Biochemical and Biophysical Research Communications 448 (2014) 129–133

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Resistin regulates the expression of plasminogen activator inhibitor-1 in 3T3-L1 adipocytes

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ARTICLE INFO

Article history:
Received 14 March 2014
Available online 22 March 2014

Keywords:
Adipocyte
Adipokine
Resistin
PAI-1
Akt
PP2A

ABSTRACT

Resistin and plasminogen activator inhibitor-1 (PAI-1) are adipokines, which are secreted from adipocytes. Increased plasma resistin and PAI-1 levels aggravate metabolic syndrome through exacerbation of insulin resistance and induction of chronic inflammation. However, the relationship between resistin and PAI-1 gene expression remains unclear.

Previously, we found that resistin regulates lipid metabolism via carbohydrate responsive element-binding protein (ChREBP) during adipocyte maturation (Ikeda et al., 2013) [6]. In this study, to clarify the relationship between expression of resistin and PAI-1, PAI-1 expression in differentiated 3T3-L1 adipocytes was measured after transfection with anti-resistin siRNA. We found that PAI-1 gene expression and secreted PAI-1 protein were significantly decreased by resistin knockdown. Furthermore, phosphorylation of Akt, which can inhibit PAI-1 expression, was accelerated and the activity of protein phosphatase 2A (PP2A) was suppressed in resistin knockdown 3T3-L1 adipocytes. In addition, the expression of glucose transporter type 4, a ChREBP target gene, was reduced and was associated with inhibition of PP2A. The addition of culture medium collected from COS7 cells transfected with a resistin expression plasmid rescued the suppression of PAI-1 expression in resistin knockdown 3T3-L1 adipocytes. Our findings suggest that resistin regulates PAI-1 expression in 3T3-L1 adipocytes via Akt phosphorylation.

1. Introduction

A number of studies have postulated that abnormal expression of adipokines aggravates components of metabolic syndrome, such as insulin resistance induced by hypoadiponectinemia, and hyperphagia induced by leptin resistance [1–4]. In metabolic syndrome, it is thought that abnormal plasma levels of certain adipokines can affect each other and complicate a patient's clinical condition. Therefore, control of adipokine expression is important for alleviation of metabolic syndrome. While the effects of individual adipokines on metabolic syndrome have been reported in many previous studies, there has been little investigation of the effects of multiple adipokines on gene expression.

In rodents, resistin is primarily secreted from adipocytes [5]. Our previous study demonstrated that secreted resistin regulates lipid accumulation in adipocytes [6]. Resistin has also been implicated in the pathogenesis of type 2 diabetes via induction of systemic insulin resistance resulting from decreased insulin sensitivity in liver and muscle [7,8].

Plasminogen activator inhibitor-1 (PAI-1) is the primary inhibitor of plasminogen activation. In hypoxia, PAI-1 expression is upregulated and provides a stromal matrix to support in growth of new blood vessels [9]. However, increased plasma PAI-1 levels in obesity are associated with type 2 diabetes and cardiovascular disease [10,11].

In this study, to clarify the relationship between resistin and PAI-1, which are both adipokines upregulated in obesity and which can aggravate metabolic syndrome, PAI-1 expression in differentiated adipocytes was measured after transfection with anti-resistin siRNA.

Abbreviations: PAI-1, plasminogen activator inhibitor-1; PI3K, phosphoinositide 3-kinase; PP2A, protein phosphatase 2A; GLUT4, glucose transporter type 4; ChREBP, carbohydrate responsive element-binding protein; PPARγ, peroxisome proliferator activated receptor γ; C/EBPα, CCAAT/enhancer-binding protein α; Smad3, sma and mad related protein 3.

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http://dx.doi.org/10.1016/j.bbrc.2014.03.076
0006-291X/

2. Materials and methods

2.1. 3T3-L1 cell culture and adipocyte differentiation

3T3-L1 pre-adipocytes were cultured and adipocyte differentiation was induced as shown in Fig. 1A and as previously described [12]. Briefly, cells were cultured in basal medium (DMEM supplemented with 10% (v/v) FBS, 10 mM HEPES, 0.2% (w/v) $NaHCO_3$, 4 mM L-glutamine, 3.5% (w/v) glucose, 0.2 mM ascorbate, 1 nM T3, and 30 μM T4) at 37 °C under a humidified atmosphere containing 5% CO_2. In this study, the day when the cells reached confluence was designated as day 0. Adipocyte differentiation was induced by incubation in differentiation medium (basal medium containing 500 μM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 1.6 μM insulin) from day 0 to day 2. Following differentiation, maturation was induced by incubation in maturation medium (basal medium containing 1.6 μM insulin and 15 μM D-biotin). Following a maturation period that started on day 2, adipocytes were analyzed on day 5.

