Tissue Factor Is Induced by Resistin in Human Coronary Artery Endothelial Cells by the NF- κ B-Dependent Pathway

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Key Words

Endothelial cells • Tissue factor • Obesity • Inflammation • Adipokines

Abstract

Objective: Atherosclerosis is characterized by endothelial inflammation and dysfunction. Adipose tissue has increasingly been recognized as an active endocrine organ secreting so-called adipokines. Among these, resistin – recently described, but not yet extensively studied – has been defined as a novel inflammatory marker in atherosclerosis. The pathophysiology underlying this interplay, however, remains to be fully characterized. The aim of the study is to determine whether resistin might affect prothrombotic characteristics of human coronary artery endothelial cells (HCAECs). **Methods and Results:** Incubation of HCAECs with resistin caused upregulation of tissue factor (TF) expression as demonstrated by FACS analysis. Moreover, TF activity was induced in a dose-dependent manner, as shown by real-time PCR and colorimetric assay. Resistin-induced TF expression was mediated by oxygen free radicals through the activation of the transcription factor nuclear factor- κ B (NF- κ B), as demonstrated by electrophoretic mobility shift assay and by suppression of TF expression by superoxide dismutase, catalase,

and the NF- κ B inhibitors PDTC and BAY 11-7082. **Conclusions:** These data confirm the hypothesis that resistin may contribute to atherothrombosis, exerting direct effects on HCAECs by promoting TF expression; thus, it represents an effector molecule able to induce a prothrombotic phenotype in cells present in the vessel wall.

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Introduction

Abdominal obesity is emerging as an important driving force behind the increased cardiovascular risk in the general population. However, the mechanisms underlying this phenomenon are still largely unknown.

Increasing evidence has assigned a central role to thrombosis in the progression and complications of atherosclerosis, and has linked it to the clinical occurrence of acute coronary syndromes [1–3]. In particular, a number of experimental and clinical studies indicate that tissue factor (TF) plays a pivotal role in the pathophysiology of

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acute coronary syndromes by triggering the formation of intracoronary thrombi following endothelial injury [4–7].

Until relatively recently, the role of adipocytes was considered to be little more than storage for fat. It is now clear that, in addition to storing calories as triglycerides, they also secrete a large variety of substances, including cytokines, chemokines and hormone-like factors (such as leptin, adiponectin and resistin) leading to a chronic pro-inflammatory state [8]. However, the direct effects of these molecules, in particular resistin, on endothelial cells are still largely unclear.

Accordingly, in the present study, we tested the hypothesis that the recently identified marker of inflammation, resistin [9, 10], might induce a prothrombotic state in human coronary artery endothelial cells via expression of TF; thus, suggesting an active role for resistin in the pathophysiology of coronary events. Furthermore, we investigated the potential involvement of the nuclear factor κ B (NF- κ B) and SP-1 intracellular pathways in this phenomenon.

Materials and Methods

Experiments were performed on human coronary artery endothelial cells (HCAECs, Cambrex Bio Science, USA). Cells were grown in EGM 2 medium (Cambrex Bio Science, USA) with endothelial cell growth supplement and 10% fetal serum. Cells were used at passages 2–5.

To investigate the effects of resistin on TF antigen and activity, cells were enzymatically harvested, counted in a hemocytometer and subcultured in 24-well plates at an initial density of about 5×10^4 cells per well; while at confluence, cell density was about 8.5×10^4 cells per well. For other sets of experiments, cells were grown in 100-mm cell plates and, at confluence, cell density was about 2×10^6 cells per plate. At confluence, cells were starved in serum-free medium for 24 h and then used in the different sets of experiments.

Preliminary studies were performed to optimize incubation time, while human recombinant resistin (PeproTech, Inc., USA) concentrations were used according to previous work [11, 12]. For human resistin (MW 11.42 kDa), the concentrations of 25, 50 and 100 ng/ml equate to 2.19, 4.38 and 8.76 nM, respectively. Endotoxin levels in the resistin preparation, as assessed by limulus assay, were <0.125 EU/ml (<12.5 pg/ml). All media and water were also tested, and the endotoxin level was found to be <0.125 EU/ml.

Effects of Resistin on TF Transcription

The effects of resistin on TF-mRNA were investigated by real-time reverse-transcription analysis, as previously described [13]. HCAECs were incubated with resistin (100 ng/ml). Thirty minutes after adding resistin, cells were washed with PBS and fresh medium (EGM 2 containing 0.1% serum) was added. Total mRNA was extracted at baseline, 30, 60, 120 and 240 min after resistin

stimulation from cell cultures using TRIzol reagent (Gibco) according to the manufacturer's instructions. Reverse transcription was performed using mMLV (Gibco), and 100 ng RNA samples from each culture condition. Samples were run in triplicate in 50- μ l reactions by using an ABI Prism 5700 sequence detector system (Applied Biosystems). Samples were incubated at 50°C for 2 min, 95°C for 10 min, and then underwent 40 cycles at 95°C for 15 s and 60°C for 1 min. Specific oligonucleotides for human GAPDH and human TF were designed on the basis of published sequences using Primer Express Software (Applied Biosystems) and validated for their specificity. SYBR-green chemistry was used to detect fluorescence and an internal standard (Applied Biosystems) was used for quantification of the message. In positive control experiments, HCAECs were incubated for 30 min with lipopolysaccharide (LPS; 50 μ g/ml) and then mRNA was extracted at 60 min. The results were analyzed using a comparative method, and the values were normalized to the GAPDH expression and converted into percentage change. Three different experiments were performed for each experimental condition.

Effects of Resistin on TF Antigen

HCAECs were incubated with resistin (25–100 ng/ml) or with LPS (50 μ g/ml) for 6 h and TF expression was detected with FACS analysis. After stimulation, cells were detached with 10 mM EDTA in 0.5% phosphate-buffered saline, collected by centrifugation, and stained for 30 min on ice in the dark with R-phycoerythrin-labeled monoclonal antibody (Pharmingen, USA) against TF, or with the appropriate isotype IgG as control. The fluorescence intensity of 10,000 gated viable cells was analyzed for each sample on the FACS Calibur Flow Cytometer (Becton Dickinson Immunocytometry Systems, USA) using Cell Quest (Becton Dickinson) acquisition software.

Six different experiments were performed for each experimental condition.

Effects of Resistin on TF Activity

HCAECs were incubated with increasing concentrations of resistin (25–100 ng/ml) for 6 h. TF activity was determined by a two-step colorimetric assay, based on the ability of TF to promote generation of coagulation FXa, as previously described [16]. Briefly, plated adherent cells were incubated with 1 nmol of recombinant human FVIIa (Novo Nordisk A/S, Denmark), followed by 100 nmol of purified human factor X (Calbiochem-Novobiochem, USA) and 5 mM CaCl_2 for 15 min at 37°C. A chromogenic substrate, specific for factor X (Cromozym X; Roche Diagnostics, Germany, 0.5 mM), was then added and incubated for 30 min at 37°C. The reaction was stopped by adding 200 μ l/ml of sample of a 30% solution of acetic acid. The change in optical density at 405 nm was quantified with a spectrophotometer.

To evaluate whether resistin-induced activity of TF resulted from de novo synthesis of this protein, another set of experiments – in which HCAECs were pre-incubated with cycloheximide (10 μ g/ml; an inhibitor of protein synthesis) or with 5,6-dichloro-1-D-ribofuranosylbenzimidazole (DRB, 10 μ g/ml; an inhibitor of DNA transcription) 30 min before adding resistin (50 ng/ml) – were carried out.

Since it has been documented that promotion of oxidative stress is a fundamental principle of resistin's mode of operation [14, 15], we conducted another set of experiments to evaluate resistin's effects on intracellular production of reactive oxygen spe-

cies (ROS). Briefly, HCAECs were loaded with 10 μ M DCFH-DA (2,7-dichlorofluorescein diacetate) in PBS for 1 h at 37°C, washed in PBS, and exposed to resistin (50 ng/ml) for 30 and 60 min. Then, the production of ROS was measured by the intensity of DCF emission at 525 nm in a fluorescence spectrometer.

