Biochemical and Biophysical Research Communications 422 (2012) 321–326

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A novel pro-inflammatory mechanism of action of resistin in human endothelial cells: Up-regulation of SOCS3 expression through STAT3 activation

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ARTICLE INFO

Article history:
Received 25 April 2012
Available online 7 May 2012

Keywords:
Resistin
Suppressor of cytokine signaling 3
Signal transducers and activators of transcription
Human endothelial cells

ABSTRACT

Resistin is a significant local and systemic regulatory cytokine involved in inflammation. Suppressors of cytokine signaling (SOCS) proteins are intracellular regulators of receptor signal transduction induced by several cytokines in a cytokine and cell specific manner. Resistin up-regulates SOCS3 expression in mice adipocytes but it is not known whether this is a common occurrence in other cells. We questioned whether resistin-induces SOCS3 in human endothelial cells and if signal transducer and activator of transcription (STAT) proteins are involved in the process. The Real-Time PCR and Western blot analysis showed that in resistin-activated HEC the gene and protein expression of SOCS3 were significantly increased. Furthermore, resistin induced activation of STAT3 as characterized by increased tyrosine phosphorylation. Resistin-induced SOCS3 expression was blocked by specific inhibitors of STAT3 signaling and by the transfection of siRNA specific for STAT3. Silencing of SOCS3 gene expression by transfection with SOCS3 siRNA reduced the expression of resistin induced-P-selectin and fractalkine in HEC. Together, our results demonstrate that in HEC (1) resistin up-regulates SOCS3 expression and activates STAT3 transcription factor; (2) the increase in SOCS3 mRNA and protein expression as well as STAT3 activation have a long-lasting effect (up to 18 h); (3) inhibition of SOCS3 function prevents resistin-induced expression of cell adhesion molecules P-selectin and fractalkine and thus activation of endothelial cells. The data uncover a new resistin-mediated mechanism in human endothelial cells and designate SOCS3 as a novel therapeutic target to modulate resistin-dependent inflammation in vessel wall diseases.

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1. Introduction

Resistin is a cysteine-rich protein belonging to the family of proteins termed FIZZ (found in inflammatory zone) or RELM (resistin-like molecules); it is a cytokine that in mice function as a molecular link between obesity and diabetes. In humans, resistin is reported as a pro-inflammatory molecule associated with both acute and chronic inflammation but its specific functions are yet to be uncovered [1]. It is assumed that resistin plays an important role in cardiovascular disease by interfering in systemic and local inflammation in the diseased vessel wall [2–4]. In endothelial cells, resistin up-regulates the expression of cell adhesion molecules and chemokines, such as ICAM-1, VCAM-1, PECAM-1 and MCP-1 [2,4]. We have recently reported that resistin up-regulates the gene and protein expression of P-selectin and fractalkine in human endothelial cells (HEC) by a mechanism involving ROS, MAPK and the transcription factors NF-kB and AP-1 [5,6]. There are data, including ours, that indicate that resistin may also interfere in cytokines signaling in HEC and could be a significant modulator of inflammation acting as a function of the specific inflammatory microenvironment [4,5].

Suppressor of cytokine signaling (SOCS) are a family of cytoplasmic proteins that carry out a negative feedback loop to attenuate signal transduction induced by cytokines or hormones such as leptin and insulin [7]. Signal transducer and activator of transcription proteins are involved in SOCS expression in various cell types, including endothelial cells [8]. It has been reported that in mice 3T3-L1 adipocytes, resistin markedly increase the expression of SOCS3 proteins, which mediate resistin antagonism to insulin and that loss of SOCS3 function impairs resistin action [9]. There are no data on resistin effects on SOCS3 expression in other cells, including vascular cells. We hypothesized that resistin action on HEC may also implicate SOCS3 protein and searched for the signaling pathways implicated. We report here that in HEC resistin induces up-regulation of SOCS3 expression, that STAT3 is the transcriptional mediator involved in the process and that inhibition of SOCS3 prevents resistin-generated expression of cell

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http://dx.doi.org/10.1016/j.bbrc.2012.04.159

adhesion molecules (P-selectin and fractalkine). As such, SOCS3 protein may be considered a novel target for future therapeutic strategies in chronic inflammatory diseases such as atherosclerosis, diabetes and arthritis.

