, there are several cellular mechanisms that counterbalance the production of O₂⁻, including enzymatic and nonenzymatic pathways (40). Among them, the best-characterized enzymatic...
pathways are SOD, which facilitates the formation of H$_2$O$_2$ from O$_2$ (19), CAT (27), and glutathione peroxidase (GPX) (10), which coordinates the conversion of H$_2$O$_2$ to water. To study potential sources of increased O$_2^\bullet$ in resistin-treated cells, we tested mitochondrial membrane potential and ATP production in resistin-treated cells. In both areas, we found a significant decrease after treatment with resistin; this suggests that resistin induces mitochondrial dysfunction. This, in turn, may be partially responsible for the increase in O$_2^\bullet$ production detected in resistin-treated cells. TTF-A, a mitochondrial inhibitor, effectively blocked the resistin-induced ROS production in HCAECs. In addition, we tested enzyme activities of CAT and SOD in HCAECs. Clearly, treatment with resistin substantially decreased the activities of both enzymes; this may impair the internal cellular response to oxidative stress in resistin-treated cells. Thus, resistin-increased O$_2^\bullet$ production may result from mitochondrial dysfunction and compromised cellular redox enzymes.

Understanding the underlying molecular mechanisms of the action of resistin can help us to develop new and effective strategies to control the detrimental effects of resistin on the vascular system. Because the action of resistin may be mediated by oxidative stress, we have tested the ability of the antioxidants SeMet, ginsenoside Rb1, and SOD mimetic MnTBAP to block resistin’s effects on resistin-treated cells. Indeed, all three antioxidants effectively blocked resistin-induced eNOS downregulation and ROS production. SeMet participates in an intramolecular transsulfuration reaction, forming selenocysteine, which, in turn, increases the activity of internal antioxidant enzymes GPX and thioredoxin reductase (1). In addition, SeMet is able to directly interact with some oxidant molecules or oxidant-generating ions (3, 43). Ginsenosides act as antioxidants by increasing internal antioxidant enzymes and acting as free-radical scavengers (11, 34, 48, 52). Previously, we have successfully used ginsenoside Rb1 to block homocysteine- and HIV protease inhibitor-induced oxidative stress and endothelial dysfunction in porcine coronary arteries (6, 54). Overall, antioxidant therapy may provide an effective strategy to prevent vascular diseases mediated by these specific risk factors, such as resistin, as well as others.

MAPKs are important in regulating cell growth, migration, and differentiation in response to various extracellular stimuli (23). Three major subfamilies of structurally related MAPKs have been identified in mammalian cells: p44/42 MAPK (ERK1/2), p38 MAPK, and JNK/stress-activated protein kinases. The pattern of MAPK activation in response to oxidative stress varies depending on the oxidant strength and cell type. Detailed regulation pathways for MAPKs are still unknown. In the present study, we demonstrated that p38 and JNK were activated in response to resistin stimulation, and we also demonstrated that the p38 inhibitor SB-239063 effectively blocked resistin-induced eNOS downregulation and ROS production in HCAECs. Because resistin increased ROS production at 120 min and induced p38 phosphorylation at 40 min, and p38 inhibitor SB-239063 effectively blocked resistin-induced ROS production, we conclude that MAPK activation may occur upstream of oxidative stress in resistin-treated HCAECs.

In conclusion, our study demonstrates that resistin, at concentrations found in obese individuals, significantly decreases eNOS expression and NO production through oxidative stress and p38 and JNK MAPK activation in HCAECs. Resistin-induced mitochondrial dysfunction and unbalanced cellular redox enzymes may be the underlying mechanisms of increased ROS production. These data, combined with the finding that resistin expression is increased in human atherosclerotic tissues, support the hypothesis that resistin may contribute to vascular disease through endothelial dysfunction. Antioxidants SeMet, ginsenoside Rb1, and MnTBAP potentially hold clinical applications in blocking resistin-induced endothelial dysfunction, thereby preventing vascular disease.

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DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES


