Autocrine effects of transgenic resistin reduce palmitate and glucose oxidation in brown adipose tissue

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Resistin has been originally identified as an adipokine that links obesity to insulin resistance in mice. In our previous studies in spontaneously hypertensive rats (SHR) expressing a nonsecreted form of mouse resistin (Retn) transgene specifically in adipose tissue (SHR- Retn), we have observed an increased lipolysis and serum free fatty acids, ectopic fat accumulation in muscles, and insulin resistance. Recently, brown adipose tissue (BAT) has been suggested to play an important role in the pathogenesis of metabolic disturbances. In the current study, we have analyzed autocrine effects of transgenic resistin on BAT glucose and lipid metabolism and mitochondrial function in the SHR- Retn vs. nontransgenic SHR controls. We observed that interscapular BAT isolated from SHR- Retn transgenic rats compared with SHR controls showed a lower relative weight (0.71 ± 0.05 vs. 0.91 ± 0.08 g/100 g body wt, P < 0.05), significantly reduced both basal and insulin stimulated incorporation of palmitate into BAT lipids (658 ± 50 vs. 856 ± 45 and 864 ± 47 vs. 1,086 ± 35 nmol/g/2 h, P = 0.01, respectively), and significantly decreased palmitate oxidation (37.6 ± 4.5 vs. 57 ± 4.1 nmol/g/2 h, P = 0.007) and glucose oxidation (277 ± 34 vs. 458 ± 38 nmol/g/2 h, P = 0.001). In addition, in vivo microPET imaging revealed significantly reduced 18F-FDG uptake in BAT induced by exposure to cold in SHR- Retn vs. control SHR (232 ± 19 vs. 334 ± 22 kBq/ml, P < 0.05). Gene expression profiles in BAT identified differentially expressed genes involved in skeletal muscle and connective tissue development, inflammation and MAPK and insulin signaling. These results provide evidence that autocrine effects of resistin attenuate differentiation and activity of BAT and thus may play a role in the pathogenesis of insulin resistance in the rat.

Resistin; brown adipose tissue; autocrine; transgenic; spontaneously hypertensive rat

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Physiol Genomics 48: 420–427, 2016. First published April 25, 2016; doi:10.1152/physiolgenomics.00122.2015.—Resistin has been found to play an important role in triglyceride and glucose clearance. Accordingly, it has been suggested that the ability of BAT to dissipate energy excess could prevent obesity, ectopic fat accumulation, and insulin resistance in peripheral tissues (29).

In humans, the development of obesity is associated with accumulation of macrophages within adipose tissue, and it is believed that resistin produced by these immunocompetent cells plays an important role in proinflammatory processes and insulin resistance of adipose tissue. On the other hand, contradictory findings have been reported regarding relationship between circulating resistin levels and obesity or insulin resistance in humans (9). Despite these interspecies differences, it has been demonstrated that even human resistin produced by macrophages can promote insulin resistance in mouse adipose tissue (25), which suggests a potentially conserved role of resistin in regulation of sensitivity to insulin action between humans and rodent models.

To analyze mechanisms of prodiabetic effects of resistin, we derived transgenic spontaneously hypertensive rat (SHR) expressing mouse resistin (Retn) under control of the adipocyte protein 2 (aP2) promoter. Transgenic mouse resistin is expressed specifically in adipose tissue but is not secreted into circulation (22–24). The transgenic SHR- Retn strain thus represents a unique model to analyze autocrine effects of resistin. In young rats (10 wk old), autocrine effects of transgenic resistin in WAT were associated with significantly reduced free fatty acid (FFA) re-esterification and increased release of FFA into circulation, ectopic fat accumulation, and insulin resistance in skeletal muscle (23, 24). In older rats (16 mo old), autocrine effects of transgenic resistin were associated predominantly with a near-total resistance of WAT to insulin action and markedly glucose intolerance (24).

Unlike WAT, the main function of BAT is nonshivering thermogenesis enabled by the expression of uncoupling protein 1 (UCP1) of the inner mitochondrial membrane (7), and this thermogenic function of BAT has a key role in protection of the mammalian organism from hypothermia. In the recent study by Bartelt et al. (2), BAT in mice exposed to cold was found to play an important role in triglyceride and glucose clearance. Accordingly, it has been suggested that the ability of BAT to dissipate energy excess could prevent obesity, ectopic fat accumulation, and associated metabolic disturbances (3).