2. Materials and methods

2.1. Cells

Human endothelial cells (HEC), the EAhy926 cell line was kindly donated by Dr. Cora Jean Edgell (Department of Pathology, University of North Carolina, Chapel Hill). The cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and grown to confluence in Petri dishes (60 mm diameter) or in tissue culture plates (6 wells).

2.2. Reagents

Human recombinant resistin was from Santa Cruz Biotechnology. The monoclonal antibodies to SOCS3, phospho-STAT3, STAT3, P-selectin, fractalkine and β-actin were from R and D Systems, secondary antibodies and all the other reagents were from Sigma Aldrich Chemie GmbH (Germany), unless otherwise stated. Proteins were detected using the Signal West Pico Chemiluminescent Substrate kit from Pierce (Rockford USA).

2.3. Experimental protocol

Confluent cultured HEC were exposed to 100 ng/ml resistin for 30 min, or 1, 2, 4, 6, 18 and 24 h; this concentration was chosen based on preliminary experiments and previous studies that established that resistin exerts its pro-inflammatory effects on HEC at 100 ng/ml [4,5,10]. Then, the cells were collected, and subsequently processed for SOCS3 mRNA and protein expression by Real Time PCR and Western blot assays, respectively. In other experiments, HEC activated by resistin for 10, 20, 30 min and the same time intervals as above were analyzed for STAT3 phosphorylation by Western blot assay. To assess the role of STAT3 in SOCS3 expression, confluent HEC were exposed to resistin in the absence or presence of STAT3 specific inhibitors, namely WP1066 (2 μM), S3I201 (20 μM) or STAT3 siRNA (100 nM) and control siRNA (100 nM). The role of SOCS3 in resistin-induced P-selectin or fractalkine expression was determined by transfection of HEC with SOCS3 siRNA before activation with resistin.

2.4. Western blot assay

The cells were washed in ice-cold PBS and solubilized in Laemli lysis buffer (60 mM Tris–Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue), heated in a boiling water bath (5 min) and then sonicated. 50 μg protein was subjected to 10–12% polyacrylamide gels electrophoresis and transferred onto nitrocellulose membranes (using a Trans Blot Semi-Dry system); the blots were blocked in 1% BSA in Tris-buffered Saline (TBS) as the blocking buffer and subsequently incubated with anti-human SOCS3 mouse monoclonal antibody (1:2000 dilution), anti-human pSTAT3 rabbit monoclonal antibody (1:1000 dilution), anti-human STAT3 mouse monoclonal antibody (1:1000 dilution), anti-human P-selectin mouse monoclonal antibody (1:500), anti-fractalkine mouse monoclonal antibody (1:500) or anti-human β-actin monoclonal antibody (1:2000 dilution) in TBS, overnight, at 4 °C. After washing, the membranes were incubated with the secondary antibody (1:2000 dilution) in TBS (1 h at room temperature) washed in TBS–0.05% Tween (wash buffer), incubated with enhanced chemiluminescence reagents (Signal West Pico Chemiluminescent Substrate kit from Pierce, 5 min) and analyzed by ImageQuant LAS 4000 (GE Healthcare); the optical density was calculated with the Scion Image software. Quantification of SOCS3 protein was determined by normalization to β-actin protein.

2.5. RNA isolation and Real Time PCR

Total cellular RNA was isolated from cultured HEC using TRIzol reagent. First-strand cDNA synthesis was performed employing 2 μg of total RNA and MMLV reverse transcriptase according to the manufacturer's protocol (Invitrogen). Quantification of SOCS3, P-selectin or fractalkine mRNA was performed by amplification of cDNA using a LightCycler 480 Real-Time PCR System (Roche), SYBR Green I chemistry and the following primers: for SOCS3 sense 5′-AAGCTGGTGTACCACTACATGC-3′ and antisense 5′-CGGTCTTCC GACAGAGATGC-3′, for P-selectin sense 5′-GGATTGTTCTGACACTCG TGG-3′ and antisense 5′-GAGGTTGGAGCAGTTCATCG-3′, for fractalkine sense 5′-AACTCGAAATGGCGGCACCTT-3′ and antisense 5′-ATG AATTACTACCACAGCTCCG-3′ and for β-actin sense 5′-CATGTACGTT GCTATCCAGGC-3′ and antisense 5′-CTCCTTAATGTCACGCACGAT-3′. The optimized amplification conditions were 2.5 mM $MgCl_2$, annealing at 62 °C and extension at 72 °C for 40 cycles. The SOCS3, P-selectin and fractalkine mRNA was normalized to β-actin mRNA level. The relative quantification was performed using the comparative C_T method and expressed as arbitrary units.

