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Biochemical and Biophysical Research Communications 348 (2006) 253–258
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Sp1 mediates repression of the resistin gene by PPARγ agonists in 3T3-L1 adipocytes ☆,☆☆

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Received 4 July 2006
Available online 18 July 2006

Abstract

Resistin is an adipokine related to obesity and insulin resistance. Expression of the resistin gene is repressed by the treatment of peroxisome proliferator-activated receptor γ (PPARγ) agonists, thiazolidinediones (TZDs). In this study, we investigated the mechanism by which TZDs inhibit the resistin gene expression. Resistin gene expression was decreased by TZD in fully differentiated 3T3-L1 adipocytes, which was abolished after treatment of cycloheximide (a protein synthesis inhibitor). TZD could not repress the expression of the resistin gene in the presence of mithramycin A (an Sp1 binding inhibitor). Sp1 binding site of the resistin promoter (−122/−114 bp) was necessary for the repression. Further investigation of the effect of TZDs on the modification of Sp1 showed that the level of *O*-glycosylation of Sp1 was decreased in this process. These results suggest that PPARγ activation represses the expression of the resistin gene by modulating Sp1 activity.

Keywords: Resistin; Sp1; PPARγ; Thiazolidinediones; *O*-glycosylation

Resistin is an adipokine and belongs to a novel class of cysteine-rich secreted proteins named FIZZ (found in inflammatory zone) or RELM (resistin-like molecule) [1]. Although, the resistin has been generally known as a mediator of obesity-associated insulin resistance [2–8], there are some contradictory data showing that the level of resistin is not related to obesity or insulin resistance [9–11].

Resistin was originally identified as an adipokine whose expression was suppressed by TZDs, synthetic PPARγ ligands, and insulin sensitizing drugs [4,12]. However, it has been reported that PPARγ regulates the expression of the resistin gene divergently [13]. Resistin expression is induced during adipogenesis and overexpression of PPARγ leads to resistin expression by stimulating adipogenesis. In contrast, the treatment of TZDs in differentiated adipocytes significantly reduces the mRNA level of resistin.

Transcription of the resistin gene is regulated by several transcription factors such as CCAAT/enhancer binding proteins alpha (C/EBPα), sterol regulatory element binding protein 1 (SREBP1), and Sp1 [14–16]. In a previous report, we have demonstrated that Sp1 regulates the resistin promoter activity and Sp1 may mediate the glucose-induced transcription of resistin [16].

Sp1 is a well-known transcription factor and three other members of Sp family have been identified [17]. Sp1 binds to GC-rich sequences and post-translational modifications like *O*-glycosylation or phosphorylation are important factors to regulate the activity of Sp1 [17]. Especially high concentration of glucose or glucosamine increases the *O*-glycosylation states of Sp1 and this modification activates Sp1 and subsequently results in the induction of some target genes such as plasminogen activator inhibitor-1 (PAI-1) and argininosuccinate synthetase [18–20]. It has been

☆ This study was supported by FPR05C1-110 of 21C Frontier Functional Proteomics project from Korean Ministry of Science and Technology.
☆☆ *Abbreviations:* PPAR, peroxisome proliferator-activated receptor; Sp1, stimulatory protein 1; TZDs, thiazolidinediones; EMSA, electrophoretic mobility-shift assay.
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doi:10.1016/j.bbrc.2006.07.048

reported that *O*-glycosylation of Sp1 by insulin stimulates translocation of Sp1 into nucleus and leads to activate transcription of target genes [21].

In this study, we demonstrated that Sp1 is involved in the repression of resistin gene expression by PPARγ agonists.

Materials and methods

Plasmids. DNA fragments containing various length of resistin promoter were inserted into pGL2-basic vectors (Promega). DNA fragments between −1946 and +11 bp, between −989 and +11 bp or between −199 and +11 bp of the resistin promoter were ligated and the resulting constructs were named as *RETN* (−1946) Luc, *RETN* (−989) Luc or *RETN* (−199) Luc, respectively. DNA sequences of the region from −122 to −114 bp of the *RETN* (−199), TCCCTCCTC, were changed to TCCATACTC in the *RETN* (−120 mt) Luc and the region from −60 to 52 bp, GGGGCCAGGG, were changed to TTGGAAAGG in the *RETN* (−60 mt) Luc. Expression vectors of Sp1 (pCMV-Sp1), PPARγ (pSPORT-PPARγ), and RXRα (pCMV-RXRα) were described previously.

Cell culture and differentiation. 3T3-L1 Adipocytes were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% calf serum (CS) (Invitrogen). Differentiation was induced by the addition of 0.5 nmol/l of 3-isobutyl-1-methylxanthine, 0.25 μmol/l dexamethasone (Sigma), and 5 μg/ml insulin to the media for 48 h. The cells were maintained in DMEM supplemented with 10% FBS and 1 μg/ml insulin for an additional 10 days with the media changed every other day.

RNA preparation, Northern blot analysis, and ELISA. 3T3-L1 Adipocytes differentiated for 10 days were incubated with troglitazone or rosiglitazone (10 μmol/l) for the indicated periods and the cells were harvested. Differentiated adipocytes were treated with troglitazone in the absence or presence of mithramycin A (100 nM) [22] for 24 h. Northern blot was carried out as described previously [23]. Membranes were hybridized with the probes for mouse resistin, PPARγ, Sp1 or GAPDH labeled with [α-^{32}P]dATP. The amount of resistin in the media was measured using the mouse resistin ELISA kit AdipoGen, Korea.

Transient transfection and reporter assay. 3T3-L1 preadipocytes were maintained in DMEM supplemented with 10% FBS. Cells were seeded at 12-well plates and transfected with 0.6 μg reporter vectors, 0.15 μg pCMV-Sp1, 0.1 μg pSPORT-PPARγ, 0.05 μg pCMV-RXRα, and 0.1 μg pCMV-β-galactosidase using LipofectAMINE and Plus reagent (Invitrogen). To transfect the same amount of DNA in each well, pcDNA was used. Cells were treated with troglitazone for 40 h and then harvested. Luciferase and β-galactosidase activities were measured according to the manufacturer's instruction (Promega).

Nuclear extracts and electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared from 3T3-L1 adipocytes as described previously with mild modifications [24]. Double-stranded oligonucleotide representing the −134 to −100 bp region (upper strand sequence: 5′-GCA GGAGGGAAATCCCTCCTCTGGGACCTCTAG-3′ and down strand sequence: 5′-CTCTAGAGGTCCCAGAGGAGGGATTTCCCTCC-3′) of the resistin gene was labeled with [α-^{32}P]dATP using Klenow DNA polymerase (Ambion). Labeled probe was incubated with nuclear proteins (5 μg) in 10 mmol/L Hepes (pH 7.9), 50 mmol/L KCl, 0.1 mmol/L EDTA, 0.25 mmol/L DTT, 0.1 mg/ml poly(dI–dC), 0.01% Nonidet P-40, and 10% glycerol at room temperature for 15 min. For supershift assay, antibody (2 μg) against Sp1 (sc-17824) or Sp3 (sc-644) (Santa Cruz Biotechnology) was incubated with nuclear extracts for 15 min on ice before the addition of the labeled probe. The reaction mixtures were electrophoresed on 5% acrylamide gel in 0.5 × TBE buffer. The gel was dried and then exposed to the X-ray film.

Immunoprecipitation and immunoblot analysis. Differentiated 3T3-L1 adipocytes were treated with troglitazone, rosiglitazone (10 μmol/L) or glucosamine (5 mM) for the indicated period. Cells were harvested in lysis buffer (20 mM Tris–HCl, pH 7.4, 5 mM $Na_2P_2O_7$, 100 mM NaF, 2 mM Na_3 VO_4, 1% NP-40, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM PMSF). Cell extracts (1 mg) were sonicated and cell debris was removed by centrifugation. Cell lysates were incubated with 4 μg of anti-Sp1 antibody, 20 μl of protein A- (Sigma), and protein G–agarose (Amersham) at 4 °C. The beads were collected and extensively washed with phosphate-buffered saline (PBS). SDS–PAGE sampling buffer was added to the beads and the samples were boiled. Electrophoresis and immunoblot were performed with anti-Sp1 antibody, anti-*O*-GlcNAc antibody (RL2, Affinity BioReagents) (MMS-240R, Covance) or PPARγ antibody (Santa Cruz Biotechnology).

Statistical analysis. Statistical differences between experimental groups were determined by Student's *t* test using InStat (GraphPad Software). Values $p < 0.05$ were considered significant.

Results

Role of PPARγ in the repression of resistin expression by thiazolidinediones

Troglitazone was treated to the differentiated adipocytes and cells were harvested at different time points. Troglitazone decreased the level of resistin mRNA in a time-dependent manner until 24 h (Fig. 1A). Rosiglitazone, another

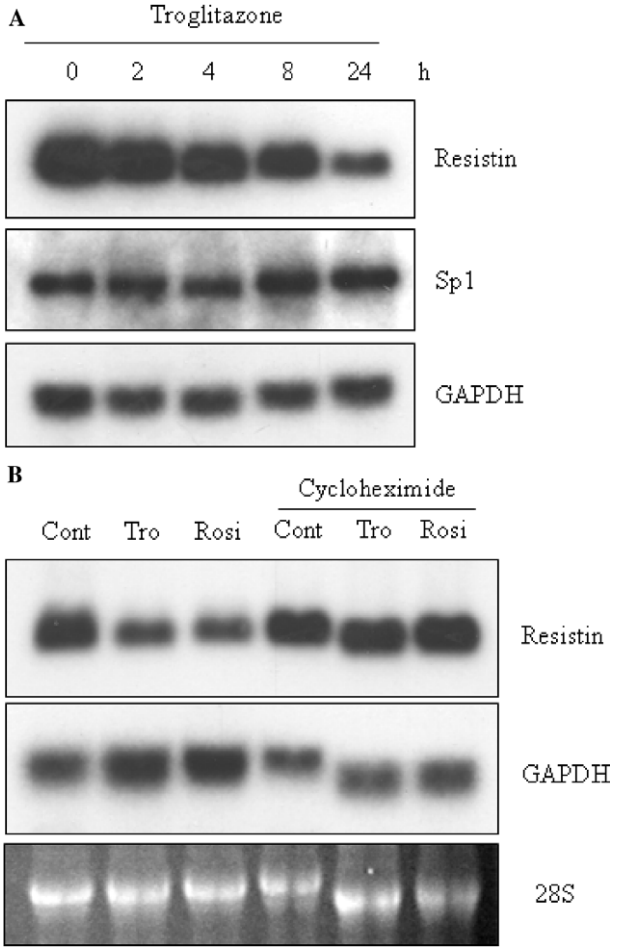

Fig. 1. Effect of TZDs on the expression of resistin in differentiated adipocytes. (A) 3T3-L1 preadipocytes were differentiated for 10 days and treated with troglitazone for the indicated time and total RNA was isolated. RNA (20 μg) was probed with cDNA fragments of resistin, Sp1 or GAPDH. (B) Differentiated adipocytes were treated with 10 μM troglitazone (Tro) or rosiglitazone (Rosi) in the absence or presence of cycloheximide (5 μM) for 24 h. Total RNA was isolated and Northern blot was performed with resistin or GAPDH probes.

member of TZDs, also repressed the level of resistin expression in a similar manner (data not shown). To test whether the effect of TZDs on the expression of resistin requires protein synthesis, cycloheximide, an inhibitor of protein synthesis, was added to the cells during the treatment of TZDs. Effect of TZDs on the resistin mRNA level was abolished when cells were exposed to cycloheximide (Fig. 1B).

Effect of mithramycin A on the expression of resistin

It has been reported that PPARγ represses the expression of some genes by modulation of Sp1 activity. We reported that Sp1 plays a role in the expression of the resistin gene. The treatment of mithramycin A, an inhibitor of Sp1 binding to DNA, decreased the expression of resistin (Fig. 2A). To determine whether Sp1 is involved in the transcriptional repression of resistin by TZDs, mithramycin A was added when cells were treated with troglitazone. Resistin mRNA level was not decreased by troglitazone in the presence of mithramycin A (Fig. 2A). Similar results were obtained from the resistin protein levels measured by ELISA (Fig. 2B).