2.2. Transfection of siRNA into 3T3-L1 cells

RNA interference was performed as previously described [6]. Briefly, anti-resistin siRNA and negative control siRNA were purchased from Life Technologies, Carlsbad, CA, USA. Transfection with siRNA was performed using Lipofectamine RNAiMAX and Opti-MEM (both from Life Technologies) according to the manufacturer's instructions. Following adipocyte differentiation, starting on day 2, 3T3-L1 cells were incubated with 50 nM of each siRNA in maturation medium and cultured until day 5.

2.3. RNA extraction and quantitative RT-PCR

Total RNA extraction and reverse transcription for real-time PCR were performed as previously described [6]. Real-time PCR was performed using an ABI StepOne Real-Time PCR System (Life Technologies) and TaKaRa SYBR premix Ex Taq II (TaKaRa Bio Inc, Kyoto, Japan) according to the manufacturer's instructions. The primer sequences used for this study were as follows: resistin sense: 5′-TCA CTTTTCACCTCTGTGGATATGAT-3′, anitisense: 5′-TGCCCCAGGT GG TGTAAA-3′, PAI-1 sense: 5′-TCAGCCCTTGCTTGCCTCAT-3′, anitisense: 5′-GCATAGCCAGCACCGAGGA-3′, GLUT4 sense: 5′-GTATGTT GCGGATGCTATGG-3′, antisense: 5′-GGAAGGTGAAGATGAAGAAG C-3′, β-actin sense: 5′-GACGGCCAGGTCATCACTATTG-3′, antisense: 5′- CCACAGGATTCCATACCCAAGA-3′, respectively. β-Actin mRNA was used as an internal control. Relative mRNA expression was determined using the $2^{-\Delta\Delta Ct}$ method.

2.4. ELISA

PAI-1 released from 3T3-L1 cells was measured using the Mouse PAI-1 Total ELISA Kit (Innovative Research Inc, Novi, MI, USA) according to the manufacturer's instructions.

2.5. Western blotting

Western blotting was performed as previously described [6]. Briefly, proteins were isolated from 3T3-L1 cells using RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 1% (v/v) nonidet P-40, 0.5% (w/v) sodium deoxycholate, and 0.1% (w/v) SDS). After determination of protein concentration using the BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions, proteins were separated on an SDS–PAGE gel and transferred to a polyvinylidene difluoride membrane. The primary antibodies used for this study were as follows: anti-Akt, anti-phospho-Akt (Ser473), anti-β-actin (Cell Signaling Technology, Danvers, MA, USA), and anti-PP2A along with a PP2A Immunoprecipitation Phosphatase Assay Kit (Upstate, Billerica, MA, USA). After incubation with horseradish peroxidase-conjugated anti-IgG, bands were visualized using the ECL Prime Western blotting detection kit (GE Healthcare, Buckinghamshire, UK), and densitometric results were analyzed using Image J software. β-actin was used as an internal control.

2.6. Measurement of cellular PP2A activity

Cellular PP2A activity was measured using a PP2A Immunoprecipitation Phosphatase Assay Kit (Upstate) according to the manufacturer's instructions.

2.7. Transient transfection of resistin expression plasmid DNA into COS7 cells and collection of conditioned medium containing secreted resistin

Plasmid DNA encoding resistin (pResistin) was constructed for our previous study [6]. Briefly, a full-length cDNA encoding the