Moreover, TF activity was evaluated as above, but in HCAECs preincubated for 30 min in the presence of 2 different free radical scavengers, such as cell-permeable superoxide dismutase (SOD; MnTMPyP) and catalase (MnTBAP) mimetic.

Positive control experiments included endothelial cells incubated for 6 h with LPS (50 μ g/ml). Six different experiments were performed for each experimental condition.

Effects of Resistin on NF- κ B and SP-1 Activation

Furthermore, to better understand the intracellular mechanisms by which resistin induces TF on HCAECs, we carried out more experiments designed to test the hypothesis that NF- κ B and SP-1 (two transcription factors known to be involved in the regulation of TF promoter in endothelial cells [16]), might be involved in mediating this phenomenon.

The levels of NF- κ B proteins in nuclear extracts from the cells were analyzed by electrophoretic mobility shift assay (EMSA). HCAECs starved in serum-free medium were washed and incubated with resistin (25–50 ng/ml) for 60 min. Cells incubated with 50 μ g/ml LPS served as a positive control. Nuclear proteins from these cells were isolated as previously described [25], and were subjected to EMSA using a 32 P-labelled NF- κ B double-strand oligonucleotide (5'-ACTTGAGGGGACTTTCCCAGGC-3') and SP-1 double-strand oligonucleotide (5'-AATTCGGGGCGG-GACCAGGGCGGGGCTCG-3'). Nuclear proteins were incubated with oligonucleotide for 30 min, subjected to gel electrophoresis and finally autoradiographed. Control experiments included cells preincubated with pyrrolidine dithio carbamate ammonium (PDTC, 100 μ M), or with BAY 11-7082 (5 μ M), two inhibitors of NF- κ B with a different mechanism of action, for 60 min before stimulation with resistin as above.

All experiments were performed in triplicate.

Statistical Analysis

All data are presented as means $\pm$ SD, and were analyzed using one-way ANOVA followed by the Scheffe test for multiple comparisons. Values of $p < 0.05$ were considered statistically significant.

Results

Effects of Resistin on TF Transcription

TF mRNA was undetectable in unstimulated HCAECs, as expected [10]. Incubation with LPS (50 μ g/ml), as anticipated, induced significant TF mRNA upregulation. Interestingly, resistin (100 ng/ml) caused a time-dependent increase in TF mRNA levels as compared to unstimulated cells. In particular, TF mRNA levels increased at 30 min of resistin incubation, reaching the peak at 60 min (fig. 1). Conversely, resistin did not affect GAPDH mRNA levels (data not shown).

Fig. 1. Effects of resistin (100 ng/ml) on TF transcription in HCAECs assessed by real-time quantitative PCR. TF mRNA was undetectable at baseline in unstimulated HCAECs. A peak in TF mRNA levels was observed after 60 min of resistin incubation, while at 120 min TF mRNA levels started decreasing. LPS served as a positive control. Conversely, no change was detected in the GAPDH mRNA levels after resistin stimulation at different time points. * $p < 0.05$ vs. baseline. Bars represent means $\pm$ SD of 3 different experiments.

Effects of Resistin on TF Antigen and Activity

HCAECs showed undetectable TF antigen and activity at baseline, and exposure to different doses (25–100 ng/ml) of resistin induced a significant increase in both TF antigen and activity (fig. 2, 3a). In particular, resistin induced a significant increase in TF antigen and activity at a concentration of 25 ng/ml (2.3- and 10-fold over medium alone, respectively), which is relevant to the clinical plasma levels of resistin in obesity and diabetic patients [17, 18], and the maximum effect at 100 ng/ml (4.1- and 17-fold over medium alone, respectively) (fig. 2, 3a).

In parallel experiments, preincubation with cycloheximide, an inhibitor of protein synthesis, or with DRB, an inhibitor of DNA transcription, completely inhibited TF activity (fig. 3b). These data suggest that resistin is able to induce de novo synthesis of TF and these new TF molecules are then expressed in an active form on the cell surface. Interestingly, in cells preincubated with the cell-permeable SOD or with the cell-permeable catalase enzyme, resistin failed to induce TF activity – suggesting that these highly reactive oxygen-derived molecules and H₂O₂ play an important role in mediating resistin effects (fig. 3c).

