Resistin contributes to neointimal formation via oxidative stress after vascular injury

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ABSTRACT

Resistin may play a major potential role in vascular remodelling and may contribute to atherogenesis. However, the role of VSMC (vascular smooth muscle cell)-derived resistin in neointimal formation is not well understood. We hypothesize that endogenous resistin derived from VSMCs may contribute to neointimal formation after vascular injury. VSMCs from thoracic aorta of adult Wistar rats were cultured. The carotid artery from adult Wistar rats was injured by balloon catheter. Resistin significantly increased migration and proliferation of VSMCs. Resistin siRNA (small interfering RNA) and resistin antibody significantly inhibited migration and proliferation of VSMCs induced by conditioned medium from stretched VSMCs. Resistin protein and mRNA expression significantly increased at 14 days after carotid injury. Resistin siRNA and NAC (N-acetylcysteine) significantly reduced resistin protein and mRNA expression induced by balloon injury. Carotid artery injury increased ROS (reactive oxygen species) production. Treatment with NAC and resistin siRNA decreased ROS production. The neointimal area was significantly increased after carotid injury and was significantly reduced by resistin siRNA and NAC. In conclusion, resistin increases migration and proliferation of VSMCs, and expression of resistin in carotid artery significantly increases after injury. Resistin siRNA attenuates neointimal formation after carotid injury partly through an antioxidative mechanism. Resistin may play a pivotal role in the pathogenesis of neointimal thickening after mechanical injury.

INTRODUCTION

Resistin was originally described as an adipocyte-secreted peptide that induced insulin resistance in rodents. Recently, resistin was found to have a potential role in atherosclerosis because resistin increased proinflammatory cytokine expression in vascular endothelial cells [1] and promoted VSMC (vascular smooth muscle cell) proliferation [2]. In the atheroma, resistin may contribute to atherogenesis by virtue of its effects on vascular endothelial cells and VSMCs [3]. Resistin also promotes foam cell formation via up-regulation of class A scavenger receptor and down-regulation of ATP-binding cassette transporter-A1 in macrophages [4], which are crucial for atherogenesis. Patients with coronary calcification have elevated plasma resistin levels [5]. Resistin increases with obesity and atherosclerotic risk factors in patients with myocardial infarction [6]. High plasma resistin levels are also found to be a predictor of all-cause mortality independent of other risk factors in patients with acute myocardial infarction [7]. Hyper-resistinaemia was found to be associated with hypertension in patients with Type 2 diabetes [8] and associated with carotid atherosclerosis in hypertensive patients [9]. Serum resistin also is positively...
correlated with the accumulation of metabolic syndrome factors in Type 2 diabetes [10]. Patients with masked hypertension have higher plasma resistin levels [11]. All these data indicate that resistin may play a major role in atherosclerosis.

We have demonstrated that mechanical stretch induces resistin expression in cultured rat VSMCs mainly by ROS (reactive oxygen species) [12]. Resistin induced by mechanical stretch may contribute to the pathogenesis of atherosclerosis under haemodynamic overload. However, the role of SMC-derived resistin in atherosclerosis needs to be further clarified. We hypothesize that endogenous resistin derived from VSMCs may contribute to neointimal formation after vascular injury.

**MATERIALS AND METHODS**

**siRNA (small interfering RNA) design**

The siRNA duplexes targeting the rat resistin mRNA (GenBank® accession no. NM_144741) were designed. For the initial screening of the most effective siRNA duplexes, the 25-nucleotide RNAs were synthesized by *in vitro* transcription (Invitrogen). Further experiments were performed with the most efficient siRNA duplex. No significant similarities to any genes other than resistin were found using BLAST against the rat reference sequence database.

**VSMC culture**

Primary cultures of VSMCs were grown by the explant technique from the thoracic aorta of 220–260 g male Wistar rats, as described previously [13]. Cells were cultured in medium 199 containing 20% fetal calf serum, 0.1 mmol/l non-essential amino acids, 1 mmol/l sodium pyruvate, 4 mmol/l l-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C under 5% CO2/95% air in a humidified incubator. When confluent, VSMC monolayers were passaged every 6–7 days after trypsinization and were used for experiments from the third to sixth passages. These third to sixth passage cells were incubated for an additional 2 days to render them quiescent before the initiation of each experiment. VSMCs cultured on the flexible membrane base were subjected to cyclic stretch produced by Flexcell FX-2000 strain unit as described previously [14]. VSMCs were transfected with 800 ng of resistin siRNA using Effectene Transfection Reagent according to the manufacturer’s instructions (Qiagen). After incubation at 37°C for 24 h, cells were stretched for 18 h and subjected to analysis by Western blotting. The cultured VSMCs were verified by staining with smooth muscle actin and smoothelin (Santa Cruz Biotechnology).