The traditional view of BAT is that its metabolic activity is regulated by the sympathetic nervous system, thyroid hormone action, or by metabolic conditions. In addition, BAT secretes many of the same adipokines as WAT, including resistin (17, 36), and it is possible that these adipokines may also affect energy metabolism of BAT. However, the putative role of
adipokines, including resistin, in regulation of lipid and glucose metabolism in BAT is largely unknown.

In the present study, we used SHR-\textit{Retn} transgenic rats to analyze autocrine effects of transgenic resistin on BAT metabolism.

**METHODS**

**Animals.** Resistin transgenic SHR rats (hereafter referred to as SHR-\textit{Retn}) with the resistin transgene under control of the aP2 promoter were derived as previously described (23). In brief, SHR/Ola\textit{pcvc} zygotes were microinjected with a mouse resistin cDNA construct that was prepared by a reverse transcriptase PCR of mRNA from fat tissue of a BALB/c mouse. There is a 75% homology between the rat and murine amino acid sequence, and thus mouse and rat resistin are likely to have similar targets (11). Four-month-old SHR-\textit{Retn} transgenic (\(n = 6\)) and SHR controls (\(n = 6\)) were used to measure metabolic parameters. Separate groups of 4 male old SHR-\textit{Retn} transgenic (\(n = 6\)) and SHR controls (\(n = 6\)) were used for microPET analysis. Additional groups of 6 wk old SHR-\textit{Retn} transgenic (\(n = 4\)) and SHR controls (\(n = 4\)) were used for gene expression profiling. The rats had been housed in an air-conditioned animal facility (22°C and 55% humidity) and allowed free access to a standard diet and water. All experiments were performed in agreement with the Animal Protection Law of the Czech Republic and were approved by the Ethics Committee of the Institute of Physiology, Czech Academy of Sciences, Prague.

**Basal and insulin-stimulated glucose oxidation and incorporation into BAT lipids.** Following decapitation in the nonfasted state, interscapular BAT was dissected and incubated for 2 h in Krebs-Ringer bicarbonate buffer with 5 mmol/l glucose, 0.1\(\mu\)Ci \([U-14\text{C}]\) glucose/ml and 2% bovine serum albumin, and gaseous-phase 95\% \(O_2\) and 5\% \(CO_2\) in the presence (250 \(\mu\)mol/l) or in the absence of insulin in the incubation media. Glucose oxidation was determined in BAT by measuring the incorporation of \([U-14\text{C}]\) glucose into \(CO_2\) according to Vr\'\v{n}a et al. (37). For measurement of incorporation of radiolabeled glucose into lipids, at the end of incubation, BAT was removed from media, rinsed in saline, transferred into chloroform-methanol (2:1), lipids were extracted, and radioactivity measured as described previously (24).

**Palmitate oxidation and incorporation into BAT lipids.** Measurement of palmitate oxidation was performed according to Vr\'\v{n}a et al. (37). In brief, isolated BAT was incubated in Krebs-Ringer bicarbonate buffer with 0.5\(\mu\)Ci/ml of \(14\text{C}\)-palmitic acid complexed with bovine serum albumin (3 mg/ml fraction V, Sigma) and 0.3 pmol/ml nonradioactive palmitic acid. Otherwise, incubation and measurement conditions were identical to those described above for glucose incorporation.

