

Resistin Promotes Endothelial Cell Activation Further Evidence of Adipokine-Endothelial Interaction

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Background—Adipocyte-derived hormones may represent a mechanism linking insulin resistance to cardiovascular disease. In the present study, we evaluated the direct effects of resistin, a novel adipocyte-derived hormone, on endothelial activation.

Methods and Results—Endothelial cells (ECs) were incubated with human recombinant resistin (10 to 100 ng/mL, 24 hours), and endothelin-1 (ET-1) release, ET-1 mRNA expression, and nitric oxide (NO) production were assessed. Transient transfection assays were used to evaluate the effects of resistin on transcription of human ET-1 gene promoter. Furthermore, the effects of resistin on AP-1–mutated ET-1 promoter were evaluated. The effects of resistin on expression of vascular cell adhesion molecule (VCAM-1) and monocyte chemoattractant chemokine (MCP-1) were studied in addition to CD40 receptor, CD40 ligand–induced MCP-1 expression, and tumor necrosis factor receptor–associated factor-3 (TRAF3), an inhibitor of CD40 signaling. Incubation of ECs with resistin resulted in an increase in ET-1 release and ET-1 mRNA expression, with no change in NO production. Whereas treatment with resistin resulted in an increase in ET-1 promoter activity, the AP-1–mutated promoter was inactive after resistin stimulation. Additionally, resistin-treated cells showed increased expression of VCAM-1 and MCP-1, with concomitant reductions in TRAF-3 expression. Resistin did not alter CD40 receptor expression; however, increased CD40 ligand induced MCP-1 production.

Conclusions—The novel adipokine resistin exerts direct effects to promote EC activation by promoting ET-1 release, in part by inducing ET-1 promoter activity via the AP-1 site. Furthermore, resistin upregulates adhesion molecules and chemokines and downregulates TRAF-3, an inhibitor of CD40 ligand signaling. In this fashion, resistin may be mechanistically linked to cardiovascular disease in the metabolic syndrome. (*Circulation*. 2003;108:736-740.)

Key Words: endothelin ■ hormones ■ receptors

The insulin-resistant metabolic syndrome affects a large number of North Americans and is associated with increased rates of atherosclerotic cardiovascular disease.¹ Over the past few years, much effort has been made to understand the interaction between insulin resistance and endothelial dysfunction,² with particular emphasis on adipocyte-derived hormones (adipokines) and their effects on vascular homeostasis.^{3,4} The adipocyte has emerged as a key secretory organ, releasing a number of bioactive molecules such as leptin, adiponectin, tumor necrosis factor- α (TNF- α), plasminogen activator inhibitor type 1, and the recently described hormone resistin.⁵ These adipokines not only function prominently in the pathogenesis of the insulin-resistant syndrome but may also serve as important vasoactive factors, directly affecting endothelial function and vascular health.⁴ The ability of adipokines to directly affect vascular homeostasis may represent an important mechanistic basis of cardiovascular disease in patients with the metabolic syndrome.

Resistin is a recently described novel adipokine that has been suggested to play a role in the development of insulin resistance and obesity.⁵ Although much controversy surrounds the exact biological role of resistin in humans, resistin appears to be produced during adipogenesis and inhibits glucose uptake in skeletal muscle cells in animal models.⁶

In the present study, we hypothesized that the adipokine resistin exerts direct effects to promote endothelial dysfunction. To this aim, we studied the effects of human recombinant resistin on endothelial cell activation and report a direct effect of resistin to upregulate endothelin-1 (ET-1), vascular cell adhesion molecule (VCAM-1), and monocyte chemoattractant chemokine (MCP-1) while downregulating TNF receptor–associated factor-3 (TRAF3), an important inhibitor of CD40 signaling. These data suggest vascular effects of the adipokine resistin and lend credence to the concept of adipocyte–endothelial cell interaction in states of insulin resistance and obesity.