**In vitro cyclic stretch on cultured VSMCs**

The strain unit Flexcell FX-2000 (Flexcell International) consists of a vacuum unit linked to a valve controlled by a computer program. VSMCs cultured on the flexible membrane base were subjected to cyclic stretch produced by this computer-controlled application of sinusoidal negative pressure as characterized and described in detail previously [14]. A frequency of 1 Hz (60 cycles per min) was used for cyclic stretch.

**Migration assay**

The migration activity of VSMCs was determined using the growth-factor-reduced Matrigel invasion system (Becton Dickinson) following the protocol provided by the manufacturer. Migration assay was performed as described previously [15]. Resistin recombinant protein (Upstate) was added to the cultured medium in the upper chamber. Cells were incubated overnight, and the top layer of the membrane was scraped gently to remove any cells. Cells were preincubated with siRNA for 16 h before performing the migration and proliferation assays. Migration was quantified by counting the number of cells in five random ×400 fields/filter and expressed as the average of number of cells per field/filter.

**Proliferation assay**

Thymidine uptake was studied by addition of 500 nCi/ml [3H]thymidine (PerkinElmer) for 6 to 24 h. VSMCs were seeded on ViewPlate (Packard Instruments) for 60 min at a density of 5 × 10⁴ cells/well in serum-free medium and incubated overnight. Recombinant resistin (15 μg/ml) or resistin siRNA was added to the plate. The proliferation assay was performed as described previously [12]. Non-specific uptake was studied in the presence of 10 μM cytochalasin B and subtracted from the measured value. MicroScint-20 (50 μl) was added, and the plate was read with TopCount (Packard Instruments).

**Balloon injury of rat carotid artery**

Animal experiments were approved and carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication no. 85–23, revised 1996). Adult Wistar rats were anaesthetized with isoflurane (3%) and subject to balloon catheter injury of the right carotid artery as described previously [16]. Briefly, a 2F Forgarty balloon catheter (Biosensors International) was inserted through the right external carotid artery, inflated and passed three times along the length of the isolated segment (1.5–2 cm in length); then, the catheter was removed. Resistin siRNA was injected to the segment, and electric pulses using CUY21-EDIT Square Wave Electroporator (Nepa Gene) were administered with five pulses and five opposite polarity pulses at 250 V/cm, 50 ms duration, 75 ms interval using Parallel fixed platinum electrode (CUY610P2–1, 1 mm

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Blots were quantified using densitometry. Dehydrogenase (GAPDH) antibody (LabFrontier) was used. Equal protein loading of the samples was further verified by staining with polyclonal GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody (Calbiochem). For 2 h at room temperature, rabbit anti-mouse IgG was biotinylated at 1:400 dilution for 30 min and incubated to Vector Elite ABC. Biotin-avidin–peroxidas complex for 30 min. To stain the collagen, Direct Red 80 (Sigma–Aldrich) was used. Sections were then developed with diaminobenzidine and diaminobenzidine enhancer (Vector), counterstained with haematoxylin. Images were examined with a fluorescence microscope.

**Western blot analysis**

Arterial protein extracts were prepared by pulverizing the arteries under liquid nitrogen, then lysed in buffer containing 1% SDS, 1mM PMSF and 10 mg/ml leupeptin in 50 mM Tris/HCl (pH 7.6). VSMCs exposed to cyclic stretch were harvested by scraping and then centrifuged (300 g) for 10 min at 4°C. The pellet was resuspended and homogenized in a Reporter Lysis Buffer (Promega), centrifuging at 10 600 g for 20 min at 4°C. Protein from cultured VSMCs and arterial extracts were used to generate Western blots. Western blot was performed as previously described [14]. Rabbit anti-rat resistin polyclonal antibody (Chemicon) and goat anti-(mouse collagen I) polyclonal antibody (Santa Cruz Biotechnology) were used. Equal protein loading of the samples was further verified by staining with polyclonal GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody (LabFrontier). All Western blots were quantified using densitometry.