We then evaluated resistin effects on intracellular production of ROS measuring their production by the

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Fig. 2. a Effects of resistin (25, 50 and 100 ng/ml) on TF antigen in HCAECs determined by FACS analysis. Resistin induced an increase in TF antigen, with maximum response at 100 ng/ml. LPS (50 μ g/ml) served as a positive control. Each bar represents mean $\pm$ SD of 6 different experiments. * $p < 0.05$, ** $p < 0.01$ vs. baseline. **b** Representative histogram of the FACS analysis experiment for TF antigen.

Fig. 3. Effects of resistin on TF activity in HCAECs, determined by a two-step colorimetric assay based on the ability of TF/FVIIa to promote generation of coagulation FXa. **a** Effects of resistin (25, 50 and 100 ng/ml) on TF activity. Resistin induced an increase in TF activity, with maximum response at 100 ng/ml. * $p < 0.05$,

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** $p < 0.01$ vs. baseline. **b** Control experiments, performed by pre-incubating HCAECs with cycloheximide (CE) or with DRB, showed that resistin-induced TF activity required de novo mRNA transcription and protein synthesis. * $p < 0.01$ vs. baseline; ** $p < 0.01$ vs. resistin (50 ng/ml). **c** Pretreatment of HCAECs with SOD, an oxygen free radical scavenger, with the decomposing agent of hydrogen peroxide, the catalase enzyme (CAT), or with PDTC and BAY 11-7082 (inhibitors of NF- κ B activation) decreased the resistin-induced TF activity. * $p < 0.01$ vs. baseline; ** $p < 0.01$ vs. resistin (50 ng/ml). LPS (50 μ g/ml) served as a positive control. Each bar represents mean $\pm$ SD of 6 different experiments.

Fig. 4. ROS generation, expressed as relative DCF fluorescence, by HCAECs incubated with resistin (50 ng/ml). Control values are represented by unstimulated cells. Data expressed as means $\pm$ SD of 4 different experiments.

intensity of DCF emission at 525 nm in a fluorescence spectrometer. As shown in figure 4, resistin was able to induce ROS intracellular production after 30 min of incubation, while no production was seen in unstimulated cells.

Effects of Resistin on NF- κ B and SP-1 Activation

To better investigate the relation between intracellular mechanisms activated after resistin stimulation and the expression of TF induced by this adipokine, we employed EMSA on nuclear extracts obtained from cells stimulated with this molecule to analyze the NF- κ B and SP-1 pathways. As shown in figure 5a, NF- κ B was activated after incubation of HCAECs with resistin (25–50 ng/ml). In fact, resistin was able to induce a distinct shifted band in stimulated cells, similar to that observed in the LPS-treated cells (positive controls), while no shift could be observed in unstimulated control cells. Conversely, transcription factor SP-1 was only slightly activated after resistin incubation (25–50 ng/ml; fig. 5b). Starting from these observations, we tested 2 different inhibitors of NF- κ B activation, PDTC (100 μ M) and BAY 11-7082 (5 μ M) before stimulation with resistin. Interestingly, pretreatment of cells with PDTC and BAY 11-7082 inhibits the NF- κ B activation (fig. 5a), and this effect was associated with the significant reduction in TF activity (fig. 3c).

Finally, in cells preincubated with the cell-permeable SOD or with the cell-permeable catalase enzyme, resistin failed to activate NF- κ B (fig. 5a).

To investigate whether PDTC, BAY 11-7082 and SOD concentrations used in our experiments exerted toxic effects on HCAECs, trypan blue exclusion assays were performed at conclusion of the experiments, demonstrating >95% viability with no differences between control and treatment groups (data not shown).

Discussion

The main findings of the present study are: (1) resistin induces TF-mRNA transcription and de novo synthesis of functionally active TF in human coronary artery endothelial cells in culture; (2) these phenomena appear to be regulated by oxygen free radicals through activation of the NF- κ B pathway.