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Methods

Cell Culture

Human saphenous vein endothelial cells (HSVECs) were isolated by digestion with trypsin (0.2%; Fisher) and collagenase type 2 (0.1%; Worthington) and cultured in MCDB-131 complete medium (VEC Technologies, Inc) supplemented with 10% FBS (Gibco) as described previously.⁷ HSVECs at passages 2 through 5 were incubated with purified human recombinant resistin (Phoenix Pharmaceuticals, Inc) for 24 hours at 10, 50, and 100 ng/mL, respectively. Resistin was free of endotoxin, as assessed with a limulus assay (<0.1 ng/mL). Additional control studies were also performed in the presence of polymyxin-B to ensure that the observed effects on endothelial cell activation were independent of endotoxin contamination. Preliminary studies were performed to optimize both incubation time and the resistin concentrations used (data not shown). The plasma concentrations of resistin in patients with insulin resistance remain to be carefully defined, although preliminary studies suggest that mean circulating resistin levels may be ≈40 ng/mL in diabetes (versus ≈15 ng/mL in lean nondiabetic patients).^{8,9}

Cell Adhesion Molecule Expression

Endothelial cells were detached by nonenzymatic cell dissociation solution (Sigma) and were stained for CD106 (VCAM-1) or CD31 (platelet-endothelial cell adhesion molecule-1, or PECAM-1) with monoclonal R-phycoerythrin-conjugated anti-human CD106 or anti-CD31 antibodies (Pharmingen), all at a 1:5 dilution. Cells were analyzed with a Beckman Coulter EPICS XL flow cytometer with EXPO32 ADC software. The fluorescence intensity of 10 000 cells for each sample was quantified, and unstained cells were used as controls.

ET-1, Nitric Oxide, and MCP-1 Measurement

Nitric oxide (NO) production was detected by measuring its final stable equimolar degradation products, nitrite and nitrate. Total nitrite was quantified after the reduction of all nitrates with nitrate reductase (Roche). The measurement of nitrite was performed in a total volume of 0.5 mL with 480 μ L of culture supernatant, 10 μ L of nitrate reductase (10 U/mL), and 10 μ L of NADPH (5 mmol/L in Tris Cl) for 3 hours at room temperature. After the conversion of nitrate to nitrite, total nitrite was determined spectrophotometrically at 540 nm by use of the Griess reaction (sulfanilamide 58 mmol/L, naphthylenediamine 3.86 mmol/L, H₃PO₄ 0.43 mol/L). Total nitrite concentration was calculated from a standard curve constructed over the linear range of the assay and expressed as micromoles per milligram of protein. ET-1 secretion into the culture supernatant was assessed with a commercial enzyme immunoassay kit (American Research Products, Inc).¹⁰ In some experiments, we evaluated the effects of resistin-neutralizing antibodies on resistin-induced ET-1 release from endothelial cells. In these studies, endothelial cells were treated with human resistin (100 ng/mL, 24 hours) in the presence and absence of a neutralized anti-resistin antibody (Phoenix Pharmaceuticals, Inc). Resistin-neutralizing antibody (2 μ g/mL) was added 60 minutes before the addition of resistin. Culture supernatant was collected, and the secretion of MCP-1 was assessed by sandwich ELISA (R&D Systems).¹⁰

ET-1 mRNA Expression

Total cellular RNA was isolated by lysis of cultured HSVECs in guanidinium isothiocyanate followed by phenol extraction. Reverse transcription-polymerase chain reaction (RT-PCR) was performed with the Qiagen One-Step RT-PCR kit as per the manufacturer's instructions. One microgram of total RNA served as a template for each reaction. For amplification, a primer pair specific for human ET-1 (sense primer, 5'-ACAGCAGTCTTAGGCGCTGAGCTCAGC-3'; antisense primer, 5'-TGGCTAGCACATTGGCATCTATTCTCA-3') was used. The RT-PCR product was 304 bp. Reverse transcription was performed at 50°C for 30 minutes. For PCR, 30 cycles were used at 94°C for 1 minute, 50°C for 1 minute, and 72°C for 2 minutes. The RT-PCR products were

visualized on 1% agarose gels with ethidium bromide. GAPDH was amplified as a reference for quantification of ET-1 mRNA. Densitometric scanning, to quantify amounts of RT-PCR product, was performed with the use of the PDI ImageWare System. The signal intensity of each ET-1 band was normalized by that of GAPDH.

