Protein Inhibitor of Activated STAT 1 (PIAS1) Protects against Obesity-induced Insulin Resistance by Inhibiting Inflammation Cascade in Adipose Tissue

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Abstract

Obesity is associated with chronic low-level inflammation, especially in fat tissues, which contributes to insulin resistance and type 2 diabetes mellitus (T2DM). Protein Inhibitor of Activated STAT 1 (PIAS1) modulates a variety of cellular processes, such as cell proliferation and DNA damage responses. Particularly, PIAS1 functions in innate immune system and is a key regulator of inflammation cascade. However, whether PIAS1 involves in the regulation of insulin sensitivity remains unknown. Here we demonstrated that PIAS1 expression in white adipose tissue (WAT) was downregulated by c-Jun N-terminal kinase (JNK) in pre-diabetic mice models. Overexpression of PIAS1 in inguinal WAT (iWAT) of pre-diabetic mice significantly improved systemic insulin sensitivity, while knockdown of PIAS1 in wild-type mice led to insulin resistance. Mechanistically, PIAS1 inhibited the activation of stress-induced kinases and the expression of nuclear factor κB (NF-κB) target genes in adipocytes, mainly including pro-inflammatory and chemotactic factors. In doing so, PIAS1 inhibited macrophage infiltration in adipose tissue, thus suppressing amplification of inflammation cascade, which in turn improved the insulin sensitivity. These results were further verified in fat transplantation model. Our findings shed light on the critical role of PIAS1 in controlling insulin sensitivity and suggest a therapeutic potential of PIAS1 in T2DM.

Keywords: PIAS1, inflammation responses, macrophage infiltration, insulin sensitivity
Introduction

Type 2 diabetes mellitus (T2DM), accounting for 90% of all cases of diabetes, is one of the most prevalent chronic diseases worldwide (1). Over the last three decades, the number of adults with diabetes has doubled, rising from 153 million in 1980 to about 366 million in 2011 (2-3). T2DM is associated with hyperglycemia, which results from insulin resistance mainly in peripheral tissues (4). Insulin resistance is caused by many risk factors, ranging from gene to environment, with obesity as the most common contributor. The majority of individuals with early onset T2DM are obese, and there is an inverse linear relationship between Body Mass Index (BMI) and the age at which T2DM is diagnosed (5-6).

A key mechanism underlying obesity-driven insulin resistance is chronic inflammation in adipose tissue, which is characterized by the accumulation of tissue immune cells (7). Macrophage is one of the primary immune cell types and crucial in triggering inflammation in obese adipose tissue (8-10). In lean, the adipose tissue macrophages (ATMs) mainly exhibit anti-inflammation effect. In obesity, however, adipocytes along with ATMs produce a wide range of pro-inflammatory mediators including chemokine (C-C motif) ligand 2 (CCL2), interleukin (IL)-1β, IL-6 and tumor necrosis factor α (TNFα) (8-11), which in turn activate key regulators of inflammation such as c-Jun N-terminal kinase (JNK) and inhibitor of nuclear factor κB kinase (IKK)/ nuclear factor κB (NF-κB) signaling pathway (12). These stress kinases then phosphorylate insulin receptor (IR) and insulin receptor substrate 1 (IRS1) on inhibitory serine residues to impair insulin signaling in adipocytes (13-14).
Additionally, transcription factors like NF-κB further induce the expression of inflammation cytokines, forming a positive feedback of inflammation response (15). Therefore, inflammation is a crucial event in obesity-induced insulin resistance and identifying the key regulator for the coordination between inflammation and insulin signaling pathways may not only clarify the mechanisms underlying insulin resistance but also provide new strategies for T2DM treatment.

Mammalian protein inhibitor of activated STAT (PIAS) proteins are a family of transcriptional regulators that possess SUMO E3 ligase activity, which consist of four members: PIAS1, PIAS2, PIAS3 and PIAS4 (16-17). Initially, PIAS1 was named for its ability to interact with and inhibit signal transducer and activator of transcription (STAT) 1 (18), but subsequent investigations have clarified that PIAS1 regulates a variety of transcription factors through distinct mechanisms, including promoting SUMOylation of target proteins and blocking the DNA-binding activity of transcription factors (19). Based on previous studies, PIAS1 plays a critical role in such cellular processes as cell proliferation (20), cell differentiation (21-22) and immune responses (23-25). In the innate immune system, PIAS1 occupies the binding sites of STAT1 and NF-κB on the promoters of their target genes to block expression of pro-inflammatory factors, such as TNFα and macrophage inflammatory protein 2 (MIP2). PIAS1−/− mice showed increased protection against pathogenic infection and increased serum levels of pro-inflammatory cytokines (26). Thus, PIAS1 is a key regulator in inflammation responses of innate immunity. However, whether PIAS1 modulates the pathogenesis of insulin resistance remains unknown.
Our previous report clarified that PIAS1 restricted adipocytes differentiation by inhibiting C/EBPβ (22); however, the function of PIAS1 in mature adipocytes is not clear yet. In the current study, we found that PIAS1 was downregulated in the inguinal white adipose tissue (iWAT) of pre-diabetic models, including leptin-deficient (ob/ob), leptin receptor-deficient (db/db) and high fat diet (HFD) mice. Ectopic expression of PIAS1 in pre-diabetic iWAT significantly activated insulin signaling pathway and improved systemic insulin sensitivity. Further studies in adipocytes found that PIAS1 suppressed expression of pro-inflammation cytokines, such as CCL2, MIP2 and TNFα, which in turn inhibited macrophage infiltration in adipose tissue, thereby attenuating inflammatory cascade and increasing insulin sensitivity. Our results demonstrated the critical role of PIAS1 in modulating insulin sensitivity via the inhibition of inflammation response in adipose tissue.
RESEARCH DESIGN AND METHODS

Animals

Male C57BL/6J mice, ob/ob mice, db/db mice and their control littermates were purchased from the Model Animal Research Center of Nanjing University. These mice were maintained on normal diet. To produce HFD mice, 6-week-old C57BL/6J mice were fed HFD (51% kcal from fat) for 5 weeks or 12 weeks, with normal chow diet (NCD) mice as control. All studies involving animal experimentation were approved by the Animal Care and Use Committee of the Fudan University Shanghai Medical College and followed the National Institute of Health guidelines on the care and use of animals.