2.6. siRNA transfection

Twenty-four hours before transfection, exponentially growing HECs were seeded at 2×10^5 cells/well (~60% confluence) on 6-well tissue culture plates. For gene expression silencing, HEC were transfected with 100 nM siRNA specific for STAT3 or SOCS3. Control siRNA was used to determine transfection efficiency. Transfection was performed according to manufacturer's protocol using HiperFect reagent (Qiagen) and DMEM supplemented with 10% fetal bovine serum (v/v). After 24 h, HEC were stimulated with 100 ng/ml resistin for 2 h, solubilized in lysis buffer and subjected to Western Blot or qPCR analysis.

2.7. Statistical analysis of the data

Statistical processing of the data was done using the one-way analysis variance between groups (ANOVA) Program of Origin. All values were expressed as mean ± SEM. p values of <0.05 were considered statistically significant.

3. Results

3.1. Resistin up-regulates SOCS3 expression in HEC in a time-dependent manner

HEC were exposed for different time intervals to 100 ng/ml resistin and then the mRNA and protein expression of SOCS3 were determined by qPCR and Western Blot, respectively. The qPCR experiments showed that starting with 2 h, resistin induced a significant increase in SOCS3 mRNA expression in HEC, and the effect was maintained for 18 h (~1.8-fold increase over control); the up-regulation of SOCS3 gene expression decreased to control level after 24 h stimulation (Fig. 1A). Western Blot analysis of the SOCS3 protein expression in resistin-exposed HEC revealed a significant increase after 2 h stimulation (~1.4-fold). The augmented protein expression was maintained and slightly increased at 4, 6 and 18 h after resistin stimulation (~1.7 and ~1.5 folds) compared to controls; after 24 h, SOCS3 protein expression decreased to control

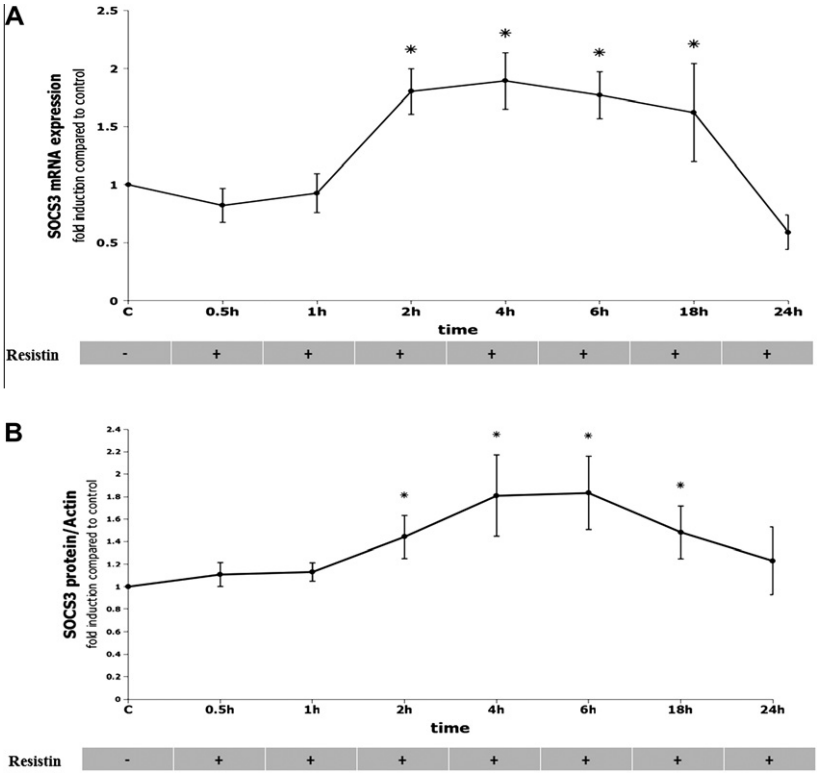

Fig. 1. Resistin up-regulates SOCS3 mRNA and protein expression in HEC as a function of time. (A) SOCS3 mRNA in HEC exposed to resistin (100 ng/ml) for 30 min, 1, 2, 4, 6, 18 or 24 h as determined by qPCR and normalized to β-actin mRNA expression. C; control cells. (B) Induction of SOCS3 protein expression in resistin-activated HEC for the same time intervals as above. SOCS3 protein expression was normalized to β-actin expression. Results are mean ± SEM ($n \geqslant 3$). *Significantly different from control (C); $p < 0.05$.

level (Fig. 1B). Remarkable, both gene and protein expression of SOCS3 increased significantly after 2 h in resistin-activated HEC.