Plasmids

To construct the human ET-1 promoter/luciferase reporter constructs, a -250/+55 promoter fragmenter was amplified by PCR from human genomic DNA (Clontech) with the oligonucleotides 5'-AAC TCG AGG GCG CAG GTT TAG CAA AGG TCT CTA ATG GG-3' and 5'-CTT AAG CTT GCT TCA GGT CCC TCA AAG CGC TGC. The resulting PCR product was digested and inserted in the Xho/*Hind*III sites of the pGL2 luciferase vector (Promega), which yielded pGL2-hET-1. Internal mutation of the ET-1 promoter AP-1 site was introduced with a site-directed mutagenesis kit (Stratagene) and the following oligonucleotides: 5'-CTG CAC GTT GCC TGT TGG GTA CTA ATA ACAC-3' and 5'-GTG TTA TTA GTA CCC AAC AGG CAA CGT GCAG-3', which led to mutation of the AP-1 site *GTGACTAA* to *GGTACTAA*. All constructs were verified by DNA sequencing analysis.

Transient Transfection Assays

HSVECs were grown to between 70% and 80% confluence on 6-well plates. Cells were transiently transfected by the Fugene 6 method (Roche) with 2 μ g of wild-type pGL2-hET-1 or mutated pGL2-hET-1 mut in the presence or absence of 100 ng/mL resistin. To correct for variability in transfection efficiency, 200 ng of p β gal control plasmid DNA (Clontech) was cotransfected in all experiments. Cell extracts were prepared 48 hours after transfection, and the luciferase and β -galactosidase assays were performed with the reporter assay kits (Clontech), respectively. Each experiment was performed at least 3 times, and each transfection was performed in triplicate.

TRAF-3 and CD40 Protein Expression

TRAF-3 and CD40 protein expression was assessed by Western blot. Briefly, endothelial cell lysates were separated by 10% SDS-PAGE gel, and the separated proteins were transferred to PVDF gel. Blots were blocked for 1 hour at room temperature with blocking buffer (5% nonfat milk in 10 mmol/L Tris, 100 mmol/L NaCl, 0.1% Tween 20, pH 7.5). Anti-TRAF 3 monoclonal antibody (Santa Cruz) at a dilution of 1:100 or anti-CD40 polyclonal antibody (Santa Cruz) at a dilution of 1:200 was reacted with the blots overnight at 4°C. After wash (3 \times 5 minutes in 1 \times TBS-0.05% Tween), the blots were incubated with horseradish peroxidase-conjugated secondary antibody at 1:3000 dilution for 1 hour at room temperature. Visualization was performed with the use of enhanced chemiluminescence. Densitometric analysis of Western blots was performed with the use of the PDI ImageWare System. Finally, to evaluate whether resistin augmented the proatherogenic effects of CD40 ligand, the effects of CD40 ligand (0.5 μ g/mL; Research Diagnostics Inc) on MCP-1 production in resistin-treated endothelial cells were evaluated.

Statistical Analysis

Group data are expressed as mean \pm SEM. Data were compared between experimental groups by 1-way ANOVA. Differences between groups were further evaluated by Fisher protected least squares differences. Differences were considered significant at a value of $P < 0.05$.

Results

In the first series of experiments, we evaluated the direct effects of human recombinant resistin (10 to 100 ng/mL) on ET-1 secretion, ET-1 mRNA, and NO release in HSVECs in culture. Incubation of HSVECs with resistin (24 hours) resulted in a significant increase in ET-1 secretion at both 50 and 100 ng/mL concentrations (Figure 1A). The effect was specific to resistin,