In vivo microPET imaging of \(18\text{F}-\text{FDG}\) uptake in BAT. \(18\text{F}-\text{FDG}\) [\(2\text{-deoxy}-2\text{-}\left(18\text{F}\right)\text{fluoro-}\text{d-glucose, fluorodeoxyglucose}\)] was used as a marker of glucose uptake. The microPET imaging was performed on the Albira microPET/CT system (Bruker BioSpin, Ettlingen, Germany). During PET/CT measurements anaesthetized (3% isoflurane in oxygen) transgenic SHR-\textit{Retn} (\(n = 6\)) and SHR controls (\(n = 6\)) (males: 350–400 g body wt) were placed head first in a prone position. After several days, the experiment was repeated after rats were exposed to cold environment (5°C) for 90 min. After the cold exposure, animals were administered 30–45 MBq \(18\text{F}-\text{FDG}\) as a bolus via tail vein catheter. After injection, animals were kept at room temperature for 60 min before PET imaging. Animal placement and scanner setting were as described for baseline acquisition. Optical reconstruction and estimation of volumes of interest (VOIs) and VOI statistics were performed using PMOD (PMOD Technologies, Zürich, Switzerland). VOIs were drawn manually around the BAT in the neck of the animals. VOI statistics were used for comparative analysis.

**Palmitate oxidation in mitochondria isolated from BAT.** Mitochondria were isolated from interscapular BAT by differential centrifugation (6) in STE medium (250 mM sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4) supplemented with BSA (2 mg/ml). Oxygen consumption by isolated mitochondria was determined at 30°C in 2 ml of K medium containing 80 mM KCl, 10 mM Tris-HCl, 5 mM K-phosphate, 3 mM MgCl\(_2\), 1 mM EDTA, pH 7.4 (19), using an Oxygen-2k (Oroboros). The rate of endogenous fatty acid oxidation was determined after addition of BAT mitochondria (0.04 mg protein/ml) to the medium supplemented with 3 mM malate 20 \(\mu\)M cytochrome c, 12.5 mM carnitine, 15 \(\mu\)M coenzyme A, 100 \(\mu\)M NAD\(^+\), 0.5 mM ATP, and 50 \(\mu\)M 2,4-dinitrophenol. When the rate of endogenous fatty acids oxidation decreased to minimum values, palmitate complexed to bovine serum albumin (6:1 molar ratio) was added to a final concentration 12.5 \(\mu\)M, and the maximal rate of palmitate oxidation was determined. The rates of oxygen consumption were expressed as pmol s\(^{-1}\) mg protein\(^{-1}\).

**Gene expression profiling.** Total RNA was extracted from BAT of SHR-\textit{Retn} transgenic and SHR control rats (\(n = 4\) per group). Quality and concentration of RNA were determined with a NanoDrop 2000 spectrophotometer (Thermo Scientific). The RNA integrity was verified on Agilent Bioanalyzer 2100, and all samples had intact RNA profiles. Affymetrix GeneChip Rat Gene 1.0 ST Array System was used for the microarray analysis following the standard protocol: 100 ng RNA was amplified with Ambion WT Expression Kit (Applied Biosystems); 5.5 \(\mu\)g single-stranded cDNA was labeled and fragmented with GeneChip WT Terminal Labeling and Hybridization (Affymetrix) and hybridized on the chip according to the manufacturer procedure. The analysis was performed in three replicates. The transcription data are MIAME compliant and deposited in the ArrayExpress database (ID # E-MTAB-3787).

**Gene expression analysis.** The expression of transgenic and endogenous \textit{Retn} gene in BAT was measured by qRT-PCR as previously described (24) (RNA was isolated from 4 rats per each group). Total RNA was extracted from BAT using Trizol reagent (Invitrogen), and cDNA was prepared and analyzed by a real-time PCR testing using Quantitect SYBR Green reagents (Qiagen) with an Option continuous fluorescence detector (MJ Research). Gene expression levels were normalized relative to the expression of peptidyl-prolyl isomerase A (\textit{Ppia}) (cyclophilin) gene, which served as the internal control, the results being determined in triplicate. Primers used for validation of differentially expressed genes, randomly selected from significant pathways, are given in Table 1.