3.2. Resistin activates STAT3 transcription factor

Since other cytokines induce SOCS3 expression via activation of STAT3 transcription factor [11,12], we questioned whether resistin increases the phosphorylation of STAT3, as well. We found that exposure of HEC to resistin for 1 h induced an increase in STAT3 phosphorylation to a value of ∼1.9-fold above the control level. This value was maintained elevated at 2, 4, 6 and 18 h of stimulation and decreased to control after 24 h (Fig. 2). Importantly, resistin did not affect the total amount of STAT3 protein for the time intervals examined.

3.3. Inhibition of STAT3 pathway reduces resistin-induced up-regulation of SOCS3 expression in HEC

To assess if STAT3 pathway is indeed implicated in resistin-induced SOCS3 expression, HEC were exposed for 2 h to inhibitors for JAK2-STAT3 pathway (WP1066, 2 μM) or for STAT3 (S3I201, 20 μM) prior to stimulation with resistin for 2 h. The results obtained by qPCR experiments revealed that both inhibitors blocked resistin–induced SOCS3 mRNA expression (Fig. 3A). Correspond-

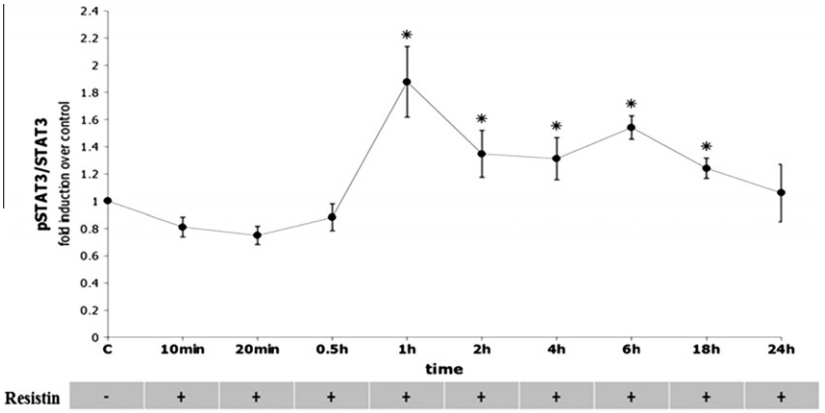

Fig. 2. Resistin induces phosphorylation of STAT3 in HEC. Confluent cells were exposed to 100 ng/ml resistin for time intervals between 10 min and 24 h (as shown) and then the protein expression of phosphorylated (pSTAT3) and total STAT3 were determined by Western blot assay. pSTAT3 protein expression was normalized to the expression of total STAT3. Results are mean ± SEM ($n \geqslant 3$). *Significantly different from control (C); $p < 0.05$.