Reagent and adipocytes differentiation

Recombinant murine TNFα was purchased from PeproTech (Rocky Hill, USA). Palmitate was purchased from Sigma-Aldrich (St. Louis, MO, USA). JNK inhibitor and NF-κB inhibitor were from Selleck Chemicals.

Two days post-confluence (designated day 0), 3T3-L1 were subjected to adipogenic differentiation as previously described (22). Adipocytes phenotype appeared on day 3 and reached maximal by day 8 post-adipogenic induction. PIAS1 overexpression or knockdown assays were performed on day 5 post-induction, and cells were harvested on day 8-9.

Generation and administration of recombinant adenovirus
Recombinant adenovirus for PIAS1 overexpression were generated using ViraPower™ Adenoviral Expression System (Invitrogen, Carlsbad, CA, USA), with LacZ recombinant adenovirus as negative control. Recombinant adenovirus for PIAS1 knockdown was produced through BLOCK-i™ Adenoviral RNAi Expression System (Invitrogen). The adenoviral expression vector pAd/Block-it encoding short hairpin RNA (shRNA) of PIAS1 was constructed, with shRNA for LacZ as control. The sequences (5’ to 3’) for shRNAs are as following: shPIAS1, CACCTTATTATTGACGGGTTGTTTA; shLacZ, AATTTAACCGCCAGTCAGGCT.

Recombinant adenovirus was produced and amplified in 293A cells, and purified using adenovirus purification kits (Sartorius, Germany). Purified adenovirus was injected twice a week subcutaneously adjacent to iWAT in 8-week-old mice for 2 weeks. For HFD mice, adenovirus administration was conducted in 18-week-old mice for 2 weeks. On day 4 after adenovirus administration, these mice were subjected to further studies.

**Metabolic parameters measurement**

For glucose tolerance test (GTT), mice were injected intraperitoneally with D-glucose (2 mg/g body weight) after an overnight fasting, and tail blood glucose levels were monitored every 0.5 h using a glucometer monitor (Roche). For insulin tolerance test (ITT), mice were injected intraperitoneally with human insulin (Eli Lilly) (0.75 mU/g body weight) after 4 h fasting, and tail blood glucose were monitored every 0.5 h. Levels of plasma insulin were detected by enzyme-linked immunosorbent assay
(ELISA) kit (Mercodia). Concentrations of CCL2, MIP2 and TNFα in iWAT or medium from adipocytes were measured by ELISA kit (USCN Life Science Inc.).

2-Deoxyglucose (2-DG) uptake assay

After iWAT-specific Ad-PIAS1 or Ad-shPIAS1 administration as described above, iWAT and epididymal white adipose tissue (eWAT) were extracted from mice and allowed to recover in KRB buffer containing 11.1 mM glucose for 1 h. After the initial recovery period, the fat depots were pre-treated with or without insulin for 0.5 h, followed by incubation with 200 µM 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG, invitrogen) for 2 h in the absence or presence of insulin. After 2-deoxyglucose uptake, the depots were rinsed and lysed. Fluorescence was measured in Envision fluorescence microplate reader and normalized to total protein concentration.

Western blotting and antibodies

Western blotting analysis was performed as previously described (22). For detection insulin signaling pathway in 3T3-L1 adipocytes, cells were harvested after stimulation with insulin (100 nM) for 5 min. For measurement of insulin signaling in iWAT, the iWAT was dissected from mice 10 min after injection with insulin. During dissection, lymph node was removed from iWAT. The primary antibodies used for western blotting were as following: IRβ, phosphorylated (p)-IRβ (Tyr1150/1151), p-IRS1 (Tyr895), AKT, p-AKT (Ser473), p-JNK (Thr183/Tyr185), p-NF-κB p65 (Ser635),
p-ERK1/2, and p-p38 MAPK from Cell Signaling Technology; NF-κB p65 and JNK from Santa Cruz Biotechnology; PIAS1 from Abcam; β-actin from Sigma-Aldrich.

**RNA extraction and quantitative PCR (qPCR)**

RNA extraction and qPCR analysis were conducted as previously described (22). Results were presented as means ± standard deviations (SD) from three independent experiments. Primers used are listed in Supplementary Table 1.

**RNA interference**

Synthetic small interfering RNA (siRNA) for PIAS1, JNK1, JNK2, and siRNA negative-control (siNC) were synthesized by Invitrogen and listed in Supplementary Table 2. 3T3-L1 cells were transfected with siRNA on day 5 after adipogenic induction.

**Chromatin Immunoprecipitation (ChIP)**

ChIP analysis was conducted as previously described (27) using anti-NF-κB p65 (Santa Cruz) or anti-PIAS1 antibody (Abcam), with rabbit IgG as a negative control. Immunoprecipitated DNA was purified and quantified by qPCR, with the DNA level in input sample as an endogenous control. The primers (5’ to 3’) for ChIP-qPCR were as following: TNFα, GCAGGTCTGTCCCTTCAC, and AGTGCCCTTCTGGCAGTT; MIP2, AGCGCAGACATCACTTCCT, and CTAGCTGCTGCCTCATTCT. 
**Macrophage migration assay**

Chemotaxis of Raw264.7 macrophages was measured using Transwell plates (Corning) with a pore size of 8.0 µm. Macrophages were loaded on upper chamber with DMEM supplemented with 0.2% BSA. The adipocytes conditioned media (ACM) were added to lower chamber. The migration cells were stained with 0.1% crystal violet and counted manually using 5-6 randomly selected areas.

**Fat transplantation**

After treated with Ad-LacZ or Ad-PIAS1 for 2 weeks, iWATs from ob/ob mice were transplanted to 8-week-old male C57BL/6J mice as previously described (28-29). Briefly, fat pads from donor mice were removed, cut into approximately 0.2 g slices, and kept in saline until transplantation. For each recipient mouse, a total of 0.8 g of the slices of fat was transplanted into subcutaneous area (i.e. below the skin on the back of host mice). Sham group had surgery, but no fat was transplanted. After recovery for 2 weeks, the indicated host mice were subjected to followed studies.