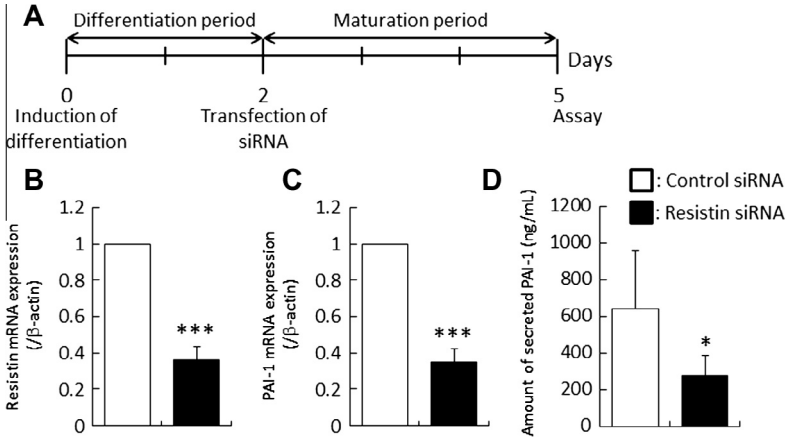

Fig. 1. Resistin knockdown influences PAI-1 expression. (A) Schedule of adipocyte differentiation and resistin knockdown. (B–D) 3T3-L1 cells were transfected with negative control (open bars) or anti-resistin (filled bars) siRNA on day 2. (B, C) Resistin mRNA and PAI-1 mRNA expression levels relative to β-actin on day 5 were normalized to those in cells transfected with negative control siRNA. (D) Effects of resistin knockdown on PAI-1 secretion. Levels of secreted PAI-1 in the culture medium on day 5 were measured by ELISA. Data are shown as means ± SD (B, C, $n = 5$; D, $n = 6$). $^{*}P < 0.05$, $^{***}P < 0.001$, versus cells transfected with negative control siRNA.

mouse resistin gene was inserted into the protein expression plasmid pcDNA3.1(+). pResistin was transfected into COS7 cells using FuGENE HD (Life Technologies). Empty plasmid DNA was used as a negative control. Twenty-four hours after transfection, the medium was changed to DMEM supplemented with 10% (v/v) FBS. This conditioned medium was collected after 48 h. The collected medium containing secreted resistin was added to 3T3-L1 cells treated with anti-resistin siRNA according to the schedule shown in Fig. 4A.

2.8. Statistical analysis

Statistical significance was determined using Student's *t*-test. Data are presented as means ± standard deviation (S.D.). *P* values <0.05 were considered to be significant.

3. Results

3.1. Decreased PAI-1 expression in resistin knockdown 3T3-L1 adipocytes

To investigate the relationship between resistin and PAI-1 expression in adipocytes, anti-resistin siRNA was transfected into differentiated 3T3-L1 cells (Fig. 1A and B). Intriguingly, PAI-1 gene expression and secreted protein content, which is associated with cardiovascular disease, were significantly reduced (Fig. 1C and D). These results indicate that resistin can regulate PAI-1 expression in 3T3-L1 adipocytes.

3.2. Acceleration of Akt phosphorylation and suppression of PAI-1 gene expression after resistin knockdown

The previous study showed that activated Akt can suppress PAI-1 gene expression [13]. In the present study, we found that Akt phosphorylation was significantly accelerated in resistin knockdown 3T3-L1 adipocytes (Fig. 2A). Furthermore, the acceleration of Akt phosphorylation continued for 24–72 h following transfection with anti-resistin siRNA (Fig. 2B). Changes in PAI-1 gene expression were also quantitated, and it was observed that PAI-1 expression was suppressed after acceleration of Akt phosphorylation (Fig. 2C).

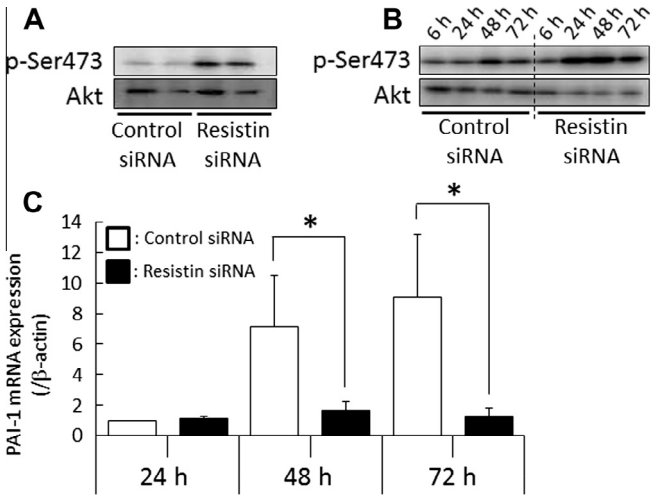

Fig. 2. Effects of resistin knockdown on Akt phosphorylation and PAI-1 expression. (A, B) Changes in Akt phosphorylation induced by resistin knockdown. Cells were transfected with negative control or anti-resistin siRNA on day 2. A representative Western blot is shown. (A) Intracellular proteins were recovered on day 5. (B) Intracellular proteins were recovered at the indicated times after transfection with siRNA. (C) Total RNA was extracted from 3T3-L1 cells at the indicated times after transfection with negative control (open bars) or anti-resistin (filled bars) siRNA. PAI-1 mRNA expression levels relative to β-actin were normalized to those in cells 24 h after transfection with negative control siRNA. Data are shown as means ± SD ($n = 3$). $^{*}P < 0.05$, versus cells in the indicated groups.