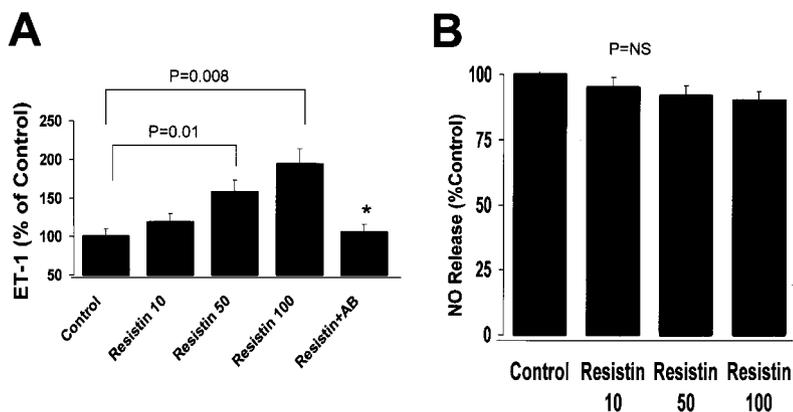


Figure 1. A, Effects of human recombinant resistin (10 to 100 ng/mL, 24 hours) on ET-1 production in cultured endothelial cells. Resistin significantly increased ET-1 release when used at both 50 and 100 ng/mL concentrations. Endothelial cells incubated with resistin (100 ng/mL) plus resistin-neutralizing antibodies (AB) exhibited no increase in ET-1 release. Experiments shown here are representative of 6 independent experiments and were performed in triplicate. * $P < 0.05$, different from resistin 50 and 100 ng/mL. B, Effects of human recombinant resistin (10 to 100 ng/mL) on NO release. Resistin did not affect NO production in endothelial cells at concentrations studied. Experiments shown here are representative of 6 independent experiments and were performed in triplicate. Statistical analysis was performed by ANOVA followed by Fisher protected least squares test.

because resistin-neutralizing antibodies completely inhibited resistin-induced ET-1 release (Figure 1A). The increase in ET-1 secretion was related in part to increased ET-1 transcript expression, because incubation of HSVEC with resistin resulted in almost a 2-fold increase in ET-1 mRNA expression (Figure 2A). Resistin did not result in changes in NO release at the aforementioned concentrations (Figure 1A) or in endothelial NO synthase (eNOS) protein expression (data not shown).

We next evaluated the effects of resistin on modulating ET-1 promoter activation. To this end, ET-1 promoter was PCR amplified, and transient transfection experiments were conducted in endothelial cells (Figure 2B). Because the transcription factor AP-1 plays a key role in the basal transcriptional activation of ET-1,¹¹ a mutation in the AP-1 site of the ET-1 promoter was generated. The effects of resistin on endothelial cells transfected with the wild type or AP-1-mutated promoter luciferase reporter construct were evaluated. Whereas resistin increased ET-1 promoter activity, the AP-1-mutated promoter was not activated, which suggests that the activation of ET-1 promoter by resistin is mediated via AP-1 (Figure 2B). In conjunction with an increase in ET-1, resistin-treated endothelial cells demonstrated an increased expression of the cell adhesion molecule VCAM-1 and augmented release of the key chemoattractant chemokine MCP-1 (Figures 3A and 3B).

To further elucidate an effect of resistin on endothelial cell activation, we evaluated the effects of resistin on TRAF-3 and CD40 expression in endothelial cells. TRAF-3 has been described recently as a novel inhibitor of CD40 ligand-induced endothelial cell inflammation.¹² Figure 4A depicts the effects of resistin to markedly downregulate TRAF-3 expression, an effect that would serve to augment the proatherogenic nature of CD40 ligand. Indeed, resistin-treated endothelial cells responded with greater increases in MCP-1 in response to CD40 ligand (Figure 3B). Resistin did not affect the expression of CD40 receptor, which suggests that endothelial cell activation through the CD40/CD40-ligand pathway occurs independently of receptor upregulation and may be related to decreased TRAF-3 expression (Figure 4B).

Discussion

The adipocyte functions as an important secretory organ, releasing a number of bioactive molecules such as leptin, adiponectin, TNF- α , plasminogen activator inhibitor type 1, angiotensinogen, and the newly described hormone resistin.^{3,5} Adipocytokines play an important role in the development of insulin resistance and obesity and exert effects at the level of the hypothalamus, muscle, and liver in an attempt to regulate energy balance. The exact biological role of resistin in humans remains equivocal.