**Statistics**

All experiments were independently repeated at least three times. Results were presented as means ± SD. Differences between two groups were assessed using unpaired two-tailed Student’s \( t \) test. Differences among more than two groups were assessed by ANOVA with post-hoc analysis for multiple comparisons. Differences were considered as significant when \( P < 0.05 \).
RESULTS
PIAS1 was downregulated in adipose tissues of pre-diabetic state

To investigate the potential role of PIAS1 in insulin sensitivity, we firstly measured PIAS1 expression in several target tissues of insulin action, including liver, eWAT, iWAT, brown adipose tissue (BAT) and muscle (Fig. 1A and B). We found that expression of PIAS1 at both message RNA (mRNA) (Fig. 1A) and protein level (Fig. 1B) was relatively higher in iWAT than in other tissues, which promoted us to investigate the function of PIAS1 in iWAT. Further investigation showed that PIAS1 has a broad distribution in different cells types from adipose tissue, for instance, adipocytes and immune cells such as macrophages (Supplementary Fig. 1). We next detected PIAS1 expression in WAT of ob/ob, db/db, and 12-week HFD mice, all of which were hyperglycemia and exhibited insulin resistance. PIAS1 was significantly decreased in both iWAT and eWAT of these pre-diabetic mice (Fig. 1C and D). Interestingly, PIAS1 was also reduced in WAT of 5-week-HFD mice (Fig. 1D), indicating that PIAS1 downregulation was an early event during the development of insulin resistance. In addition, we further evaluated PIAS1 expression in mature adipocytes and stromal vascular fraction (SVF), finding out that PIAS1 was decreased in both adipocytes and SVF of pre-diabetic mice (Fig. 1E).

Unlike PIAS1, however, other genes of PIAS family didn’t exhibit good correlation with pre-diabetic state (Supplementary Fig. 2). Briefly, there were no significant differences in the expression of other PIAS genes between wild-type and ob/ob WAT, except for PIAS2 in eWAT (Supplementary Fig. 2A and B), while
increased $PIAS2$, $PIAS3$ and $PIAS4$ were observed in db/db mice (Supplementary Fig. 2C and D). These data together suggested a distinct function of PIAS1 compared with other PIAS proteins.

PIAS1 promoted insulin sensitivity in mature adipocytes

Free fatty acid (FFA) and inflammation cytokines such as TNFα are two main mediators of obesity-induced diabetes (30). We therefore detected PIAS1 expression in 3T3-L1 mature adipocytes upon TNFα or palmitate treatment, with the result that expression of PIAS1 gradually declined depending on the dose and time course of treatment (Fig. 2A-C), which was consistent with the results observed in pre-diabetic mice.

Based on the negative correlation between PIAS1 expression and pre-diabetic state, we hypothesized that PIAS1 might be a potential regulator of insulin sensitivity. To test the presumption, we examined the effect of PIAS1 knockdown or overexpression on insulin signaling pathway, by performing RNA interference for PIAS1 (siPIAS1) or infecting adipocytes with adenovirus expressing PIAS1 (Ad-PIAS1). As expected, PIAS1 protein was significantly reduced by siPIAS1 (Fig. 2D) and effectively increased by Ad-PIAS1 (Fig. 2E). Oil red O staining indicated that neither knockdown nor overexpression of PIAS1 affect the lipid accumulation (Supplementary Fig. 3A and B), suggesting that altered expression of PIAS1 in adipocytes had no effect on adipogenesis. Importantly, phosphorylation of IRβ, IRS1 and AKT induced by insulin were significantly reduced by siPIAS1. In
PIAS1-deficient cells, TNFα could hardly further inhibit the insulin signaling (Fig. 2D). To rule out off target effect, another two sets of siPIAS1 were also used, with similar results (Supplementary Fig. 3C). Consistently, PIAS1 overexpression significantly rescued the impaired insulin signaling caused by TNFα (Fig. 2E). Taken together, these results indicated that PIAS1 significantly augmented insulin sensitivity in adipocytes.

**PIAS1 in iWAT improved systemic glucose tolerance and insulin sensitivity**

To further explore the impact of PIAS1 on insulin sensitivity in vivo, we ectopically expressed PIAS1 in pre-diabetic mice by infecting Ad-PIAS1 into iWAT. Western blotting showed that PIAS1 expression was specifically increased in iWAT, but not in other tissues like eWAT and liver (Supplementary Fig. 4A). PIAS1 overexpression didn’t affect the body weight (Supplementary Fig. 4B-D) and iWAT weight (Supplementary Fig. 4E and F), suggesting that short-term overexpression of PIAS1 had little effect on fat development. Of note, blood glucose levels were decreased by PIAS1 overexpression under both fasting and fed condition (Fig. 3A-C). However, plasma insulin level was not affected (Fig. 3A). Additionally, GTT and ITT showed that forced expression of PIAS1 in iWAT significantly improved systemic glucose tolerance and insulin sensitivity in pre-diabetic mice (Fig. 3D-F), while had no effect on plasma insulin level during GTT (Fig. 3D and F), implying that it was the improved insulin sensitivity by PIAS1 that contributed to decreased blood glucose. Consistently, insulin signaling pathway in iWAT was augmented by PIAS1
overexpression, as indicated by increased insulin-stimulated phosphorylation of IRβ, IRS1 and AKT (Fig. 3G and H). However, insulin signaling in eWAT was not significantly affected by PIAS1 overexpression in iWAT (Supplementary Fig. 4G). To better clarify function of PIAS1 in regulating insulin sensitivity, we conducted 2-DG uptake assay, finding out that PIAS1 overexpression in iWAT significantly promoted glucose uptake of iWAT upon insulin stimulation, while had little effect on eWAT (Fig. 3I).

We then depleted PIAS1 expression in wild-type mice by infecting adenovirus carrying PIAS1 shRNA (Ad-shPIAS1) into iWAT and found that PIAS1 knockdown was specifically occurred in iWAT (Supplementary Fig. 5A). No obvious change was observed in body weight (Supplementary Fig. 5B) and iWAT weight (Supplementary Fig. 5C) upon PIAS1 knockdown. Importantly, PIAS1-depleted mice developed insulin resistance. Briefly, deficiency of PIAS1 in iWAT led to much higher blood glucose (Fig. 4A), impaired glucose tolerance and insulin sensitivity (Fig. 4B), attenuated insulin signaling pathway (Fig. 4C), and reduced glucose uptake in iWAT (Fig. 4D). Collectively, PIAS1 in iWAT regulated insulin sensitivity in vivo.