For gene expression studies of BAT activation, RNA was reverse-transcribed with the SCRIPT cDNA Synthesis Kit (Jena Bioscience), using a mixture of oligo-dT and random hexamer primers. Quantitative PCR was performed on the ViiA 7 instrument (Life Technologies) with 5 \(\times\) HOT FIREPol Probe qPCR Mix Plus chemistry (Solis Biodyne). The following predesigned TaqMan gene expression assays (Life Technologies) were used for gene quantification: \(Ucp1\) (Rn00560865_m1), \(Atp5a1\) (Rn01527025_m1), \(Sdhb\) (Rn00590475_m1), \(B2m\) (Rn00560865_m1). All samples were normalized to the B2m values, and the results expressed as fold changes of cycle.

| Table 1. Primers used for validation of differentially expressed genes |
|-----------------|-----------------|-----------------|
| Gene            | Forward Primer  | Reverse Primer  |
| \textit{Cacu}2d1 | CAGCGTGGTTACAGCTGGTCAC | GGCCTGCAGATTGGGTTTT |
| \textit{Des}    | AGCCTCCGAGAACACCGGC | TCTGACATGCCGCTCATT |
| \textit{Il6}    | CATTCTTGCTGGACCGGACC | GCTGGAAATGTCTGGGGGAG |
| \textit{Vcl}    | AGTTCTCGCTGAGCAAGAGC | CATTGCCGCTGTAGACACAC |
| \textit{Ppia}   | AGGATACAGGCTCGCGACAT | TCAGTCCGCGAAACACAC |
threshold (Ct) value relative to controls using the $2^{-\Delta\Delta Ct}$ formula. We used five rats per each group. The reason for using two different genes as internal controls for quantitative PCR experiments (Ppia and R2m) was the fact that these two experiments were performed in different laboratories, using different chemistries (TaqMan vs. SYBR green).

Statistical analysis. All data are expressed as means ± SE. Differences between control and experimental groups were evaluated by paired or nonpaired t-tests as appropriate. Statistical analysis of the gene expression data was performed using the REST XL program that tests for significance by a randomization procedure (21). Statistical significance was defined as $P < 0.05$.

Gene expression data were preprocessed in Partek Genomic Suite (Partek). Analyses were performed by methods similar to those previously described (4). In brief, the transcription profiles were background corrected by the robust multiarray average method, probe-sets summarized by median polish, and quantile normalized and variance stabilized by base-2 logarithmic transformation. Analysis of variance yielded transcripts differentially expressed between analyzed samples (within LIMMA) (27). Storey’s $q$ values (30) were used to select significant differentially expressed genes ($q < 0.05$). All statistical analyses were performed in R and within Bioconductor (8). Differentially expressed genes were selected for GSEA (Gene Set Enrichment Analysis) (31). We performed GSEA on genes that mapped to KEGG pathways (10) and have defined Gene Ontology Enrichment Analysis) (31). We performed GSEA on genes that mapped to KEGG pathways (10) and have defined Gene Ontology terms (1) using the Fisher test and approach of Tian et al. (34). For the purpose of the GSEA, transcripts with $P < 0.05$ were considered differentially expressed. The GSEA uses classical Fisher’s exact test and examines the significance of the association to the pathway between the group of deregulated genes and all genes. This method does not take into account the direction of deregulation and only identifies that pathways are changed.

To identify the significantly perturbed pathways, we performed SPIA (Signaling Pathway Impact Analysis) (32) analysis on KEGG pathways: genes with $P < 0.05$ were considered differentially transcribed. Where appropriate, the resulting statistical $P$ values were corrected for multiple testing by false discovery rate method (5). The SPIA method considers overrepresentation of differentially expressed genes, as well as the function of every gene in a given pathway and the magnitude of gene expression changes. SPIA takes into account the direction of deregulation, and it can be the reason why these two methods produce different results. In addition, the SPIA can indicate that the signal pathway is activated or repressed.

RESULTS

Autocrine effects of transgenic resistin reduce relative weight of BAT and impair lipid and glucose metabolism in BAT. In our previous experiments, we focused on the effect of transgenic nonsecreted resistin in WAT (22–24). Resistin expression in our model is driven by aP2 promoter, which is known to be predominantly expressed in adipocyte lineage (26) and in addition also in macrophages (14). Concerning adipocytes, aP2 is expressed not only in white fat cells but also in brown fat (13, 26). In this study, we have therefore focused our attention on BAT. As can be seen in Fig. 1A, expression of transgenic resistin in BAT was ~30% compared with endogenous rat resistin, a ratio similar to that observed in WAT (22).