3.3. Suppression of PP2A activity and reduction of GLUT4 expression by resistin knockdown

Akt phosphorylation is regulated by several mechanisms. Akt is principally phosphorylated by activated phosphoinositide 3-kinase (PI3K) and phosphoinositide-dependent kinase-1, which are activated by insulin signaling. However, our previous study showed that intracellular lipid content in adipocytes was reduced by resistin knockdown [6]. This result is inconsistent with the fact that insulin signaling increases intracellular lipid content. Therefore, we hypothesized that Akt in resistin knockdown 3T3-L1 adipocytes was phosphorylated through an insulin-independent mechanism. To clarify the mechanism of acceleration of Akt phosphorylation, expression of protein phosphatase 2A (PP2A), a phosphatase for numerous kinases, was quantitated. Expression of PP2A was not affected by resistin knockdown (Fig. 3A). However, interestingly, PP2A activity was suppressed after treatment with anti-resistin siRNA (Fig. 3B). PP2A is activated by xylulose 5-phosphate, which is synthesized from glucose via the hexose monophosphate shunt [14]. We next examined the expression of glucose transporter type 4 (GLUT4) in resistin knockdown 3T3-L1 adipocytes. A previous study demonstrated that the expression of GLUT4 is regulated by carbohydrate responsive element-binding protein (ChREBP), a key transcription factor for lipogenic genes [15]. In addition, we previously observed that transcriptional activity of ChREBP was suppressed in resistin knockdown

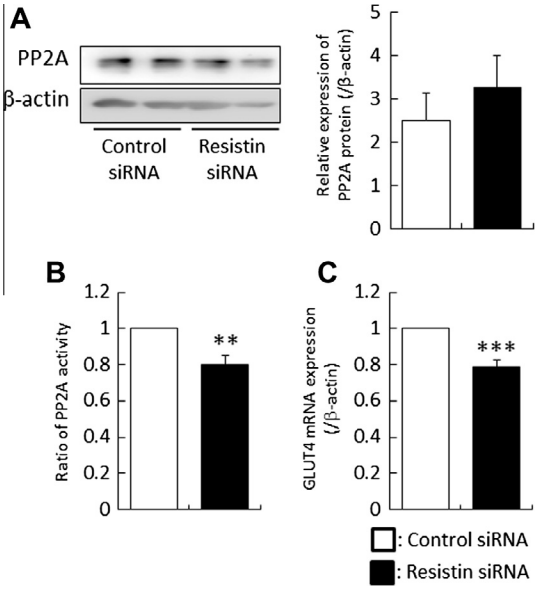

Fig. 3. Suppression of PP2A activity and reduced GLUT4 expression in resistin knockdown 3T3-L1 adipocytes. Cells were transfected with negative control (open bars) or anti-resistin (filled bars) siRNA on day 2. (A) PP2A levels in differentiated 3T3-L1 cells on day 3. Intracellular proteins were recovered on day 3 and subjected to Western blotting. Relative band intensities of PP2A were normalized to those of cells transfected with negative control siRNA. (B) Relative PP2A activity in differentiated 3T3-L1 cells on day 3. Intracellular proteins were recovered on day 3 and analyzed using a PP2A Immunoprecipitation Phosphatase Assay Kit as described in the Section 2. (C) GLUT4 mRNA expression levels relative to β-actin on day 3 were normalized to those of cells transfected with negative control siRNA. Data are shown as means ± SD (A, B, $n = 3$; C, $n = 4$). $^{**}P < 0.01$, $^{***}P < 0.001$, versus cells transfected with negative control siRNA.

3T3-L1 adipocytes [6]. Consistent with these previous studies, expression of GLUT4 was suppressed by anti-resistin siRNA treatment. These results suggest that activation of Akt is partially regulated by suppression of PP2A activity in resistin knockdown 3T3-L1 adipocytes.