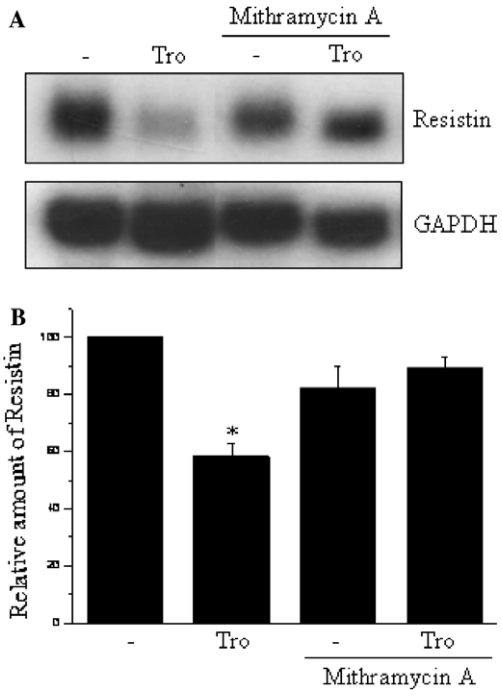

Fig. 2. Mithramycin A interferes with the effect of troglitazone on the expression of the resistin gene. Differentiated adipocytes were treated with troglitazone in the absence or presence of mithramycin A (100 nM) for 24 h. (A) Northern blot analysis. Total RNA (20 μg) was probed with resistin or GAPDH cDNA fragments labeled by [α-^{32}P]dATP. (B) ELISA of resistin in the media. The amount of resistin in the media after the treatment of troglitazone or mithramycin A was measured by resistin ELISA kit. The amount of resistin in the absence of troglitazone and mithramycin A was set to one. Bars represent means ± SEM of four independent experiments. $^{*}p < 0.05$ compared with control without troglitazone.

Mapping of cis-acting elements in the resistin promoter involving the effect of TZDs

To identify the *cis*-acting element that mediates transcriptional repression by TZDs, transient transfections and reporter assays were performed with several mouse resistin promoter-luciferase constructs. Since differentiated adipocytes hardly accept plasmid DNAs, we used 3T3-L1 preadipocytes for these experiments. Both Sp1 expression vector and PPARγ expression vector were transfected into preadipocytes since PPARγ gene expression and Sp1 binding activity in preadipocytes are much lower than those in fully differentiated adipocytes (Fig. 3A). Without the overexpression of Sp1 or PPARγ, troglitazone did not change the transcriptional activity of resistin promoter. When both Sp1 and PPARγ were overexpressed, troglitazone substantially decreased the promoter activities of all constructs tested, *RETN* (−1946), *RETN* (−989), and *RETN* (−199) Luc. Since the region between −199 and +11 bp of the resistin promoter still had the major *cis*-acting element, we searched Sp1 binding sites within this region. A possible Sp1 binding site (−122/−114 bp) was destroyed in *RETN* (−199) Luc and the resulting construct was named *RETN* (−120 mt) Luc. Overexpression of Sp1 did not increase the promoter activity of *RETN* (−120 mt) Luc, indicating that the region at −122/−114 bp was the Sp1 binding site. In addition, troglitazone did not suppress the promoter activity of the *RETN* (−120 mt) Luc construct, indicating that the Sp1 binding site at −122/−114 bp was necessary for the repression by troglitazone (Fig. 3B). In contrast, mutations at another G/C-rich region at −60/−52 bp did not interfere with the effect of troglitazone.

Effect of TZDs on the binding ability of Sp1 proteins to DNA

EMSA was performed with nuclear extracts from adipocytes and oligonucleotide containing the Sp1 binding site at −122/−114 bp of the resistin promoter as a probe. Since it was well known that Sp1 and Sp3 bound to the same DNA sequence, we used antibodies specific to these proteins. Sp1 and Sp3 from adipocyte nuclear extracts bound to the probe, which was proved by supershifts of the bands after the addition of specific antibodies against Sp1 or Sp3 (Fig. 4). Next, we tested whether the binding activity was affected by TZDs. When cells were treated with troglitazone or rosiglitazone, there was no remarkable change in the binding activity to the Sp1 binding site, and only slight reduction was observed (Fig. 4).