Relative weight of interscapular BAT isolated from SHR-Retn transgenic rats was significantly reduced compared with SHR controls ($0.071 \pm 0.05$ vs. $0.091 \pm 0.08$ g/100 g body wt, $P < 0.05$). On the other hand, relative weight of epididymal WAT pad was similar in SHR-Retn vs. SHR control rats (data not shown). Figure 1B shows that expression of a nonsecreted form of the mouse transgenic resistin was associated with a significantly reduced oxidation of both palmitate and glucose. In addition, SHR-Retn transgenic rats vs. SHR controls exhibited a substantially reduced incorporation of palmitate but not glucose into BAT lipids (Fig. 1C). As can also be seen from data in Fig. 1C, incremental (i.e., insulin stimulated minus basal) palmitate uptake was similar in SHR-Retn vs. SHR rats, suggesting that expression of transgenic resistin did not impair the insulin sensitivity of BAT regarding its ability to stimulate fatty acids incorporation into lipids.

BAT activation status. To determine the activation status of BAT in SHR vs. SHR-Retn animals, we measured expression levels of representative genes associated with BAT differentiation and activation. As a marker of differentiated BAT we used UCP1 and Cidea. UCP1 is obligatory for BAT thermogenesis, and its expression is almost exclusively limited to BAT (12, 22). In rodents, Cidea is also highly expressed in BAT, but not in WAT, thus distinguishing between these two fat depots (20, 38). As can be seen from Fig. 2, Ucp1 as well as Cidea gene expression was significantly downregulated in SHR-Retn transgene (Fig. 2, A and B). Similarly, expression of Pparg, encoding an important transcription factor mediating...
mitochondrial biogenesis in BAT as well as activating UCP1 expression (12), was also downregulated in SHR-Retn vs. SHR controls (Fig. 2C). On the other hand, expression of Fabp4 (fatty acid binding protein 4) gene, commonly known as aP2, which is highly expressed in brown and white adipocytes and their precursors (26) was not affected in the SHR-Retn transgene (Fig. 2D).

**Fig. 2. BAT activation status in SHR (n = 5) and SHR-Retn (n = 5) animals analyzed by expression levels of representative genes associated with BAT differentiation and activation. Statistical significance *P < 0.01 and **P < 0.001.**

In vivo microPET imaging of $^{18}$F-FDG uptake in BAT. PET statistics for baseline acquisition of SHR controls resulted in an average $^{18}$F-FDG uptake of $191 \pm 32$ kBq/ml, while in SHR-Retn the baseline $^{18}$F-FDG uptake was $208 \pm 36$ kBq/ml. After an acute cold exposure (1.5 h at 4°C), the average $^{18}$F-FDG uptake was measured as $351 \pm 50$ kBq/ml for SHR controls and $232 \pm 50$ kBq/ml for SHR-Retn transgenic rats.

**Fig. 3. In vivo microPET imaging of $^{18}$F-FDG uptake in BAT.**

A: average values of $^{18}$F-FDG uptake in BAT at room temperature, while after cold exposure, transgenic rats incorporated significantly less $^{18}$F-FDG into BAT. *Statistical significance $P < 0.01$. B: representative microPET images. A: BAT; B: heart.
coenzyme A, 100·H9262 of carnitine cycle by addition of 3 mM malate, 12.5 mM carnitine, 15·7). The rate of endogenous fatty acid oxidation was determined after induction transcriptional profiling. *P

Fig. 5. Validation of gene expression profiles obtained by Affymetrix transcriptional profiling in BAT by quantitative real-time PCR in SHR controls (n = 4) vs. SHR-Retn transgenic rats (n = 4). The directional differential expression of selected genes was in agreement with Affymetrix transcriptional profiling. *P < 0.05.