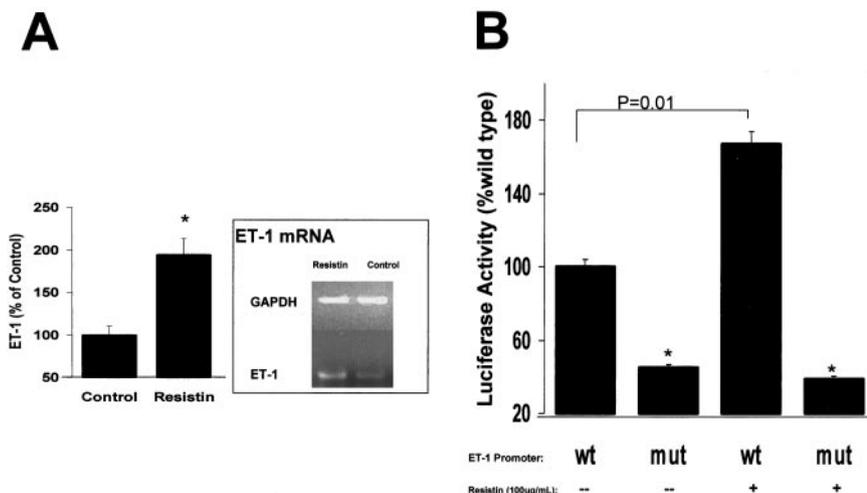


Figure 2. A, Effects of human recombinant resistin (100 ng/mL, 24 hours) on ET-1 mRNA expression in human endothelial cells. Marked upregulation of ET-1 transcript was noted in resistin-treated cells. Experiments shown here are representative of 6 independent experiments performed in duplicate. * $P < 0.05$, different from control. B, Resistin induces ET-1 promoter activity via AP-1 site. Endothelial cells were transiently transfected with wild-type (wt) or AP-1-mutated ET-1 (mut) promoter luciferase construct, and cells were treated with and without resistin (100 ng/mL). Luciferase activities were measured and normalized against an internal cotransfected β -galactosidase standard. Resistin treatment resulted in induction of ET-1 promoter; however, AP-1-mutated promoter was no longer activated, which suggests that resistin induces ET-1 promoter

activity via AP-1 site. Experiments shown are representative of 5 independent experiments performed in duplicate. * $P < 0.05$, different from wild type without resistin and wild type with resistin.

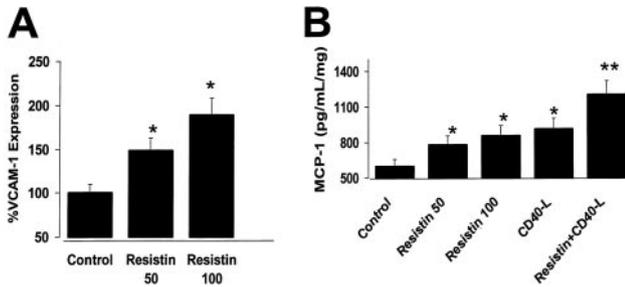


Figure 3. A, Effects of resistin on VCAM-1 expression, determined by flow cytometry. Marked upregulation of VCAM-1 in resistin-treated endothelial cells (50 and 100 ng/mL, 24 hours). Experiments shown are representative of 5 independent experiments performed in duplicate. * $P < 0.05$, different from control. B, Effects of resistin (100 ng/mL) and CD40 ligand (1 μ g/mL; CD40-L) on endothelial cell MCP-1 production. Both resistin and CD40-L increase MCP-1 production in endothelial cells. Notably, in resistin-treated cells, CD40-L incites greater increases in MCP-1, which suggests that CD40-L-mediated endothelial cell activation is enhanced by resistin. Experiments shown are representative of 5 independent experiments performed in duplicate. * $P < 0.05$, different from control, and ** $P < 0.05$, different from control, resistin, and CD40-L.

Patients with the metabolic syndrome of insulin resistance are at an increased risk of cardiovascular disease,¹ and emerging evidence points to a common origin of metabolic and cardiovascular processes.² An important point of convergence of cardiovascular and metabolic pathways is the endothelium, with evidence suggesting that insulin resistance directly promotes endothelial dysfunction,¹³ which is an early and integral part of atherosclerotic vascular disease.^{14,15} In this vein, recent evidence points toward adipokines as intermediaries that link insulin resistance to endothelial dysfunction and vascular decompensation. As such, circulating adipocyte-derived hormones may impair endothelial function by altering vasoactive factor production and inciting endothelial cell activation, with resultant propensity for cardiovascular disease. For example, adiponectin, another adipocyte-derived hormone, serves to improve insulin sensitivity, with decreased levels associated with obesity and the metabolic syndrome. Adiponectin has been demonstrated to favorably affect endothelial function and vascular homeostasis,¹⁶

whereas leptin, another adipocytokine, potently induces ET-1 release in endothelial cells.¹⁷ These observations suggest that a physiologically relevant interaction exists between the adipocyte and vascular control mechanisms (Figure 5).