**Real-time PCR**

For analysis of resistin mRNA, real-time PCR was performed. The carotid arteries were harvested, stripped of adventitia and then pooled for RNA extraction. Total RNA was isolated as described previously [14]. Reverse transcription and real-time PCR was performed as described previously [14]. The cDNA produced by reverse transcription was used to generate rat resistin cDNA probe by PCR. The PCR primer sequences used have been described previously [12].

**Immunohistochemical analysis**

The carotid artery was harvested and fixed in 10% formaldehyde and sliced into 5-μm-thick paraffin sections. For immunohistochemical stain, the slides were postfixed in 4% paraformaldehyde for 20 min, treated in 3% hydrogen peroxide/PBS for 25 min, blocked in 5% normal rabbit serum for 20 min, blocked with biotin/avidin for 15 min each and incubated with FITC-conjugated goat polyclonal anti-resistin antibody (Chemicon), CD68 antibody (AbD Serotec) or mouse monoclonal anti-(smooth muscle cell actin) antibody (Calbiochem). For 2 h at room temperature, rabbit anti-mouse IgG was biotinylated at 1:400 dilution for 30 min and subjected to Vector Elite ABC. Biotin–avidin–peroxidas complex for 30 min. To stain the collagen, Direct Red 80 (Sigma–Aldrich) was used. Sections were then developed with diaminobenzidine and diaminobenzidine enhancer (Vector), counterstained with haematoxylin. Images were examined with a fluorescence microscope.

**Statistical analysis**

All results are expressed as means ± S.D. Statistical significance was evaluated by ANOVA, followed by the Tukey–Kramer comparison test. A value of P < 0.05 was considered to denote statistical significance.

**RESULTS**

**Selection and characterization of effective anti-resistin siRNAs**

Three siRNAs were designed and generated by in vitro transfection to target-defined positions within the coding region of the rat resistin mRNA (Table 1). The potencies of the three siRNAs relative to a randomized control siRNA were compared at the translational level by Western blot analysis. To better discriminate the effect of different siRNAs on resistin protein level, we performed cyclic mechanical stretch of cultured VSMCs, which up-regulated resistin expression. We have previously demonstrated that resistin was maximally induced after 20% cyclic stretch of 6 h [12]. We used 6 h of cyclic stretch to select the most potent siRNA in the present study. As shown in Figures 1(A) and 1(B), the resistin siRNA-3 was the most potent resistin silencer capable of reducing resistin protein expression below its basal level, giving an overall inhibition of 70% at 6 h. Control with scrambled siRNA of resistin siRNA-3...
Table 1  Sequence and target localization within the rat resistin mRNA of the siRNAs (siRNA-1–siRNA-3) designed to screen for effective resistin gene silencing

The 25-nucleotide RNA sequences shown are arranged with complementary homology, yielding the corresponding siRNA duplexes. scsiRNA indicates absence of homology with respect to the resistin mRNA sequence and was used as scrambled siRNA. Numbers indicate the position of the targeted region within the resistin mRNA sequence from the ATG initiation codon.

<table>
<thead>
<tr>
<th>Anti-resistin siRNA</th>
<th>Targeted region within resistin mRNA*</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>siRNA-1</td>
<td>159–183</td>
<td>5′-UCUUGCUAGGCGCUUCUAACUGGG-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5′-CCCAUGGAUGACCGCAUGAGA-3′</td>
</tr>
<tr>
<td>siRNA-2</td>
<td>172–196</td>
<td>5′-UCUUGAUGGCUUUCGUGAGG-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5′-CCACAGGAACUCAUGAACAAGA-3′</td>
</tr>
<tr>
<td>siRNA-3</td>
<td>335–359</td>
<td>5′-ACACAUUGUAUGAAGCUACCC-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5′-GGACGUCCGUGAGGAUACAAUGUGU-3′</td>
</tr>
<tr>
<td>scsiRNA</td>
<td>335–359</td>
<td>5′-GGAGGCUGAGUAUAGUAACGGCUGU-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5′-ACAGCCGUUACUAUACUGAGCAGGCUC-3′</td>
</tr>
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Resistin increases migration of VSMC

The cultured VSMCs expressed smooth muscle actin and smoothelin (see Supplementary Figure S2 at http://www.clinsci.org/cs/120/cs1200121add.htm). Smoothelin is a marker for the highly differentiated smooth muscle cells. These markers were not detected in endothelial cells. When the same numbers of VSMCs were cultured, rat VSMCs migrated significantly through the filter membrane after exogenous addition of recombinant resistin (20 μg/ml) compared with the control group without treatment (see Supplementary Figure S3 at http://www.clinsci.org/cs/120/cs1200121add.htm).