Gene expression profiles in BAT. To search for molecular mechanisms of transgenic resistin effects, global gene expression profiling was performed in BAT from SHR-Retn transgenic vs. SHR control rats. Altogether, we detected 194 genes showing significant differential expression (q < 0.05) (Supplemental Table S1), and 3,727 genes were differentially expressed at a nominal P < 0.05 value.1 The directional expression of the selected genes was confirmed by real-time PCR (Fig. 5). Table 2 shows the significant pathways revealed by SPIA or GSEA analyses on KEGG pathways. SPIA revealed that transgenic expression of Retn was associated with deregulated dilated cardiomyopathy, complement and coagulation cascades, focal adhesion, and influenza A KEGG pathways. These pathways contain deregulated genes that are part of other specific pathways. Thus expression of transgenic Retn was associated with upregulation of skeletal muscle development genes (e.g., Tmp1, Myl2, Tpm2, Myl3, Tnn1, Tm, Myh7, Tpm3, Myh6), connective tissue development genes (e.g., Thbs4, Col3a1, Col1a1, Itgb6, Col1a2, Itga4, Col5a2, Col4a5, Col5a1), complement and coagulation cascade genes (e.g., Fgγ, C1qα, Clqε, F2r, C1qα, Clr, C7, Fga), and deregulated genes from overlapping Toll-like receptor signaling (Jun, Pik3r1, Irf7, Tlr7, Pik3r3, Rela, Mapk12, Myd88, Mapk26, Pik3r5, Pik3cd, Pik3cb, Mapk8), and interleukin 1 signaling pathways (Jun, Pik3r1, Rela, Pycard, Myd88, Mapk26, Mapk8) as well as nuclear factor kappa B signaling pathway (Pik3r1,

Not surprisingly, the cold exposure led to induction of BAT energy turnover measured as 18FDG uptake. This uptake was increased by 1.83 times in SHR rats, while in SHR-Retn transgenic rats, the 18F-FDG uptake was increased only slightly by a factor 1.11 (Fig. 3).

Palmitate oxidation in mitochondria isolated from BAT. We first tested the maximum oxidative capacity of isolated BAT mitochondria. For this we used glycerol-3-phosphate as a substrate, which is known to induce the maximum flux of electrons through the respiratory chain in BAT (16). We found equal rates in SHR and SHR-Retn transgenic rats, indicating that the total capacity and thus the amount of enzymes of mitochondrial oxidative phosphorylation do not differ between strains (data not shown). Neither mitochondrial oxidation of endogenous free fatty acids nor oxidation of exogenous palmitate differed between the groups. The rates for endogenous free fatty acids were 3,075 ± 290 pmol oxygen/s/mg protein in the SHR and 3,090 ± 284 in SHR-Retn rats. The rates of palmitate oxidation were 5,577 ± 428 pmol oxygen/s/mg protein in SHR rats and 6,317 ± 542 in SHR-Retn rats (Fig. 4).

1 The online version of this article contains supplemental material.
BROWN ADIPOSE TISSUE AND TRANSGENIC RESISTIN IN THE SHR

Table 2. List of genes from KEGG pathways determined by SPIA and GSEA analyses showing effects of transgenic resistin in BAT