3.4. Treatment with conditioned medium containing secreted resistin and expression of PAI-1

Finally, we investigated whether the expression of PAI-1 in resistin knockdown 3T3-L1 adipocytes could be rescued by the addition of medium containing secreted resistin according to the schedule shown in Fig. 4A. We found that although resistin expression was suppressed, induction of Akt phosphorylation was also suppressed (Fig. 4B and C). Furthermore, PAI-1 gene expression and protein secretion were rescued by the conditioned medium (Fig. 4D and E). These results suggest that resistin regulates the expression of PAI-1 in 3T3-L1 adipocytes.

4. Discussion

The elevated plasma concentrations of resistin and PAI-1 observed in conjunction with obesity have been regarded as risk factors for type 2 diabetes and cardiovascular disease [16–20]. Furthermore, previous studies have demonstrated that plasma resistin levels are positively correlated with PAI-1 levels [21,22]. However, it is still unclear whether resistin and PAI-1 upregulate each other or whether obesity independently increases the levels of both adipokines.

In this study, downregulation of PAI-1 was observed in resistin knockdown 3T3-L1 adipocytes. Moreover, PAI-1 expression was rescued by treatment with conditioned medium containing secreted resistin. To confirm whether the regulation of PAI-1 expression by resistin was restricted in 3T3-L1 adipocytes, anti-resistin siRNA was transfected into another type of adipocytes ST-13 cells (Supplemental Fig. 1A). However, PAI-1 gene expression increased 2.5-fold in resistin knockdown ST-13 adipocytes (Supplemental Fig. 1B). In addition, there was no effect on Akt phosphorylation unlike 3T3-L1 adipocytes (Supplemental Fig. 1C). 3T3-L1 pre-adipocyte derived from the mouse embryonic fibroblast. On the other hand, ST-13 pre-adipocyte was established from the mouse adult primitive mesenchymal cells [23]. In spite of using the same sort of adipocyte, differences in cell origins might be the cause of these discrepancies [24,25]. It is yet unclear whether the regulation of PAI-1 expression by resistin is restricted to 3T3-L1 adipocytes at the moment. Therefore, the further examination will be required.

Peroxisome proliferator activated receptor γ (PPARγ) is known as an important transcription factor for PAI-1 during adipogenesis [26]. However, our previous study showed that resistin knockdown after adipocyte differentiation had no effect on expression of PPARγ or CCAAT/enhancer-binding protein α (C/EBPα). PPARγ and C/EBPα are able to mutually induce expression of one another, forming a positive feedback loop [27]. Therefore, it was thought that neither the expression nor the activity of these transcription factors was affected by resistin knockdown. Akt phosphorylation was significantly accelerated in resistin knockdown 3T3-L1 adipocytes (Fig. 2A). A previous study showed that activated Akt can