Effect of TZDs on the glycosylation of Sp1

Next we tried to investigate how TZDs affect Sp1 activity. Since TZDs did not change the mRNA level of Sp1 (Fig. 1A) or did not significantly affect the binding activity

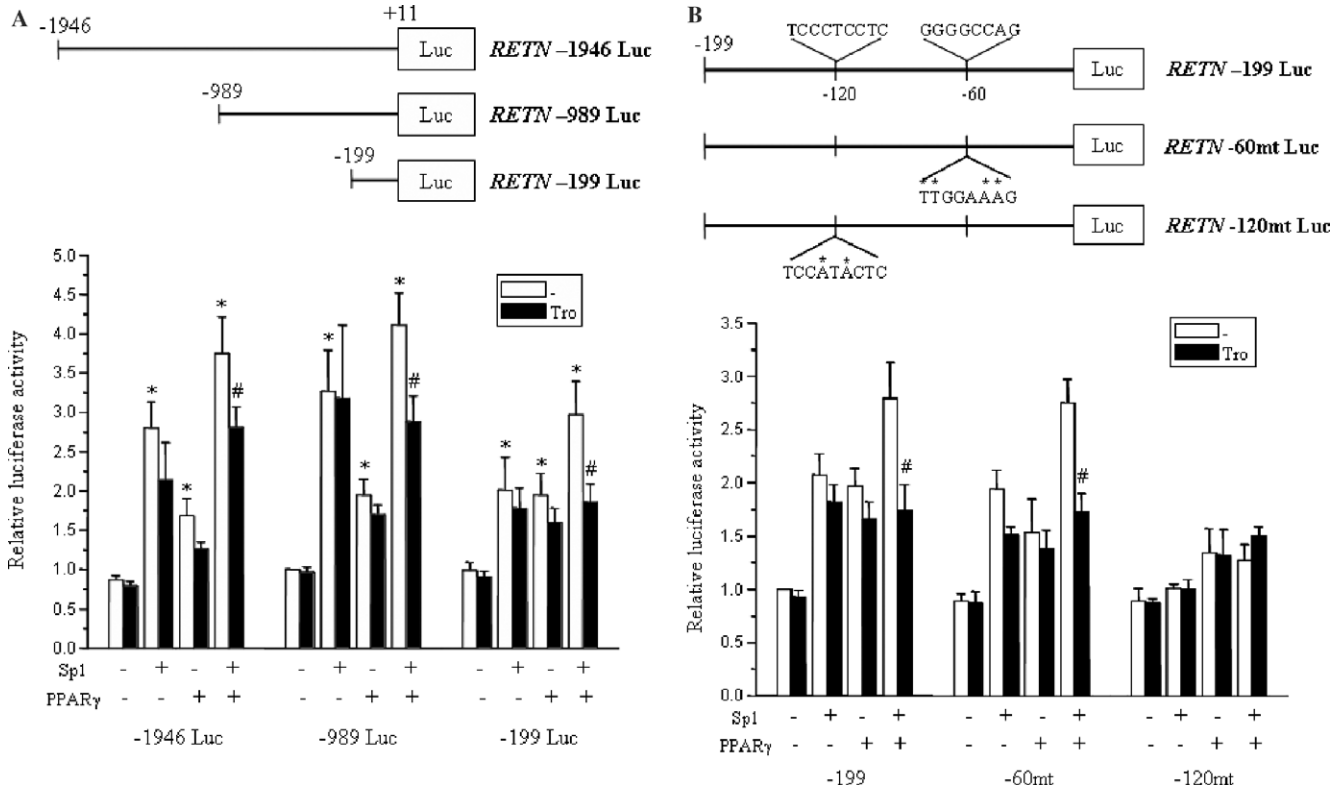

Fig. 3. An Sp1 binding site in the resistin promoter is involved in the repression of resistin expression by troglitazone. (A) 3T3-L1 preadipocytes were transfected with one of the mouse resistin promoter-luciferase constructs and expression vectors of Sp1, PPARγ, RXRα or β-galactosidase as indicated. To transfect the same amount of DNA in each well, pcDNA was used. Cells were treated with troglitazone for 40 h. Luciferase activity was normalized by β-galactosidase activity. Activity of *RETN* −989 Luc in the absence of PPARγ or Sp1 overexpression was set to one and other activities were expressed relative to this value. Bars represent means ± SEM of six independent experiments. $^{*}p < 0.05$ compared with the value of each construct in the absence of PPARγ or Sp1 overexpression. $^{\#}p < 0.05$ compared with the value in the absence of troglitazone. (B) Two reporter constructs containing mutations around −60 or −120 bp of the resistin gene were generated by site-directed mutagenesis. Cells were transfected and analyzed as described in (A). Bars represent means ± SEM of five experiments. $^{\#}p < 0.05$ compared with the value in the absence of troglitazone.