PIAS1 inhibited inflammatory infiltration in iWAT

In obesity, inflammation in adipose tissue is a major contributor to insulin resistance (7). We therefore determined a potential role of PIAS1 in adipose tissue inflammation. PIAS1 overexpression in iWAT of pre-diabetic mice significantly decreased the mRNA level of a serial of inflammation cytokines such as CCL2, IL-1β, TNFα and
MIP2, as well as F4/80, a critical marker of macrophage (Fig. 5A and B). In order to
determine which fraction of iWAT was significantly regulated by PIAS1, we isolated
SVF and mature adipocytes from iWAT, finding out that pro-inflammatory genes were
decreased in both SVF and mature adipocytes (Fig. 5C). Consistently, PIAS1
overexpression decreased the protein level of CCL2, TNFα and MIP2 in iWAT (Fig.
5D), whereas PIAS1 knockdown increased them (Fig. 5E). Importantly, these
phenomena were restricted to iWAT. Expressions of inflammation genes in eWAT
were not affected by PIAS1 alteration in iWAT (Supplementary Fig. 4H and I, and
5D).

NF-κB is known as a pro-inflammatory transcriptional factor, which drives
expression of inflammation genes (31). We then performed ChIP assay, observing that
overexpression of PIAS1 inhibited the binding of p65 NF-κB to the endogenous
promoters of TNFα and MIP2, which was accompanied by increased occupation of
PIAS1 on these promoters (Fig. 5F and G). This might be a major cause for the
impaired expression of pro-inflammation genes. Moreover, PIAS1 overexpression
inhibited activation of JNK and p65 in pre-diabetic iWAT as indicated by their
phosphorylation modification (Fig. 5H), both of which were pro-inflammatory, while
PIAS1 knockdown was able to promote their activation (Fig. 5I). More importantly,
ectopic expression of PIAS1 significantly decreased macrophage infiltration into
iWAT as indicated by F4/80 staining (Fig. 5J). This was most likely due to the
impaired CCL2 level showed above, which is a key chemokine for macrophage.
These data collectively suggested that PIAS1 attenuated inflammation response in
PIAS1 in adipocytes inhibited macrophage migration in vitro

We then determined the effect of adipocytes PIAS1 on macrophage infiltration in vitro. Firstly, we detected the inflammation cascade in 3T3-L1 mature adipocytes, with the results that PIAS1 overexpression inhibited mRNA level of pro-inflammatory cytokines and chemokines (Fig. 6A), whereas PIAS1 knockdown promoted them (Fig. 6B), as well as the activation of p65 and JNK (Fig. 6C). Consistently, these cytokines secreted by adipocytes were significantly inhibited by PIAS1 overexpression and promoted by PIAS1 knockdown (Fig. 6D). In addition, we found that PIAS1 inhibited the binding of p65 NF-κB to the promoters of TNFα and MIP2, even under the condition of TNFα induction (Fig. 6E). As to the expression of CCL2, TNFα was considered as a mediator that links level of CCL2 to PIAS1 (Supplementary Fig. 6).

To further clarify the role of PIAS1 in macrophage migration, we detected adipocytes conditioned media (ACM)-induced chemotaxis in Transwell plates. Migration of Raw264.7 macrophages to Ad-PIAS1 ACM was significantly decreased compared with Ad-LacZ ACM, while siPIAS1 ACM induced much more macrophages chemotaxis than siNC ACM did (Fig. 6F). These results together emphasized the crucial role of adipocyte PIAS1 in inhibiting macrophage chemotaxis.

PIAS1 regulated insulin sensitivity in fat transplantation model

To further verify the role of PIAS1 in regulating inflammation response and insulin
sensitivity, we established fat transplantation model, in which Ad-PIAS1 or Ad-LacZ treated iWATs from ob/ob were transplanted into host mice (Fig. 7A). We found that there were no significant differences in body weight (Fig. 7B), fasting blood glucose level (Fig. 7C), and insulin level (Fig. 7D) among three groups as shown. However, blood glucose under fed condition was higher in transplantation group, while there were little differences between iWAT-Ad-LacZ and iWAT-Ad-PIAS1 transplantation groups (Fig. 7D). Remarkably, systemic glucose tolerance and insulin sensitivity were impaired in iWAT-Ad-LacZ transplantation group compared with Sham, primarily due to the bad fat from ob/ob, while iWAT-Ad-PIAS1 transplantation mice had improved glucose tolerance and enhanced insulin sensitivity in comparison to iWAT-Ad-LacZ group (Fig. 7E). Consistently, insulin signaling was better activated in iWAT-Ad-PIAS1 fat graft than iWAT-Ad-LacZ graft (Fig. 7F). Additionally, inflammation infiltration was decreased in iWAT-Ad-PIAS1 fat graft compared with iWAT-Ad-LacZ graft, as indicated by expression of inflammation-related genes, such as CCL2, TNFα and F4/80, and F4/80 staining (Fig. 7G and H).

Role of JNK signaling in regulation of PIAS1 expression

The results above indicated that PIAS1 was downregulated in inflammatory and pre-diabetic state, and overexpression of PIAS1 alleviated obesity-induced insulin resistance. Next, we sought to identify the signaling pathway that regulated PIAS1 expression. NF-κB and JNK signaling pathway were two key mediators downstream of inflammatory cytokines (7), we therefore determined whether PIAS1 could be
regulated by NF-kB or JNK, finding out that JNK inhibitor SP600125 promoted the
eexpression of PIAS1, whereas NF-kB inhibitor had little effect on it (Fig. 8A). In
addition, PIAS1 expression was gradually enhanced depending on the dose and time
course of SP600125 treatment (Fig. 8B and C). And inhibition of JNK by SP600125
reversed the downregulation of PIAS1 caused by TNFα treatment (Fig. 8D). It is
known that there are three different JNK genes, JNK1, JNK2, and JNK3. JNK1 and
JNK2 had a broad distribution, whereas JNK3 mainly localized in neurons rather than
adipose tissues (32). We therefore transfected siRNAs specific for JNK1 or JNK2 into
3T3-L1 adipocytes, separately or together. Both siJNK1 and siJNK2 could effectively
reduce expression of themselves, and had no mutual influence (Fig. 8E). We found
that both siJNK1 and siJNK2 increased PIAS1 protein level (Fig. 8F); and when they
were used together, PIAS1 expression was further augmented, even rescued impaired
PIAS1 expression caused by TNFα treatment (Fig. 8G). We then evaluated the
regulation of PIAS1 by JNK in vivo. Consistently, PIAS1 expression in iWAT of
ob/ob mice was significantly increased by SP600125 (Fig. 8H). Moreover, we found
that JNK was much more activated in iWAT of pre-diabetic mice as indicated by the
increased p-JNK (Fig. 8I and J), which was negatively correlated with the expression
of PIAS1. All these results suggested that JNK was a vital mediator for PIAS1
downregulation.
Discussion