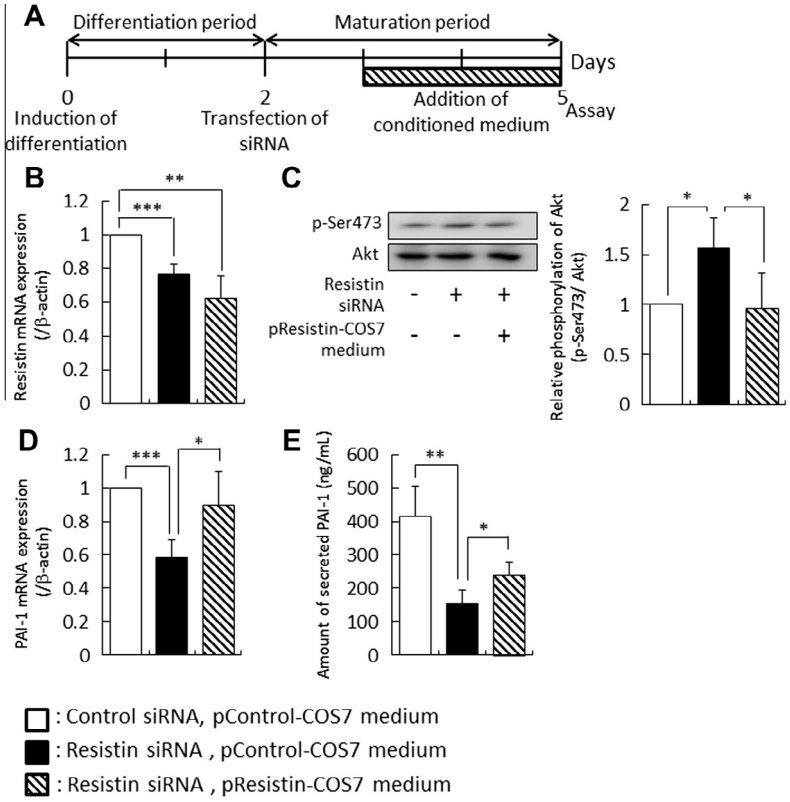

Fig. 4. Effects of the addition of conditioned medium containing resistin on PAI-1 expression in resistin knockdown 3T3-L1 adipocytes. (A) Schedule of adipocyte differentiation, resistin knockdown, and addition of the conditioned medium. (B) Resistin mRNA expression levels relative to β-actin on day 5 were normalized to those of cells transfected with negative control siRNA. (C) Effects of the addition of conditioned medium on Akt phosphorylation. Intracellular proteins were recovered on day 5. A representative Western blot is shown. Relative band intensities of p-Ser473 Akt were normalized to those of cells transfected with negative control siRNA and supplemented with conditioned medium not containing resistin. (D, E) Effects of the addition of conditioned medium on PAI-1 expression. (D) PAI-1 mRNA expression levels relative to β-actin on day 5 were normalized to those of cells transfected with negative control siRNA and supplemented with conditioned medium not containing resistin. (E) Levels of secreted PAI-1 in the culture medium on day 5 were measured by ELISA. Data are shown as means ± SD ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, versus cells transfected with negative control siRNA and supplemented with conditioned medium not containing resistin.

suppress PAI-1 gene expression [13]. To investigate whether Akt phosphorylation is directly involved in the relationship between resistin and PAI-1 expression, we added an Akt inhibitor to resistin knockdown 3T3-L1 adipocytes. We found that there was no difference in PAI-1 expression in negative-control siRNA and anti-resistin siRNA treated cells. However, since Akt phosphorylation is important for adipogenesis, the addition of an Akt inhibitor reduced intracellular lipid content and altered the expression of some genes (data not shown). Sma and mad related protein 3 (Smad3), which is activated by transforming growth factor β, is also known to be a transcription factor for PAI-1. Phosphorylated Akt inhibits nuclear translocation of Smad3 by binding to Smad3 directly [28]. Therefore, further studies are needed to investigate the role of Smad3 in resistin knockdown 3T3-L1 adipocytes, such as by quantitative analysis of nuclear translocation and transcriptional activity.

The present study showed that acceleration of Akt phosphorylation is partly regulated by suppression of PP2A activity in resistin knockdown 3T3-L1 adipocytes. It has been reported that PI3K, which is important for adipogenesis, is activated by an insulin-independent mechanism in adipocytes [29]. Since Akt phosphorylation is significantly accelerated in resistin knockdown 3T3-L1 adipocytes, not only the suppression of PP2A, but also another mechanism, such as the activation of PI3K via an insulin-independent pathway, may activate Akt. Further study is required for better understanding of the mechanism of activated Akt in resistin knockdown 3T3-L1 adipocytes.

Because the aim of this study was to clarify the relationship between resistin and PAI-1 expression, PAI-1 expression was quantitated following transfection with anti-resistin siRNA and the addition of conditioned medium containing secreted resistin to differentiated adipocytes. We found that resistin regulates PAI-1 expression in 3T3-L1 adipocytes. This finding implies that control of resistin function may be useful to predict and alleviate not only type 2 diabetes but also cardiovascular disease through regulation of PAI-1 expression. To elucidate the possibility of the PAI-1 expression control by regulation of resistin, further studies such as in vivo resistin knockdown are needed. In addition, the possibility remains that other adipokines may influence one another's expression. Better strategies for alleviation of metabolic syndrome may result from further investigation of the effects of other adipokines on adipokine gene expression.

Acknowledgments

This work was supported in part by the Kyoto Pharmaceutical University Fund for the Promotion of Scientific Research. The authors are grateful to Dr. Tsutomu Oikawa for kindly provision of a pre-adipocyte cell line ST-13. The authors also thank the Honjo International Scholarship Foundation (HISF), Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.076.

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