of Sp1 to DNA, we examined whether TZDs affect the post-translational modification status of Sp1. Troglitazone was treated to the adipocytes and then cell lysates were subjected for immunoprecipitation with anti-Sp1, antibody. Sp1 protein levels were not changed by the treatment of troglitazone, but *O*-glycosylation status of Sp1 was decreased (Fig. 5). Effect of troglitazone on the level of *O*-GlcNAc-modified Sp1 occurred in a time-dependent manner: the level of *O*-GlcNAc-Sp1 started to decline 4 h after the treatment of troglitazone and the progressive decrease was observed over 48 h. In addition, we tested glycosylation status of total cell lysates by immunoblot with *O*-GlcNAc antibody to figure out whether TZDs could affect the glycosylation of other proteins. TZDs did not affect the level of glycosylation state of proteins (data not shown). To investigate whether the reduction of Sp1 glycosylation by TZDs was induced by the change in the expression level of *O*-GlcNAc transferase (OGT) or *O*-GlcNAcase (OGA) genes, mRNA levels of OGT and OGA genes were measured. Troglitazone did not affect the expression level of either OGT or OGA genes (data not shown).

Discussion

It was shown that TZD treatment in fully differentiated adipocytes significantly reduced the mRNA level of resistin. We previously found that 6-month treatment of rosiglitazone in patients with type 2 diabetes mellitus significantly decreased plasma resistin concentration [25].

In this study, we found that repression of resistin gene expression by TZD was abolished by treatment of mithramycin A (an Sp1 binding inhibitor). Moreover, elimination of Sp1 binding site in the resistin promoter totally abolished the repressive activity of troglitazone, indicating that Sp1 binding site is important for this effect. In this study only one Sp1 binding site around −120 bp of the resistin gene has been identified, however, it is possible that several other Sp1 sites are dispersed in the resistin promoter and they act cooperatively. EMSA results prove that Sp1 and Sp3 bind to the −122/−114 bp region of the resistin gene. Sp3, however, hardly induced the resistin promoter activity in the transient transfection experiments (data not shown). Therefore the effect of TZDs through this *cis*-acting element may be mainly caused by the binding of Sp1. EMSA

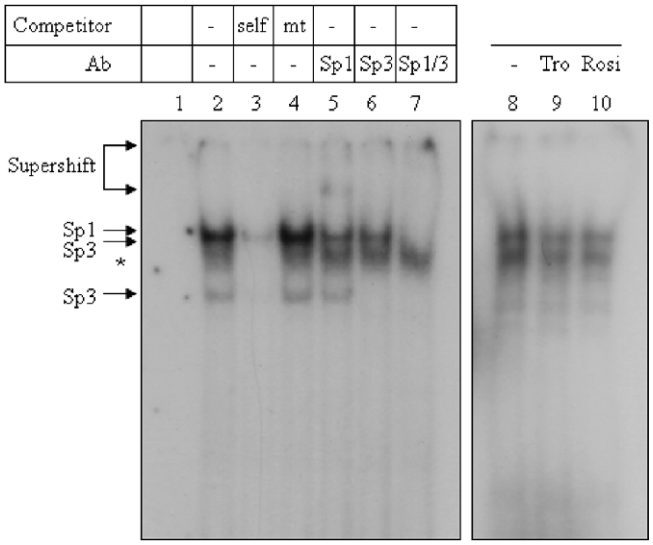

Fig. 4. Binding of Sp1 and Sp3 to the proximal promoter region of the mouse resistin gene. Oligomer representing the −134 to −100 bp region of the resistin gene was labeled with [α-^{32}P]dATP and used as a probe. Nuclear extracts were prepared from differentiated 3T3 L1 adipocytes. Free probe alone was shown in the lane 1. Five micrograms of the nuclear proteins was incubated with the probe in the absence (lane 2) or presence (lanes 3 and 4) of competitors at 100-fold molar excesses. Antibodies against Sp1 (lane 5), Sp3 (lane 6) or both (lane 7) were added to the nuclear extracts before the addition of the probe. Nuclear extracts were prepared from the cells treated with troglitazone (lane 9) or rosiglitazone (lane 10) 48 h and 5 μg of proteins was used for EMSA. Free probe, Sp1, Sp3 or supershift bands are marked as arrows. Asterisk (*) represents a band that is specific to this probe but not related with Sp1 or Sp3.