In the current study, we propose a model in which PIAS1 plays a role in controlling insulin sensitivity by inhibiting inflammatory cascade (Fig. 8K). In pre-diabetic state, PIAS1 was significantly downregulated by pro-inflammatory factors in adipocytes as a consequence of activated JNK. Decreased PIAS1, on the contrary, gave rise to augmented NF-κB and JNK signaling, which produce much more inflammatory cytokines, thereby inducing macrophage infiltration. All these inflammatory cascades caused by PIAS1 downregulation further led to insulin resistance. Importantly, PIAS1 overexpression in iWAT was able to improve obesity-induced insulin resistance. Accordingly, the reciprocal regulation between PIAS1 and inflammatory response constitute a feedback loop likely to determine systemic insulin sensitivity. Our study is the first to demonstrate a novel role of PIAS1 in the regulation of insulin sensitivity.

Adipose tissue is a highly active metabolic site of insulin action. Particularly, chronic inflammation in adipose tissue is an essential cause of obesity-induced insulin resistance (30; 33). In the insulin-sensitive adipose tissue of lean, adipocytes secret anti-inflammatory cytokines (34). As an individual becomes obese, adipocytes hyperplasia and hypertrophy occurs, releasing inflammation cytokines and chemokines that attracts pro-inflammation macrophage to adipose tissue (35). These cytokines secreted by adipocytes and macrophage further activate JNK and NF-κB, leading to amplification of inflammation cascade. As a result, insulin resistance occurs (7). In the current study, a notable observation was that modulation of PIAS1 expression only in iWAT is sufficient to alter whole body insulin sensitivity. The
glucose uptake assay in iWAT supported our hypothesis. Hence, we thought that metabolic improvement in iWAT could facilitate the relief of systemic metabolic dysfunction. Interestingly, PIAS1 overexpression also promoted insulin signaling pathway in cultured adipocytes, in which macrophages were absent. This is primarily due to autocrine effect of adipocytes. Briefly, insulin signaling of adipocytes was attenuated by the inflammation cytokines secreted by themselves.

In addition to its expression level, phosphorylation of PIAS1 plays key role in inflammation cascade. Ser90 and Ser522 are two known residues that could be phosphorylated. It is reported that phosphorylated PIAS1, but not the Ser90A mutant, becomes rapidly interacted with the promoters of NF-κB target genes in macrophage, thereby inhibiting expression of these inflammation genes (24). Moreover, phosphorylation of PIAS1 on Ser522 promotes its transrepression activity on NF-κB and inhibits inflammation (36). Thus, phosphorylation of PIAS1 is critical to suppress inflammation cascade. It is interesting to investigate whether phosphorylation of PIAS1 facilitates insulin sensitivity through its anti-inflammatory function.

The regulation of PIAS1 expression has seldom been studied so far. The current study determined that PIAS1 was aberrantly expressed in pre-diabetic mice, and that pro-inflammatory factors could downregulate PIAS1 expression through JNK. Interestingly, as PIAS1 overexpression was able to inhibit the activation of JNK, there might be a feedback loop between PIAS1 and JNK regulation. JNK signaling pathway is a key mediator of metabolic stress responses caused by obesity, which is activated in iWAT of obesity-induced diabetes (37-38). However, whether JNK directly
modulates PIAS1 expression are still unclear and needs further investigation.

In addition to being a transcriptional suppressor, PIAS1 functions as a SUMO E3 ligase and promote the SUMOylation of proteins, which in turn affects the location, stability or activity of the target proteins (39-40). Some proteins in insulin signaling pathway could be modified by SUMO. For example, oncogene AKT, a mediator of growth-factor signaling, plays essential roles in cell proliferation and tumorigenesis (41). It has been recently reported that AKT could be SUMOylated. SUMOylation of AKT is required for its kinase activity and essential for cell growth and tumorigenesis as well (42). It is possible that PIAS1 could directly induce SUMOylation of the proteins in insulin signaling pathway like IR or IRS, thereby modulating insulin sensitivity. In our studies, however, the endogenous co-immunoprecipitation (Co-IP) assay was conducted and no obvious interaction between PIAS1 and IR or IRS was observed (data not shown). More experiments such as Co-IP assays to detect the interaction between exogenously expressed proteins and SUMOylation assays should be performed to examine the possibility.

The increasing prevalence of T2DM is really a large burden globally, heightening an urgent need to investigate diabetes pathogenesis and develop efficient strategy to prevent and control diabetes. Our findings identified PIAS1 as a key contributor of insulin sensitivity. Upregulation of PIAS1 improved insulin resistance caused by obesity. Thus, PIAS1 may be a potential diagnostic and therapeutic target for T2DM.
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Figure legend:

FIG. 1. PIAS1 expression was negatively correlated with the pre-diabetic state in fat of mice
A. qPCR analysis of PIAS1 mRNA expression in mouse liver, eWAT, iWAT, BAT and muscle tissues (n=3). Data were normalized to the PIAS1 mRNA level in liver. B. Western blotting was used to detect PIAS1 protein level in the mouse liver, eWAT, iWAT, BAT and muscle tissues (n=3), with quantification data by ImageJ software on the bottom. C. qPCR analysis showed PIAS1 mRNA in the iWAT and eWAT of pre-diabetic mice, including ob/ob mice (n=4) and their WT littermates (n=4), db/db mice (n=4) and their WT littermates (n=4), NCD mice (n=3) and HFD mice (n=3). Data were normalized to the PIAS1 mRNA level in control mice of each group. D. Western blotting analysis of PIAS1 protein level in the iWAT and eWAT of ob/ob mice (n=3), db/db mice (n=3), HFD mice (n=3) and their control mice (top). And quantification of relative PIAS1 level normalized to β-actin (bottom). E. Western blotting analysis of PIAS1 protein level in the SVF or adipocytes fraction from the iWAT and eWAT of obese and normal mice. * P < 0.05; ** P < 0.01; *** P < 0.001.