The latest addition to the adipokine family is the peptide resistin,⁵ named for its ability to cause resistance to insulin and link obesity to diabetes. Resistin belongs to a family of resistin-like molecules, and the resistin mRNA encodes a 114-amino acid polypeptide that contains a 20-amino acid signal sequence.¹⁸ In mice, resistin is expressed predominately in white adipose tissue; however, it is found abundantly in serum, which suggests a role of resistin as a circulating factor capable of modifying responses at distinct organ sites. Resistin levels are markedly elevated in obese mice and lowered by peroxisome proliferator-activated receptor- γ agonists like rosiglitazone. Additionally, antagonism of resistin action (with resistin antibodies) improves insulin sensitivity and lowers plasma glucose, which supports an important role of resistin in the pathogenesis of insulin resistance. Although the link between resistin, obesity, and insulin resistance appears to be strong in mice, the exact physiological relevance of resistin in humans remains debatable, and plasma levels of resistin across different populations have not been defined clearly. Preliminary observations suggest that mean plasma levels in patients with diabetes are ≈ 40 ng/mL.^{8,9}

The present study reports for the first time the direct vasoactive effects of human recombinant resistin in cultured endothelial cells. Resistin-treated endothelial cells responded by greater production of ET-1 and augmented ET-1 mRNA expression. ET-1 is a potent endothelium-derived vasoactive factor that engenders endothelial dysfunction, and it has been suggested to play an important role in the development of diverse cardiovascular diseases,^{10,19} including obesity-associated hypertension. Using transient transfection experiments, we demonstrated that resistin upregulates ET-1 promoter activity via the AP-1 site. ET-1 promoter is regulated by AP-1 and GATA-2, which interact synergistically.²⁰ The present results suggest an AP-1-dependent mechanism yet do not allow us to make any conclusions about GATA-2 in mediating the effects of resistin on ET-1 promoter activity. Our results with resistin and AP-1 are similar to those observed with leptin and ET-1 promoter activity reported by Quehenberger et al.¹⁷ In their studies, stimulation of endothelial cells with leptin resulted in increased ET-1 promoter activity as long as at least 1 potential AP-1 binding site was present.

Although resistin did not affect the basal release of endothelium-derived NO, it significantly augmented the expression of the cell adhesion molecule VCAM-1 and the chemoattractant chemokine MCP-1, key processes in early atherosclerotic lesion formation.²¹ In addition to the aforementioned effects, resistin-treated cells expressed lower levels of TRAF-3, a key inhibitor of CD40 signaling in endothelial cells.¹² CD40 receptor/CD40 ligand has emerged as a key determinant of endothelial cell activation and atherosclerosis.¹² CD40-mediated transcription factor activation is critically dependent on TRAFs, and recent elegant studies by Urbich et al¹² have defined the central role of TRAF-3 as a potent inhibitor of CD40 ligand-mediated endothelial cell activation. Upregulation of TRAF-3 represents a novel mechanism of endothelial protection and potently retards endothelial dysfunction evoked by CD40 ligand. The observation that resistin inhibits TRAF-3 expression, albeit preliminary, is provocative because it suggests a potential for exaggerated