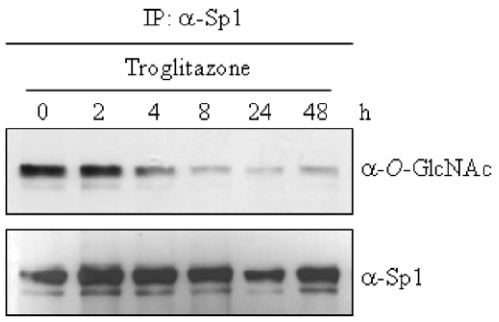

Fig. 5. Effect of TZDs on the *O*-glycosylation status of Sp1. Differentiated adipocytes were treated with troglitazone for the indicated periods and the cell lysates were prepared. Cellular proteins (1 mg) were immunoprecipitated with anti Sp1 antibody (4 μg) and then subjected to SDS–PAGE. Anti *O*-GlcNAc or Sp1 antibodies were used for immunoblot.

result also shows that treatment of TZDs slightly decreases the binding activity to this region. However, it is not likely that this difference is enough to cause the significant change in the level of resistin expression by TZDs.

It has been reported that TZDs suppress the expression of some target genes such as thromboxane receptor, KDR or type1 angiotensin II receptor genes through modulating Sp1 activity in smooth muscle cells or endothelial cells [26–28]. In these studies, the authors have suggested that there is a physical interaction between Sp1 and PPARγ. In our study, however, we cannot detect any direct interaction between Sp1 and PPARγ using coimmunoprecipitation experiments in adipocytes (data not shown). On the other hand, our results clearly show that TZDs decrease the level of *O*-glycosylation of Sp1, suggesting that TZDs decrease Sp1 activity by modulation of glycosylation status of Sp1. Further study is required to figure out how activation of PPARγ decreases the glycosylation status of Sp1 and how the change of glycosylation status of Sp1 affects expression of the resistin gene. Nevertheless we propose a model to explain the regulation of the resistin gene expression by TZDs. Sp1 is a general transcription factor, but the expression of Sp1 is significantly increased during adipocyte differentiation. Therefore Sp1 plays a quite important role in the resistin expression in adipocytes. Activation of PPARγ induces an unknown gene(s), and this protein is involved in the reduction of Sp1 glycosylation. The change in glycosylation status modulates recruiting of repressors or activators to Sp1, and finally the transcriptional activity is repressed. In addition, it is possible that PPARγ directly regulates the expression of a corepressor that interacts with Sp1. Since recent report shows that Sp1 can interact with some corepressors, it will be interesting whether treatment of TZDs affects the interaction of Sp1 with these corepressors [29].

In this study, we used 3T3-L1 preadipocytes for transient transfection and reporter assay since it is very difficult to transfect plasmid vectors in fully differentiated adipocytes. Transfection of Sp1 expression vectors in preadipocytes induced resistin promoter activity, which is quite consistent with our previous observation [16]. Interestingly, transfection of PPARγ expression vectors in preadipocytes also increased resistin promoter activity as shown in Fig. 3. Considering that addition of TZD did not increase the activity further and no direct interaction between Sp1 and PPARγ in adipocytes, it is less likely that overexpression of PPARγ directly increases resistin promoter activity. PPARγ is a master gene for adipocyte differentiation, and overexpression or activation of PPARγ stimulates the induction of several adipocyte specific transcription factors such as C/EBPα and SREBP [30,31]. *Cis*-acting elements for C/EBPα or SREBP have been identified in the resistin promoter [15,30,31]. Therefore PPARγ overexpression itself in preadipocytes might stimulate resistin expression indirectly through the induction of these adipogenic transcription factors in early stage of differentiation. Nevertheless, TZD significantly repressed the resistin promoter activity in preadipocytes with Sp1 and PPARγ overexpression as well as in fully differentiated adipocytes.

In summary, we demonstrate that Sp1 plays an important role in the suppression of the resistin gene by PPARγ ligands. We have identified an Sp1 binding site in the resistin gene necessary for the TZDs' effect. In addition, treatment of TZDs decreases the *O*-glycosylation status of Sp1 and this process may be involved in the repression of Sp1 activity.

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