FIG. 2. PIAS1 regulated insulin signaling pathway in adipocytes
A-B. On day 8 of adipogenic induction, 3T3-L1 adipocytes were treated with 0, 10, 20, 40 ng/ml TNFα (A) or 0, 100, 200, 400 µM palmitate (B) for 12h. Western blotting analysis of PIAS1 protein level upon induction at the indicated dose. C. 3T3-L1 adipocytes were incubated with 20 ng/ml TNFα. After treatment with TNFα for the indicated time, cells were harvested and PIAS1 expression was measured by western blotting. D, E. On day 5 after adipogenic induction, PIAS1 expression was artificially altered in 3T3-L1 adipocytes. For knockdown of PIAS1 expression, adipocytes were transfected with siPIAS1 (D); for overexpression of PIAS1, adipocytes were infected with Ad-PIAS1 (E). On day 8 after adipogenic induction, the indicated cells were treated with TNFα (20 ng/ml) for 12h, followed with 100 nM insulin stimulation for 5 min. These cells were then subjected to western blotting assay to determine the protein level of p-IRβ, IRβ, p-IRS1, p-AKT, AKT, PIAS1 and
FIG. 3. Forced expression of PIAS1 in iWAT improved systemic insulin sensitivity

Male ob/ob, db/db, HFD mice were infected with Ad-LacZ or Ad-PIAS1 twice a week for 2 weeks via subcutaneous injection adjacent to iWAT, followed by measurement of blood glucose and serum insulin level (A-C), performance of GTT and ITT assay (D-F), examination of insulin signaling pathway (G), and evaluation of glucose uptake (I) on day 4 after adenovirus administration. A. Blood glucose (left) and serum insulin level (right) were monitored in ob/ob mice (n=4/group). B-C. Blood glucose levels were measured in db/db mice (B) and HFD mice (C) (n=4-5/group). D-F. GTT and ITT assays were conducted in ob/ob mice (D), db/db mice (E) and HFD mice (F) (n=4-5/group), with insulin level measured as well during GTT (middle of D and F). Two-way RM-ANOVA was used to assess the difference between groups. G. These mice were euthanized after insulin stimulation (0.75 mU/g body weight) for 10 min via intraperitoneal injection. Western blotting analysis of the protein level of p-IRβ, IRβ, p-IRS1, p-AKT, AKT, PIAS1 and β-actin in iWAT of ob/ob mice (left) and HFD mice (right). H. Quantification of relative phosphoprotein levels normalized to respective total kinase protein content or β-actin. I. The iWAT and eWAT from Ad-PIAS1 or Ad-LacZ subcutaneous treated HFD mice (n=3/group) were extracted and subjected to 2-DG uptake assay in the absence and presence of insulin stimulation. Data were normalized to Ad-LacZ-treated group in basal condition. All these data shown were representative of at least three independent experiments, with the number of mice included in each group of each experiment indicated. * P < 0.05; ** P < 0.01; *** P < 0.001.

FIG. 4. Deficiency of PIAS1 in iWAT gave rise to impaired insulin sensitivity

Male C57BL/6J mice were infected with Ad-shLacZ or Ad-shPIAS1 twice weekly for 2 weeks via subcutaneous injection adjacent to iWAT, followed by test of the insulin
sensitivity on day 4 after adenovirus administration. A. The blood glucose was measured under both the fasting and fed condition (n=5-6/group). B. GTT and ITT analysis (n=5-6/group). Two-way RM-ANOVA was used to assess the difference between groups. C. Western blotting analysis of the protein level of p-IRβ, IRβ, p-IRS1, p-AKT, AKT, PIAS1 and β-actin in iWAT after insulin stimulation. D. The iWAT and eWAT from Ad-shPIAS1 or Ad-shLacZ treated mice (n=3-4/group) were extracted and subjected to 2-DG uptake assay in the absence and presence of insulin stimulation. Data were normalized to Ad-shLacZ-treated group in basal condition. The data shown were representative of at least three independent experiments, with the number of mice included in each group of each experiments indicated. * P < 0.05; ** P < 0.01; ***P < 0.001.

FIG. 5. PIAS1 regulated inflammatory responses in iWAT
A-B: qPCR analysis of the mRNA level of the inflammation-related genes in the iWAT of ob/ob (A) and HFD mice (B) (n=3-4/group). The data were normalized to the mRNA level in Ad-LacZ treated group. C. The iWAT of ob/ob mice infected with Ad-LacZ or Ad-PIAS1 was separated into SVF (left) and mature adipocytes (right). qPCR analysis of the mRNA level of the inflammation-related genes in SVF and mature adipocytes (n=3/group). The data were normalized to the mRNA level in Ad-LacZ treated group. D-E. ELISA assay was used to measure the protein level of CCL2, MIP2 and TNFα in the iWAT of HFD mice (n=3/group) upon PIAS1 overexpression (D) or in the iWAT of wild-type mice upon PIAS1 knockdown (E). Data were presented as cytokines levels per µg of total extractable protein. F-G. ChIP-qPCR assay was carried out to test binding activity of NF-κB and PIAS1 on the promoters of TNFα and MIP2 in the iWAT of ob/ob (F) and HFD mice (G). The data were expressed as the percentage of input and normalized to Ad-LacZ treated group. H. Western blotting analysis of the p-JNK, t-JNK p-p65, and t-p65 in the iWAT of ob/ob mice upon PIAS1 overexpression. I. Western blotting analysis of the p-p65 and p-JNK in the iWAT of wild-type C57BL/6J mice upon PIAS1 knockdown. J. F4/80 staining was conducted in the indicated iWAT of ob/ob mice (top) and HFD mice.
The data shown were representative of at least three independent experiments, with the number of mice included in each group of each experiments indicated. * $P < 0.05$; ** $P < 0.01$.