Over the past few years, it has become clear that inflammation plays an important pathophysiological role not only in the development of atherosclerosis, but also in determining its complications, such as the occurrence of acute coronary syndromes [1–3]. Inflammatory mechanisms involved in the development of atherosclerosis can be triggered by multiple factors, such as LDL, oxygen reactive species, activated blood cells, interleukins and IFN- γ [19]. In addition, increasing importance has been assigned to selected markers of systemic inflammation [20], such as CRP, as predictors of major cardiovascular events [21–23].

In recent years, a great deal of work has aimed to study adipose tissue. Increasing evidence indicates that adipose tissue is an important source of cytokines [24] and that adiposity contributes to a proinflammatory environment [25].

Several lines of evidence have indicated that adipose-derived products might be involved in cardiovascular disease, since their effects on cells of vessel walls – such as endothelial and smooth muscle cells – have been extensively documented [26]. In particular, it has been recognized that resistin, one of the most recently described cytokines produced by the adipose tissue and by macrophages and monocytes [27, 28], is able to induce the expression of adhesion molecules and the production of some cytokines in human umbilical vein endothelial cells [11]. These effects on endothelial cells were observed also in a recent paper by Li et al. [14], where the authors showed that resistin was able to affect the protein expression of PI3Kp85 α to stimulate the release of PAI-1, vWF

Fig. 5. Effects of resistin on nuclear translocation of NF- κ B and SP-1. **a** Effects of resistin on nuclear translocation of NF- κ B in HCAECs determined by electrophoretic mobility shift assay. Nuclear extracts were prepared from untreated cells or treated with increasing concentrations of resistin (25 and 50 ng/ml) and LPS (50 μ g/ml) as positive control. Resistin was able to induce a distinct shifted band in stimulated cells, while no shift could be observed in unstimulated control cells. Preincubation of HCAECs with SOD, an oxygen free radical scavenger, with the decomposing agent of hydrogen peroxide (the catalase enzyme, CAT) or with PDTC and BAY 11-7082 (inhibitors of NF- κ B) reduced resistin-induced activation of NF- κ B. Panel = result of a representative experiment; bar graph = summary of data from 3 separate experiments (means $\pm$ SD). * $p < 0.01$ vs. baseline; ** $p < 0.01$ vs. resistin (50 ng/ml). **b** Effects of resistin on nuclear translocation of SP-1 in HCAECs determined by electrophoretic mobility shift assay. Transcription factor SP-1 was only slightly activated after resistin incubation (25–50 ng/ml). Panel = result of a representative experiment; bar graph = summary of data from 3 separate experiments (means $\pm$ SD). * $p < 0.05$, ** $p < 0.01$ vs. baseline.

and ET, and to downregulate eNOS – thus, finally leading to endothelial dysfunction. Finally, Kralisch et al. [29] recently demonstrated that secretory products from adipocytes impair endothelial functions, and that the NF- κ B pathway is involved in modulating these effects. However, in that paper, the authors used the entire fraction of the different adipocytokines produced by primary differentiated adipocytes to stimulate ECs in vitro, i.e. they did not analyze them separately. Thus, they did not investigate the relative role played by the different adipokines, with both pro- and anti-atherosclerotic properties, in modulating the observed effects. Moreover, we have recently demonstrated that resistin induces human coronary artery smooth muscle cell proliferation in a dose-dependent fashion through activation of ERK (extracellular signal-regulated kinase 1/2) and PI3K (phosphatidylinositol 3-kinase) pathways [12]. Again, Jung et al. [30] demonstrated that resistin induces vascular smooth muscle cell migration. Taken together, these data point out that this protein does not only represent an inflammatory marker, but that it is also an important inflammatory modulator.