Conditioned medium collected from stretched VSMCs significantly increased migration of VSMC, similar to the effect of recombinant resistin. Addition of resistin siRNA and resistin antibody significantly inhibited migration of VSMCs induced by conditioned medium (Supplementary Figure S3). The migration activity of VSMC was similar in the control and scrambled siRNA-treated groups. Addition of NAC also significantly inhibited the migration of VSMCs induced by conditioned medium.

Resistin increases proliferation of VSMCs

The [3H]thymidine incorporation was significantly higher in the resistin-treated group than in the control group (Supplementary Figure S4 at http://www.clinsci.org/cs/120/cs1200121add.htm). Resistin siRNA and resistin antibody significantly decreased the [3H]thymidine incorporation induced by conditioned medium. Scrambled siRNA did not affect the proliferation of VSMCs induced by conditioned medium. Addition of NAC also significantly inhibited the [3H]thymidine incorporation induced by conditioned medium.

Resistin siRNA reduces resistin protein and mRNA expression after carotid artery injury

To demonstrate the efficiency of siRNA delivery into the carotid artery by electric pulses, control siRNA was labelled with Cy3 (cyanine 3) at the 5′-side (Invitrogen). In the carotid artery transfected with siRNA by electric pulses, successful transfection was evidenced by green staining under the fluorescence microscope that was observed at the area of intimal thickening 14 days after balloon injury (Supplementary Figure S4). After 7 and 14 days of balloon injury, resistin protein expression was significantly increased compared with the control group (Figure 2). Delivery of resistin siRNA into the carotid artery after balloon injury decreased resistin protein expression in a dose-dependent manner. The most potent dose of resistin siRNA to reduce resistin protein expression after balloon injury was 20 μM (Figure 3). There was no inhibitory effect of different concentrations of scrambled resistin siRNA on resistin expression.
Resistin siRNA attenuates arterial injury

Resistin siRNA reduces intimal thickening and increases lumen size after carotid artery injury

Balloon injury of carotid artery for 14 days significantly increased the thickness of the intimal area compared with the control group without injury (Figure 3A). Delivery of resistin siRNA significantly reduced the thickness of the intimal area induced by balloon injury. The thickness of intimal area was reduced by 50%. Control siRNA did not reduce intimal area induced by balloon injury. Treatment with NAC had similar effect to resistin siRNA. Balloon injury of carotid artery for 14 days significantly reduced vessel lumen size compared with the control group without injury (Figure 3B). Delivery of resistin siRNA and treatment with NAC significantly increased vessel lumen size after balloon injury. The lumen size was increased by 38%. Control siRNA did not change lumen size after balloon injury. Immunohistochemical double staining demonstrated that there were strong resistin-positive labelled cells at 14 days after carotid injury, while the intimal layer in the sham group did not express resistin (Figure 4). Resistin siRNA decreased the resistin and CD68 labelling signal intensity after carotid artery injury. Scrambled siRNA did not alter the expression of resistin labelling after carotid artery injury. Treatment with NAC also decreased the resistin and CD68 labelling in the carotid artery after balloon injury. To investigate any modification in the vascular structure and in the arterial composition after carotid injury with resistin siRNA, we detected collagen in the arterial wall by immunohistochemical staining. Balloon injury significantly increased the collagen I content in the intimal area, and treatment with resistin siRNA and NAC significantly attenuated the collagen I content induced by balloon injury (Figure 5). These data indicate that resistin and oxidative stress mediate the neointimal formation after carotid injury by increased collagen I formation.
ROS mediates resistin expression in carotid artery after injury

As shown in Figure 6, carotid artery injury increased ROS production when determined using the ROS assay with a fluorescence microscope. Treatment with NAC decreased ROS production. Carotid artery injury also increased resistin protein expression, and treatment with NAC decreased resistin expression.