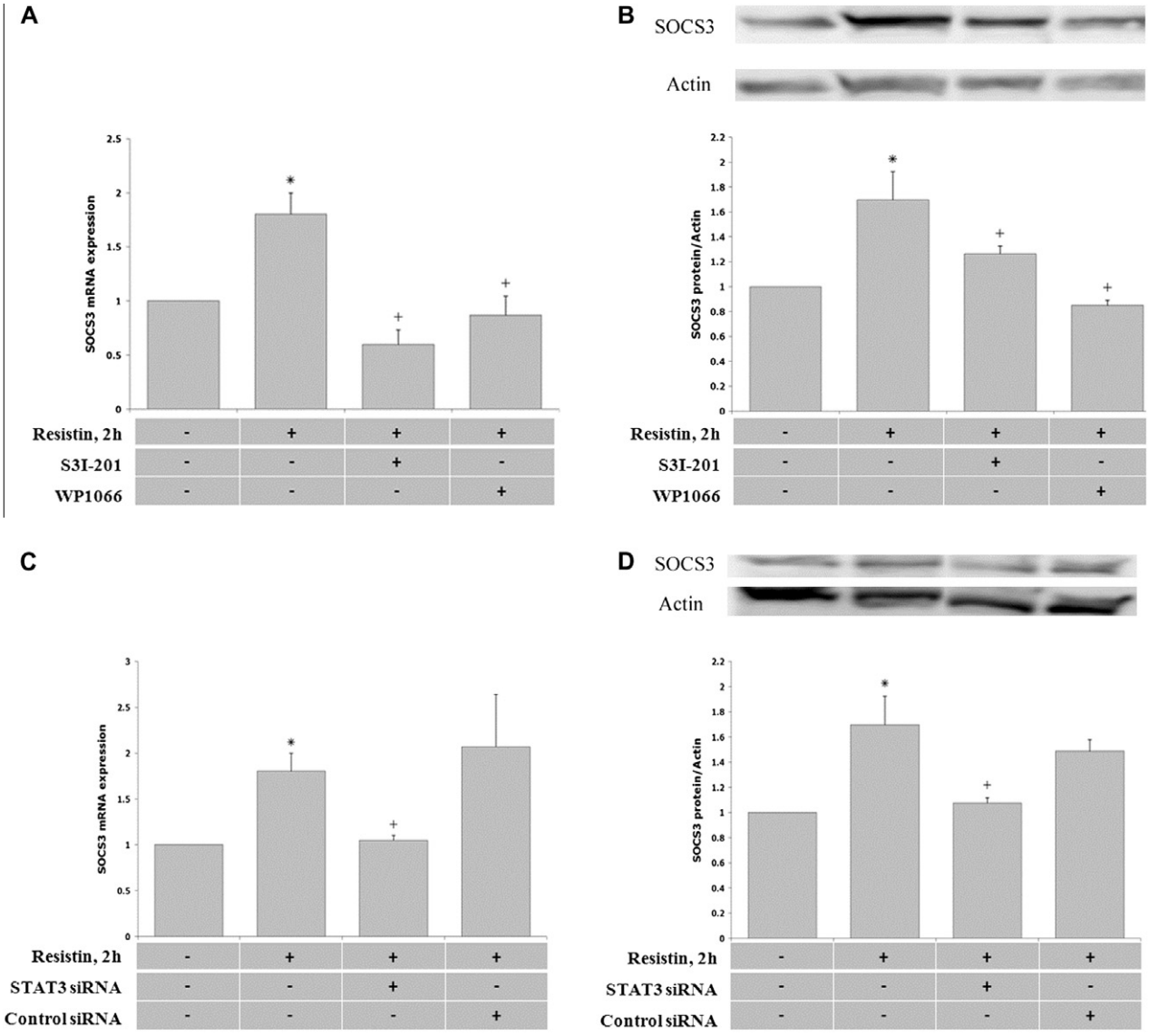

Fig. 3. STAT3 signaling pathway participates in resistin-induced SOCS3 expression in HEC. (A) Effect of JAK2/STAT3 and STAT3 inhibitors (WP1066 and S3I201 respectively) on the SOCS3 mRNA induced by resistin in HEC. Cells were treated with WP1066 and S3I201 for 2 h before stimulation for with resistin (2 h). Total cellular RNA was isolated and reverse-transcribed and the gene expression measured by qPCR. SOCS3 mRNA expression was normalized to β-actin mRNA. (B) Effect of JAK2/STAT3 and STAT3 inhibitors on SOCS3 protein expression induced by resistin in HEC. Cells were pre-treated (2 h) with WP1066 or S3I201 prior resistin stimulation. Cellular lysates were subjected to Western blot assay. SOCS3 protein expression was normalized to β-actin. (C) Silencing of STAT3 inhibits SOCS3 mRNA in resistin-activated HEC. The cells were transfected with STAT3 siRNA, or control siRNA and further exposed to resistin (2 h, 100 ng/ml). Quantification of mRNA was done by Real Time PCR and normalized to β-actin mRNA. (D) Blocking STAT3 transcription factor (as above) diminishes to control level SOCS3 protein expression induced by resistin in HEC. The SOCS3 protein was normalized to β-actin protein. Note the lack of effect of control siRNA. Results are mean ± SEM ($n \geqslant 3$). *Significantly different from untreated cells; + significantly different from resistin-activated HEC; $p < 0.05$.

ingly, SOCS3 protein expression induced by resistin in HEC was blocked by pre-incubation of cells with JAK2-STAT3 and STAT3 inhibitors (Fig. 3B).