In the present study, we have demonstrated that incubation with resistin induces TF-mRNA transcription and de novo synthesis of functionally active TF. In particular, we have demonstrated here that resistin exerts its effects on TF antigen in a similar magnitude with previous findings from our group and others [7, 13]. Of particular pathophysiological interest was the finding that these TF molecules were functionally active, as demonstrated by the parallel increase in TF-procoagulant activity, which was detectable on the surface of stimulated cells. This phenomenon appears to be mainly related to the synthesis of new TF molecules, since cycloheximide and DRB (inhibitors of protein synthesis and mRNA transcription, respectively) completely inhibited the resistin effects on TF expression. This observation might have important pathophysiological consequences, considering that endothelial cells are at the interface between the vessel wall and circulating blood coagulation factors; thus, resistin seems to be able to induce a ‘procoagulant’ phenotype in endothelial cells through TF expression.

Recent publications suggested that resistin causes endothelial dysfunction through oxidative stress and downregulation of eNOS [14, 15]. In addition, oxidative stress seems to also play a pivotal role in the activation of NF- κ B pathways [31]. In line with these previous reports, we have demonstrated that resistin-induced TF activity in HCAECs is mediated by oxygen free radicals and that the

transcription factor NF- κ B is the potential link in modulating these phenomenon. Indeed SOD (a scavenger for oxygen free radicals) and catalase (a decomposing agent of hydrogen peroxide) significantly reduced NF- κ B activation as well as TF activity. In addition, resistin effects on TF activity were significantly reduced by PDTC and BAY 11-7082, two NF- κ B inhibitors. Taken together, these results allow us to suggest a hypothetical axis, resistin-TF, in which oxygen free radicals represent the second cytoplasmatic messenger, and NF- κ B is the nuclear effector of TF gene transcription.

The promoter of human TF contains 5 binding sites for specificity protein 1 (Sp1), 3 sites for epidermal growth response-1 (Egr-1), 2 sites for activator protein-1 (AP-1) and 1 site for NF- κ B. Among these binding sites, Sp1 sites seem to be involved in constitutive expression of the basal level of TF, whereas Egr-1, AP-1 and (mainly) NF- κ B sites are chiefly accountable for inducible expression of TF [16]. In line with these previous observations, we found that resistin-induced TF expression was modulated by NF- κ B activation, while SP-1 was not affected.

NF- κ B seems to be activated by several stimuli during the atherosclerotic process where it actively participates in this process potentially leading to plaque disruption and acute coronary events [32]. Previous evidence has already suggested that resistin induces NF- κ B activation in macrophages and promotes proinflammatory cytokines expression [33]. However, in the present study, we demonstrated for the first time that activation of the NF- κ B pathway by resistin causes expression of TF in HCAECs.

Taken together, the findings of the present study permit us to add new evidence to the possible role of resistin in cardiovascular disease. In fact, increased circulating levels of resistin have been observed in patients with heart failure [34] and in patients with several components of the metabolic syndrome, including hypertension and diabetes mellitus [35], diseases usually associated with atherosclerosis and, potentially, with acute coronary events. Resistin levels are also related to prevalent [36] and premature [37] coronary artery disease. Finally, there is also some evidence that resistin protein accumulates in carotid atherosclerotic plaques and not in the wall of normal mammary arteries; thus, suggesting a role in linking inflammation and atherosclerotic process [37].

Our results point out that resistin might have a pivotal role in the pathophysiology of these cardiovascular

events. Therefore, we might speculate that this adipokine contributes to the complication of the atherosclerotic plaque and the occurrence of acute coronary syndromes through expression of TF, and thus promoting an 'endothelial pro-thrombotic phenotype'.

A potential limitation of the present study might be that we performed some experiments by testing resistin concentrations higher than those usually observed in the plasma of healthy subjects (average 20 ng/ml) [9, 17]. However, this limitation should be overcome considering that elevated plasma concentrations of this peptide have been measured in several pathological conditions [34–37] and that these concentrations might only loosely reflect its levels within the arterial wall, where this adipokine might be present in amounts sufficiently high to exert relevant cellular effects [37].

In conclusion, the present study, although in vitro, describes the close relationship between resistin and thrombosis, providing for the first time support for the view that this adipokine might indeed have an important role in the pathophysiology of acute coronary syndromes. Although future studies are required to clarify whether these mechanisms are also important in the clinical setting, this report supports an emerging new role for resistin in the pathogenesis and progression of cardiovascular disease.

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