**FIG. 6. PIAS1 in the adipocytes suppressed the chemotaxis of macrophage**

On day 5 after adipogenic induction, PIAS1 was silenced by siPIAS1 or ectopically expressed by Ad-PIAS1. The indicated cells were then harvested on day 8 post-induction and subjected to further studies. A. qPCR analysis of the mRNA level of inflammation genes. Data were normalized to the mRNA level in the cells treated with Ad-LacZ. B. qPCR analysis of the mRNA level of inflammation genes. Data were normalized to the mRNA level in the cells treated with siNC. C. The indicated cells were harvested and western blotting was conducted to measure the level of the indicated proteins. D. Cellular supernatant of the indicated cells was collected and then subjected to ELISA assay to detect the concentration of inflammation cytokines. E. The indicated cells were harvested after stimulation with TNFα for 12 h. ChIP-qPCR assay was performed to measure binding activity of NFEκB on the promoters of TNFα and MIP2. The data were normalized to the IgG control. F. The conditioned media, prepared by incubating the indicated adipocytes in DMEM supplemented with 0.2% BSA for 12 h, were added to the lower chamber. Macrophages were loaded on the upper chamber and allowed to migrate for 12 h at 37°C. The migratory cells on the lower surface of the membrane were counted manually using 5-6 randomly selected areas. * $P < 0.05$; ** $P < 0.01$; ***$P < 0.001$.

**Fig. 7. PIAS1 modulated insulin sensitivity in fat transplantation models**

A. Schematic design of fat transplantation. After 2-week Ad-LacZ or Ad-PIAS1 treatment, iWATs from ob/ob mice were transplanted into the subcutaneous area (i.e. below the skin on the back of host mice) of wild-type C57BL/6 mice, with Sham (surgery, but no fat was transplanted) as a control (A). After recovery for 2 weeks, the indicated host mice were subjected to followed studies (B-H). B-D. The body weight (B), fasting blood glucose (C), blood glucose (left) and insulin level (right) under fed
condition (D) were evaluated (n=4/group). E. GTT and ITT assays were conducted among three groups (n=4/group), with two-way RM-ANOVA post-hoc analysis to assess the difference. F. Indicated fat grafts were dissected from transplantation mice upon insulin stimulation, and subjected to western blotting to measure the level of p-IRS1 and p-AKT. G-H. The fat grafts were used to evaluated the inflammation response as indicated by mRNA level of inflammation genes (G) (n=4/group), and the F4/80 staining (H). Data were presented as means ± SD. For transplantation groups vs. Sham group: # P < 0.05; for iWAT-Ad-PIAS1 transplantation group vs. iWAT-Ad-LacZ transplantation group: * P < 0.05; ** P < 0.01.

FIG. 8. PIAS1 was downregulated by JNK signaling in pre-diabetic state

A. The 3T3-L1 mature adipocytes were treated with JNK inhibitor SP600125 and NF-κB inhibitor QZN for 12 h, with DMSO as a control. Western blotting analysis of the protein level of PIAS1. B. The 3T3-L1 mature adipocytes were treated with JNK inhibitor SP600125 at the indicated dose. Western blotting analysis of the protein level. C. The 3T3-L1 mature adipocytes were treated with JNK inhibitor SP600125 (20 µM) for the indicated time. Western blotting analysis of the protein level at the indicated time points. D. The 3T3-L1 mature adipocytes were pre-treated with SP600125 (20 µM) for 1 h, then incubated with TNFα (20 ng/ml) for 24 h. Cells were harvested and subjected to western blotting to test the protein level of PIAS1. E-G. siJNK1 or siJNK2 was transfected into 3T3-L1 mature adipocytes, separately or together. qPCR analysis of JNK1 and JNK2 expression (E). Western blotting analysis of PIAS1 level (F-G). H. SP600125 was injected once every other day subcutaneously adjacent to iWAT in ob/ob mice for a week, with DMSO as control. Western blotting was used to detect the PIAS1 expression in the indicated iWAT. I. Western blotting analysis of the p-JNK level in the iWAT of ob/ob, db/db, HFD mice and their respective control. J. Quantification of p-JNK level in the iWAT of ob/ob, db/db, HFD mice and their respective control. K. In the pre-diabetic state, PIAS1 was significantly decreased by pro-inflammatory factors in adipocyte via activated JNK. Downregulated PIAS1 triggered augmented NF-κB and JNK signaling, which produce much more
inflammatory cytokines and induced macrophage infiltration, thereby impairing insulin sensitivity. * $P < 0.05$; ** $P < 0.01$. 

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FIG. 6. PIAS1 in the adipocytes suppressed the chemotaxis of macrophage
Fig. 7. PIAS1 modulated insulin sensitivity in fat transplantation models
FIG. 8. PIAS1 was downregulated by JNK signaling in pre-diabetic state.
Supplementary table 1: qPCR primer pairs used in this study

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<th>Gene</th>
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Supplementary table 2: siRNA used in this study

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Cell culture
The 3T3-L1 preadipocytes were maintained and propagated in Dulbecco’s modified Eagle medium (DMEM) with 10% calf serum. The 293A cells and Raw264.7 macrophages were maintained and propagated in DMEM with 10% fetal bovine serum (FBS).

Oil Red O staining
The adipocytes were washed with cold PBS and fixed for 10 min with 3.7% formaldehyde. Oil Red O (0.5% in isopropanol) was diluted with water (3:2), filtered through a 0.45 µm filter, and incubated with the fixed cells for 4 h at room temperature. The cells were washed with water, and stained fat droplets in the adipocytes were examined by light microscopy and photographed.

Isolation of SVF and adipocytes from adipose tissues
SVF cells and adipocytes were isolated by enzymatic digestion (collagenase VIII; Sigma). The digested tissues were filtered through a 100 µm mesh filter to remove debris and then centrifuged. The adipocytes floated above the supernatant. The cellular pellet involving the SVF was resuspended with an ammonium chloride lysis buffer to remove red blood cells. The isolated adipocytes and SVF cells were washed twice with PBS, and subjected to further investigation.

Immunofluorescence study
iWAT or eWAT were excised, fixed with 4% paraformaldehyde overnight, embedded in paraffin and cut into sections. For immunofluorescence staining, sections were incubated with PIAS1 (Abcam) or F4/80 (Abcam) antibodies to test whether macrophages in adipose tissues expressed PIAS1. For immunofluorescence staining in adipocytes, the mature adipocytes were isolated from iWAT and eWAT as described, immediately fixed and smeared on slides, then permeabilized before staining with PIAS1 antibody (abcam), with nile red staining to show the lipid droplets. All the fluorescence images were taken and analyzed with a Leica confocal microscope (Leica TCS SP5, Leica, Germany).