To further assess if STAT3 is a transcriptional mediator of SOCS3 induction by resistin, we set-up experiments to evaluate if silencing STAT3 by STAT3 siRNA affects SOCS3 expression. To this purpose HEC were transfected with STAT3 siRNA before resistin stimulation (2 h) and then SOCS3 mRNA and SOCS3 protein expression were determined. The experiments showed that silencing STAT3 gene expression in resistin-stimulated HEC blocked SOCS3 mRNA (Fig. 3C). In control experiments, in which HEC were transfected with negative control siRNA, the resistin-dependent up-regulation of SOCS3 mRNA was not influenced (Fig. 3D). Moreover, silencing STAT3 gene expression in HEC transfected with STAT3 siRNA before resistin activation blocked SOCS3 protein as well (Fig. 3D).

3.4. Loss of SOCS3 function impairs resistin-induced expression of endothelial cell adhesion molecules

We have previously reported that resistin induces a significant increase in fractalkine and P-selectin adhesion molecules expression in HEC [5,6]. To elucidate whether SOCS3 protein interferes in this process, the cells were transfected with SOCS3 siRNA for gene expression silencing prior to resistin activation. After 6 and 18 h, fractalkine and P-selectin gene and protein expression were evaluated by qPCR and Western blot assays, respectively.

The experiments showed that blocking SOCS3 gene expression prevented P-selectin gene expression whereas the effect was not detected in cells transfected with scrambled siRNA (Fig 4A). Correspondingly, Western Blot analysis revealed that SOCS3 gene silencing led to a decrease in P-selectin protein expression compared to control cells that were transfected with control siRNA (Fig 4B).

Similarly, SOCS3 gene expression silencing had the same effect on fractalkine gene and protein expression in resistin-activated HEC (Fig 4C and D).

4. Discussions

We have previously shown that resistin activates human endothelial cells (inducing the up-regulation of cell adhesion molecules), interferes in cytokines signaling and could contribute by specific mechanisms to the tailoring of the micro-inflammatory milieu [5,6]. In this study we searched for the new mechanism(s) underlying resistin inflammatory effects on vascular endothelium. The experiments showed that (1) exposure to resistin up-regulates SOCS3 mRNA and protein expression in HEC; (2) the increase in SOCS3 mRNA and protein in resistin- activated HEC is significantly augmented starting with 2 h of cell stimulation and the effect persists up to 18 h; (3) the activation of STAT3 signaling pathway is implicated in the process; (4) STAT3 activation is significant after 1 h of cell stimulation and continues for 18 h and (5) silencing of SOCS3 impedes resistin-induced expression of endothelial cell adhesion molecules in endothelial cells.

The role of SOCS3 as a mediator of the cellular effect of resistin has been reported thus far for mouse adipocytes, only. The studies have shown that in these cells resistin increases SOCS3 protein expression and that SOCS3 is the cellular mediator in the capacity of resistin to antagonize insulin action [9]. In endothelial cells, SOCS3 was described to be induced by IL6 and IFN gamma with a maximum at ~2 h for mRNA and 4 h for the proteins, after which they rapidly decrease to the baselines level [12]. Interestingly, our data revealed that, like IL6/IFN gamma, resistin generated up-regulation of SOCS3 expression in HEC at 2 h but in contrast, the SOCS3 gene and protein expression was extended for up to 18 h. This may indicate either a peculiar feature of resistin signaling in endothelial cells or/and the contribution of other inflammatory mediators induced by resistin in these cells. It has been shown that resistin stimulates IL-6 expression in mouse endothelial cells [13] and since IL-6 induces SOCS3 expression [12], we presume that the combined effect of these cytokines contribute to prolonged expression of SOCS3 in HEC; further experiments are needed to clarify this matter.