Figure 4 Resistin siRNA reduces resistin protein labelling after carotid injury
Far-left-hand panels, representative cross-sections of carotid artery stained by haematoxylin at \( \times 100 \). Other panels, immunohistochemical stain of the intimal area was performed at 14 days after carotid injury with or without siRNA treatment. Labelling of resistin and CD68 decreased after resistin siRNA therapy, and control siRNA did not change the labelling induced by carotid injury. The resistin-positive labelling cells were VSMCs, which were proved by positive anti-smooth muscle (SM) cell actin staining.
DISCUSSION

In the present study, we found that resistin directly promoted the migration and proliferation of VSMCs, which contributes to the development of neointimal formation in restenosis and is an accelerated arteriopathy in response to vascular injury. We have previously demonstrated that haemodynamic forces can play a significant role in the modulation of resistin expression of VSMCs [12]. In the present study, resistin was enhanced in the intimal area after carotid injury. Taken together, these data indicated that resistin might play an important role in remodelling during vascular disease. The injury model was performed in the carotid artery, and the VSMCs were cultured from the thoracic aorta. The VSMCs in different area of vessels may have different characteristics. In this study, the in vitro findings provide the basic mechanism to explain the effect of atherogenesis of resistin. However, it is not known whether the in vitro results using VSMCs from thoracic aorta can be applied to the VSMCs from carotid artery.

Previous studies have demonstrated that hypoxia is an important aetiological factor in pulmonary hypertension and atherosclerosis [18,19]. Mechanical stress and hypoxia increase resistin expression in VSMCs [12,19], indicating a role of resistin in atherosclerosis. In the present study, knock down of endogenous resistin by siRNA after carotid artery injury resulted in decreased neointimal formation. Our experiment provides evidence for a prorestenotic role of endogenous resistin derived from VSMCs because the local delivery of resistin siRNA most likely targets arterial SMCs. The involvement of resistin in atherosclerosis has been observed in clinical studies. Shin et al. [8] have demonstrated that serum resistin is independently associated with increasing carotid intima media thickness in treated hypertensive patients, and Takata et al. [5] have demonstrated that hyperresistinaemia would contribute to the pathogenesis of hypertension in patients with Type 2 diabetes.

siRNA has become the tool of choice for gene function study after the gene silencing effect of RNA interference in mammalian cells has been confirmed [20]. Synthetic siRNAs leverage the naturally occurring RNA interference process in a manner that is consistent and predictable with regard to extent and duration of action.

Figure 5  Effect of resistin siRNA on carotid artery collagen I protein expression
(A) Representative Western blots for collagen I in the injured carotid artery for 14 days with or without resistin (Res.) siRNA and NAC. (B) Quantitative analysis of collagen I protein levels. *P < 0.001 compared with control; **P < 0.001 compared with 14 days. Scr., scrambled.

Figure 6  Carotid artery injury increases ROS production
Immunohistochemical stain of the intimal area was performed at 14 days after carotid injury with or without NAC treatment. Labelling of ROS and resistin decreased after treatment with NAC. The ROS and resistin-positive labelling cells were VSMCs, which were proven by positive anti-smooth muscle (SM) cell actin staining.
confirmed that resistin siRNA effectively inhibited the negative effect of scrambled siRNA. We further demonstrated a dose–response of the resistin inhibitor and was chosen as the therapeutic agent. We designed three resistin siRNAs to study the silencing of atherosclerosis and restenosis warrants further study. We have demonstrated previously that hypoxia also generates ROS in VSMCs [19]. In this study, we confirmed that oxidative stress is involved in the neointimal formation after carotid artery injury. The antioxidant and free-radical scavenger NAC reduced ROS formation and reduced intimal thickness induced by balloon injury. The resistin expression was increased after carotid injury and was reduced by NAC. The migration and proliferation of VSMC induced by resistin and conditioned medium after cyclic stretch was attenuated by NAC. These results indicate that oxidative stress is involved in the neointimal formation by increased resistin expression. In the present study, we have demonstrated that resistin siRNA and NAC have similar effects on intimal area and lumen size. These results indicate that antioxidant effect may be the sole mechanism of action of resistin siRNA. Our study confirmed the importance of the modulation of oxidative stress by resistin.