Flow cytometry staining
SVF cells and mature adipocytes were isolated as described. Then the cells were resuspended in PBS with 1% FBS and surface markers were stained with fluorescence-labeled antibodies. For intracellular protein staining, cells were fixed and permeabilized by Fixation & Permeabilization fixed kit (eBioscience), and then stained with PIAS1 antibody (abcam). The data were analyzed with FlowJo software. For SVF cells, CD45, F4/80 and PIAS1 were stained. Percentages of PIAS1+ cells and PIAS1+ F4/80+ cells were shown by gating to CD45+ cells. For mature adipocytes, CD45 and PIAS1 were stained. CD45 staining was to exclude contamination of immune cells. Mean fluorescence intensity (MFI) of PIAS1 staining was shown by gating to CD45- cells.
Supplementary FIG. 1. Measurement of PIAS1 expression in adipocytes and macrophages from adipose tissues.

A-C. Immunofluorescence analysis of PIAS1 expression in adipocytes and macrophages. A. Representative images showed PIAS1 expression in iWAT (left) and eWAT (right) from WT or ob/ob mice. B. Representative images for PIAS1 and F4/80 staining in the iWAT from WT or ob/ob mice. White arrowheads showed the co-localization of PIAS1 and F4/80, indicating that macrophages in adipose tissues expressed PIAS1. C. Mature adipocytes were isolated from iWAT and eWAT of WT mice as described, and subjected to immunofluorescence analysis, with nile red to stain the lipid droplets. Representative images showed PIAS1 expressed in mature adipocytes. Scale bars in all the images above were 25µm. D-F. FACS analysis of PIAS1 expression in adipocytes and macrophages. D. Schematic design of FACS analysis. SVF cells and adipocytes were isolated by enzymatic digestion, and stained by indicated antibodies. E. SVF cells were stained by fluorescence-labeled antibodies for CD45, F4/80 and PIAS1. Flow cytometry representation of gated CD45<sup>+</sup> immune cells stained with F4/80 and PIAS1. Percentages of PIAS1<sup>+</sup> cells and PIAS1<sup>+</sup>F4/80<sup>+</sup> macrophages were shown. F. Mature adipocytes were stained by fluorescence-labeled antibodies against CD45 and PIAS1, with CD45 staining to exclude immune cell contamination. Flow cytometry representation of gated CD45<sup>−</sup> cells stained with PIAS1. ** P < 0.01
Supplementary FIG. 2. The expression of PIAS2, PIAS3 and PIAS4 in WAT of ob/ob and db/db mice

A-D. qPCR analysis was used to measure PIAS2, PIAS3 and PIAS4 mRNA expression in iWAT (A) and eWAT (B) of ob/ob mice, iWAT (C) and eWAT (D) of db/db mice. Data were normalized to the mRNA level in WT mice respectively. The data shown were representative of at least three independent experiments. * P < 0.05; ** P < 0.01; ***P < 0.001.
Supplementary FIG. 3. The effect of PIAS1 on lipid accumulation and insulin signaling pathway

A, B. Knockdown or overexpression of PIAS1 were conducted in the 3T3-L1 adipocytes on day 5 post-induction. Oil red O staining was performed to evaluate the lipid accumulation in PIAS1-deficient cells (A) or PIAS1 overexpression cells (B) on day 8 post-induction. C. Another two sets of siPIAS1 were transfected into 3T3-L1 adipocytes on day 5 post-induction. On day 8 post-induction, cells were stimulated with or without 100 nM insulin for 5 min before harvest. The protein levels of p-IRβ, p-IRS1, PIAS1 and β-actin was detected by western blotting.
Supplementary FIG. 4. PIAS1 overexpression in iWAT had little effect on the body weight and iWAT development, as well as the inflammation response and insulin signaling in eWAT

Male ob/ob, db/db, HFD mice were infected with Ad-LacZ or Ad-PIAS1 twice a week for 2 weeks via subcutaneous injection adjacent to inguinal fat pad. A. Western blotting was used to detect the protein level of PIAS1 in iWAT, eWAT and liver of ob/ob mice (left) and HFD mice (right) after adenovirus injection. B-D. The body weight of ob/ob mice (B), db/db mice (C) and HFD mice (D) were measured before and after adenovirus injection respectively (n=4-5/group). E-F. The weight of iWAT (left) and the percentage of iWAT weight relative to the body weight (right) in ob/ob mice (E) and HFD mice (F) were measured after adenovirus injection (n=3/group). The data shown were representative of at least three independent experiments. G. Insulin signaling pathway was detected in eWAT from ob/ob or HFD mice with iWAT-Ad-PIAS1 induction. H-I. qPCR analysis of inflammation genes in eWAT from ob/ob (H) or HFD mice (I) with iWAT-Ad-PIAS1 induction (n=3-4/group).
Supplementary FIG. 5. PIAS1 knockdown in iWAT had no effect on the body weight and iWAT development

Male C57BL/6J mice were infected with Ad-shLacZ or Ad-shPIAS1 twice a week for 2 weeks via subcutaneous injection adjacent to iWAT. A. Western blotting was used to detect the protein level of PIAS1 in iWAT, eWAT and liver after adenovirus injection. B. The body weight was measured before and after adenovirus injection respectively (n=5-6/group). C. The weight of iWAT (left) and the percentage of iWAT weight relative to the body weight were determined after adenovirus injection (n=4-5/group). The data shown were representative of at least three independent experiments. D. qPCR analysis of inflammation genes in eWAT from Ad-shLacZ treated mice (n=4/group).
Supplementary FIG. 6. PIAS1 regulated CCL2 expression through TNFα
A. 3T3-L1 adipocytes were treated with TNFα (20ng/ml) for 12 h, and qPCR analysis of CCL2 expression. B. PIAS1-deficient adipocytes were treated with neutralizing antibody anti-TNFα (100ng/ml) for 12 h, with qPCR analysis of CCL2 expression. ** P < 0.01; *** P < 0.001