Since it has been previously shown that SOCS3 is a target gene for STAT3 transcription factor in endothelial cells [14], we carried out experiments to assess whether resistin activates STAT3 pathway. Our data showed that after 1 h of stimulation, resistin activated STAT3 transcription factor, (as characterized by increased tyrosine phosphorylation) and the effect lasted longer (up to 18 h) compared to the action of Il-6 or IFN-gamma (2–4 h respectively) [12]; these data may indicate the possibility of multipart effect of resistin in the process. Moreover, our results showed that the enhanced SOCS3 expression (mRNA and protein) induced by resistin was blocked by specific inhibitors of STAT3 signaling and by the transfection of siRNA specific for STAT3. These data indicate that STAT3 pathway represents a central regulator of SOCS3 expression in resistin-activated HEC. The similar kinetics of SOCS3 mRNA and protein expression and STAT3 activation suggest that STAT3 transcription factor is the main resistin-inducible transcription factor responsible for sus-

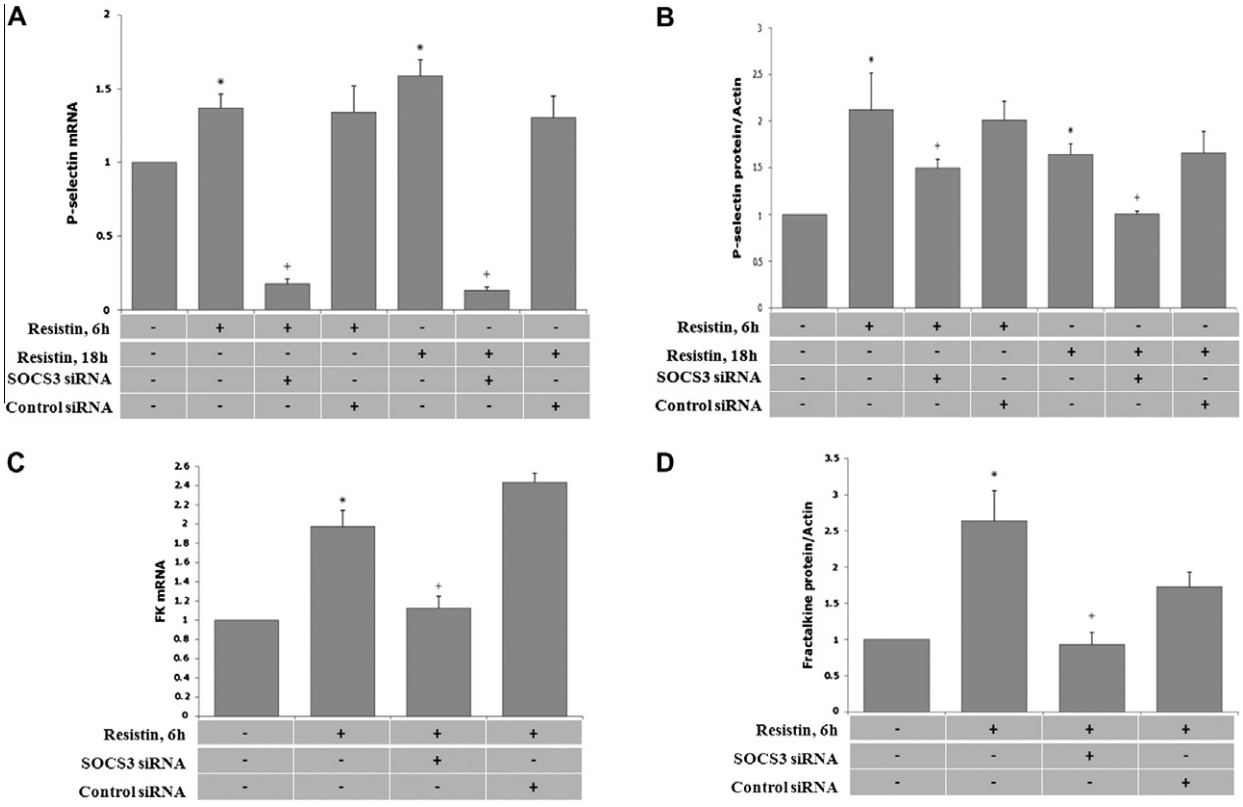

Fig. 4. SOCS3 proteins impede resistin-induced expression of P-selectin and fractalkine (Fk) in HEC. The cells were transfected with SOCS3 siRNA or control siRNA, stimulated with resistin for 6 or 18 h and then P-selectin and fractalkine gene and protein expression were determined by qPCR and Western blot, respectively. (A) P-selectin mRNA increases notably upon cell exposure to resistin and is reduced significantly in cells transfected with SOCS3 siRNA; no change is detected in cells transfected with control siRNA. (B) P-selectin protein expression is reduced significantly in HEC transfected with SOCS3 siRNA as compared to control siRNA. (C) Silencing SOCS3 gene expression inhibits fractalkine mRNA in resistin-stimulated HEC. Quantification of mRNA was done by qPCR and normalized to β-actin mRNA. (D) Blocking SOCS3 gene expression (via transfection) decreases fractalkine protein expression induced by resistin in HEC. Fractalkine protein expression was normalized to β-actin. Results are mean ± SEM ($n \geqslant 3$). *Significantly different from untreated cells; + significantly different from resistin-activated HEC; $p < 0.05$.

taining SOCS3 expression induced by resistin. In contrast, in the studies on mice adipocytes no increase in phosphorylation of STATs in response to resistin was detected [9]. The difference between our results and those obtained on mice adipocytes might be due to species or cell type-specific effects of resistin.

It is not clear how resistin activates STAT3 signaling pathway in HEC and whether the effects of resistin are mediated by one or several receptors in vascular cells. In monocytes, there are indications that resistin uses TLR4 for its pro-inflammatory effects [15]. We have preliminary data indicating that TLR4 may be involved in resistin-induced SOCS3 expression (data not shown). We may presume that resistin-induced STAT3 phosphorylation in HEC is due to a direct effect on JAK/STAT pathway activation or acts through TLR4 associated adaptor proteins or through cross talk among receptors.