Endothelium plays a pivotal role in all vascular modifications, from dysfunction of vasodilation to alteration of arterial structure and compositions as well as in the arterial stiffness and atherosclerotic plaque modifications. In our study, we did not observe the role of endothelium in the resistin effect to the neointimal formation after balloon injury because the balloon injury model disrupts the endothelium.

The toxic effect of resistin siRNA was not investigated in the present study. However, the resistin siRNA was not chemically modified. siRNAs are highly effective without any chemical modifications, resulting in a dramatically lower toxicity profile [24]. The off-target effect of siRNA is a major biological restriction [21,22]. We used scrambled siRNA to confirm the specificity of our designed siRNA. The off-target effect of resistin siRNA may be minimal. Innate immunity or interferon response may be stimulated by siRNA [25]. Innate immunity is associated with atherosclerosis. In this study, we measured serum interferon-γ levels after applying resistin siRNA. The serum interferon-γ levels were similar between siRNA-treated animal and control animals (results not shown). The macrophage content by CD68 labelling in the arterial wall was decreased by resistin siRNA. These results imply that local delivery of resistin siRNA to the injured carotid artery does not aggravate the immune response in our study model. The immune response does not contribute to the attenuation of neointimal formation by resistin siRNA in the carotid injury model.

We have demonstrated previously that resistin expression is regulated by cyclic stretch of VSMCs by increases in angiotensin II and ROS production [12]. Oxidative stress is involved in the signalling pathway of resistin induction. AngII (angiotensin II) plays an important role in atherosclerosis. We have shown that stretched VSMC secrete resistin via AngII production [12]. Angiotensin receptor blocker attenuated the secretion of resistin by AngII in VSMC. These results indicate that AngII may play a role in the resistin induction to form neointima in the carotid artery injury model. We have previously demonstrated that hypoxia also generates ROS in VSMCs [19]. In this study, we confirmed that oxidative stress is involved in neointimal formation after carotid artery injury. The antioxidant and free-radical scavenger NAC reduced ROS formation and reduced intimal thickness induced by balloon injury. The resistin expression was increased after carotid injury and was reduced by NAC. The migration and proliferation of VSMC induced by resistin and conditioned medium after cyclic stretch was attenuated by NAC. These results indicate that oxidative stress is involved in the neointimal formation by increased resistin expression. In the present study, we have demonstrated that resistin siRNA and NAC have similar effects on intimal area and lumen size. These results indicate that antioxidant effect may be the sole mechanism of action of resistin siRNA. Our study confirmed the importance of the modulation of oxidative stress by resistin.

AUTHOR CONTRIBUTION

Kou-Gi Shyu participated in the design of the study and drafted the manuscript. Li-Ming Lien and Bao-Wei Wang made substantial contributions to the conception and design of the study, acquisition of data, and analysis and interpretation of the results. Peiliang Kuan made substantial contributions to the conception and design of the study. Hang Chang oversaw the study and gave final approval of the version to be published.

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Figure S1 Resistin siRNA did not affect the resistin expression on basal VSMCs without stretch
*P < 0.001 compared with control with or without resistin siRNA (n = 4 per group).

Figure S2 Cultured VSMCs but not endothelial cells (EC) express smoothelin and smooth muscle (SM) actin
Similar findings were observed in another three independent experiments.

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Figure S3  Effect of resistin on migration and proliferation activity of VSMCs

(A) VSMCs that migrated through filter were stained. (B) Migration of VSMCs was quantified by staining and counting the number of cells that migrated to the bottom of the filter in five fields under a ×400 high-power field (HPF). *P < 0.001 compared with resistin (Res.). CM, conditioned medium. (C) Proliferation of VSMCs was quantified by [3H]thymidine incorporation assay. **P < 0.05 and *P < 0.001 compared with conditioned medium (CM) (n = 4 per group). Dp44mT, di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone (an iron chelator).
Figure S4  Expression of siRNA in the intimal area of carotid artery after balloon injury
Far-left panels, representative cross-sections of carotid artery stained by haematoxylin. Other panels, positive expression is identified as a green colour under a fluorescence microscope at cross-sectional area of intima detected 14 days after carotid injury. Magnification, ×100. DAPI, 4',6-diamidino-2-phenylindole.