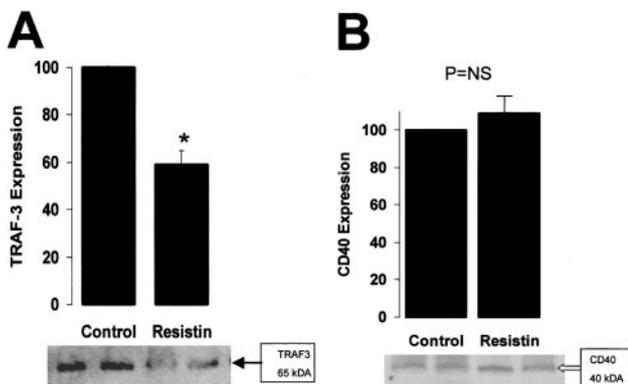


Figure 4. Effects of resistin (100 ng/mL, 24 hours) on TRAF-3 expression determined by Western blotting (A). Marked downregulation of TRAF-3 by resistin is observed. TRAF-3 is an inhibitor of CD40 ligand-induced endothelial cell activation. No effects of resistin on CD40 receptor expression were observed (B). Experiments shown are representative of 4 independent experiments performed in triplicate.

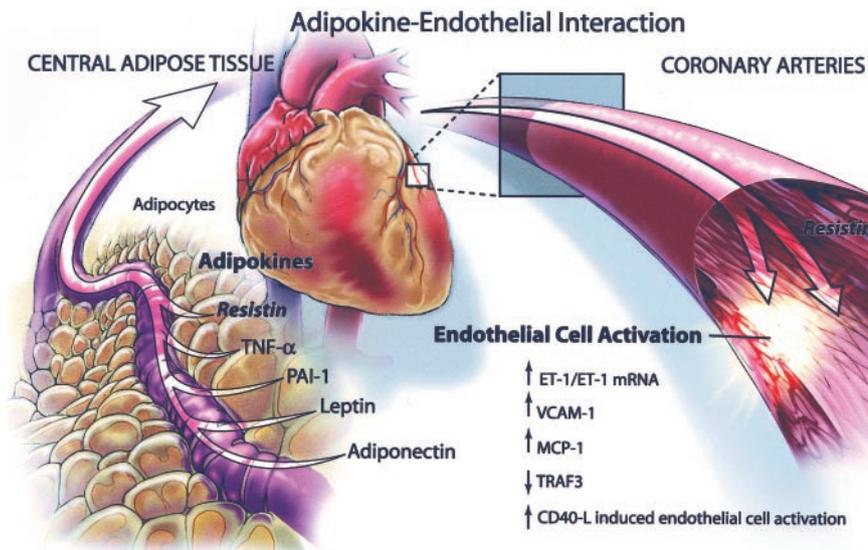


Figure 5. Illustrated model of adipokine-endothelial cell interaction. Adipocyte produces numerous hormones and cytokines such as TNF- α , plasminogen activator inhibitor type 1 (PAI-1), leptin, adiponectin, and newly described molecule resistin. These factors play an important role locally (within adipose tissue) but may also have important effects as circulating bioactive factors. Ability of adipokines such as resistin to directly modulate endothelial function and incite endothelial activation may represent an important link between insulin resistance and cardiovascular disease.

CD40 ligand-mediated endothelial activation and marked endothelial dysfunction in the presence of elevated resistin levels. Indeed, CD40 ligand-mediated MCP-1 expression was higher in endothelial cells treated with resistin (Figure 3B). Resistin treatment did not change CD40 receptor expression, which suggests that endothelial activation is not a result of increased receptor expression.

Study Limitations

The finding that nitrogen oxides in the conditioned medium were unchanged after resistin treatment may not necessarily exclude an effect of resistin on the NO synthase (NOS) pathway. The amount of nitrogen oxides in the conditioned medium is a reasonable reflection of endothelial NOS-derived NO production in healthy endothelial cells. However in "activated" cells, most of the NO is produced from inducible NOS, and hence, without assessment of NOS isoforms, an effect of resistin on NOS cannot be excluded. Additional studies aimed at evaluating the effects of resistin on asymmetric dimethylarginine, another feature of the activated endothelium, are warranted.

In summary, the novel adipokine resistin exerts direct effects to induce ET-1 promoter activity via the AP-1 site, promotes ET-1 release, upregulates adhesion molecules and chemokines, and downregulates TRAF-3, a newly identified inhibitor of CD40 ligand signaling. These data reinforce the vasoactive potential of adipocyte-derived hormones and suggest that adipocyte-endothelium cross talk may be an important mechanism of cardiovascular disease in the metabolic syndrome.

Acknowledgments

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