Interestingly, our data indicate that SOCS3 expression and activated STAT3 co-exist up-to 18 h stimulation of endothelial cells with resistin. The kinetic experiments revealed that in resistin-activated HEC, STAT3 maximal activation occurred at 1 h stimulation and SOCS3 expression was significant after 2 h of incubation. After SOCS3 protein production a slight decrease in STAT3 phosphorylation was detected, but at a level that did not reach a statistical significance. In the case of other cytokines, such as IFN-gamma and Il-6, the increase in SOCS3 protein was accompanied by a reduction of STAT3 phosphorylation, suggesting a regulatory role for SOCS3 in STAT3 signaling in endothelial cells [12]. The divergence between these data and our results could be due to differences in cytokines action (IFN-gamma, Il-6 vs. resistin) and may be explained by the possibility that resistin-induced up-regulation of SOCS3 does not reach the level to completely shut off STAT3 activation. As a consequence, SOCS3 and activated STAT3 coexist for an extended time period and could prolong and enhance the inflammatory process in endothelial cells. This assumption is sustained by our results showing that blocking SOCS3 before stimulation with resistin, reduced HEC activation, namely inhibited the expression of resistin–induced endothelial cell adhesion molecules (fractalkine and P-selectin). The data corroborate well with the reported incomplete suppression of STAT3 by SOCS-3 in patients affected by chronic inflammatory diseases, such as rheumatoid arthritis in which resistin is recognized as an important contributor in these pathologies [16,17]. As such, the extended coexistence of SOCS3 and activated STAT3 induced by resistin may have a role in the perpetuation of the inflammatory process. In conclusion, the novel findings reported here are that in human endothelial cells (a) resistin up-regulates SOCS3 expression by activation of STAT3 pathway; (b) resistin-induced SOCS3 expression does not shut off STAT3 activation and (c) SOCS3 acts as a mediator in resistin-induced cell adhesion molecules. The results indicate the notable role of SOCS3 in maintaining the pro-inflammatory state of endothelium and designate SOCS3 as a novel therapeutic target to modulate resistin-dependent inflammation in vessel wall diseases.

Acknowledgments

The authors are indebted for excellent technical help of Mrs. Gabriela Mesca and Ioana Manolescu. This work was supported by a grant of the Romanian National Authority for Scientific Research, CNCS – UEFISCDI, project number PN-II-ID-PCE-2011-3-0928, by Romanian Academy and by CARDIOPRO, a project funded by ERDF and Romanian Government, project ID 143, SMIS 2667. V Simion acknowledges the financial support of European Social Fund Fellowship Program POSDRU/107/1.5/S/82839. The financial support of European Social Fund-''Cristofor I. Simionescu'' Postdoctoral Fellowship Programme (ID POSDRU/89/1.5/S/55216), Sectorial Operational Programme Human Resources Development 2007–2013 to M Calin is gratefully acknowledged.

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