<table>
<thead>
<tr>
<th>SPIA on KEGG Pathways</th>
<th>FWER</th>
<th>Deregulated Genes (P &lt; 0.05)</th>
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<tbody>
<tr>
<td>Dilated cardiomyopathy</td>
<td>2.85e-06</td>
<td>↑ Cacna2d1, ↑ Cacn1, ↑ Cacnb1, ↑ Igfbp6, ↑ Tmpl1, ↑ Des, ↑ Igf1, ↑ Myl2, ↑ Cacnb6, ↑ Adcy4, ↑ Cacng3, ↑ Igf6, ↑ Tpm2, ↑ Myl3, ↓ Tncl, ↑ Cacng7, ↑ Tm, ↑ Actb, ↑ Igf4, ↓ Myh6, ↓ Iga3, ↓ Tpm3, ↓ Adcy3, ↓ Sgcd, ↓ Adcy2, ↓ Ahrbl, ↓ Tpm4, ↓ Lama1, ↓ Iga2b, ↓ Tgb2, ↓ Igb1, ↓ Lama2, ↓ Iga11, ↓ Sgc6, ↓ Hm6</td>
</tr>
<tr>
<td>Complement and coagulation cascades</td>
<td>0.00093</td>
<td>↓ Tpml1, ↓ F3, ↓ Serpin1e, ↓ F13a1, ↓ Fgg, ↓ Plaur, ↓ Map1l, ↓ Klkb1, ↓ Clq1b, ↓ Clq1c, ↓ Serpin1l, ↓ Plau, ↓ F2r, ↓ Cphb2, ↑ C6, ↑ C1qa, ↑ C1r, ↑ C7, ↑ Fgr</td>
</tr>
<tr>
<td>Focal adhesion</td>
<td>0.015</td>
<td>↓ Jun, ↓ Pik3r1, ↓ Pdgfb, ↓ Vcl, ↓ Tshb4, ↓ Col3a1, Col1a1, ↓ Pik3r3, ↓ Igfb6, ↑ Myl2, ↑ Col1a2, ↑ Cysc, ↑ Actn2, ↑ Igf1, ↑ Mylpf, ↑ Actn3, ↓ Igfa6, ↑ Fli1, ↑ Flnc, ↑ Pp1r12b, ↑ Actb, ↓ Igf4, ↓ Dock1, ↓ Flnb, ↓ Igfa3, ↓ Vegfa, ↓ Crk, ↓ Pak1, ↓ Col5a2, ↓ Sh2a, ↓ Ibp, ↓ Col4a5, ↓ Pik3r5, ↓ Prkaa, ↓ Fn1, ↓ Actn4, ↓ Col5a1, ↓ Pik3a3, ↓ Iga2b, ↓ Lamc3, ↓ Egfr, ↓ Map4k3, ↓ Igb1, ↓ Lamda, ↓ Iga11, ↓ Col6a4</td>
</tr>
<tr>
<td>Influenza A</td>
<td>0.017</td>
<td>↓ Jun, ↓ Oas1b, ↓ Pik3r1, ↓ Ilof, ↓ Ccl2, ↓ 1r7, ↓ T7e, ↓ Tsgf10, ↓ Pik3r2, ↓ Oas1a, ↓ Pml, ↓ Nlrp3, ↓ Mrx2, ↓ Ddx58, ↓ Irf9, ↓ Oas2, ↓ Rasd2, ↓ Rti-dm, ↓ Trim25, ↓ Actb, ↓ Rela, ↓ Pycard, ↓ Makpl2, ↓ Stau2, ↓ Mavs, ↓ Adar, ↓ Myd88, ↓ Map2k6, ↓ Pik3r5, ↓ Il12a, ↓ Prkaa, ↓ Pik3cd, ↓ Pik3cb, ↓ Af2, ↓ Nkbbi, ↓ Il1b, ↓ Rti-ba, ↓ Mapk8, ↓ Nup98, ↓ J33</td>
</tr>
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GSEA on KEGG Pathways | FDR | Deregulated Genes (P < 0.05) |
<table>
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<tr>
<td>Insulin signaling</td>
<td>0.008</td>
<td>↓ Irs2, ↓ Pik3r1, ↓ Sh2b2, ↓ Pparaq1a, ↑ Pkh3, ↑ Irs1, ↓ Pik3r3, ↓ Pppl3a, ↓ Soc2a, ↓ Pygm, ↓ Pkh1a, ↓ Prkar2b, ↓ Prkau2, ↓ Fbp2, ↓ Gck, ↓ Prkag3, ↓ Mtor, ↓ Hk2, ↓ Pck1, ↓ Crk, ↓ Lipg, ↓ Glys2, ↓ Slc2b, ↓ Pik3r5, ↓ Pik3cb, ↓ Pik3cd, ↓ Pkp2b, ↓ Mapk8, ↓ Rhog</td>
</tr>
<tr>
<td>MAPK signaling</td>
<td>0.001</td>
<td>↓ Nra4a1, ↓ Jun, ↓ Dusp5, ↓ Fos, ↓ Dusp4, ↓ Pdgfb, ↑ Dusp7, ↓ Dusp2, ↓ Dusp6, ↓ Rarg1f1, ↓ Dusp10, ↓ Cacna2 g1, ↓ Cacnb1, ↓ Cacnb6, ↓ Dusp8, ↓ Cacna2d1, ↓ Me2c, ↓ Arbb2, ↓ Ddih3, ↓ Cacng6, ↓ Map3k1l, ↓ Cacng3, ↓ Srf, ↓ Flnc, ↓ Pla2g2d, ↓ Tab2, ↓ Chepl1, ↓ Cacng7, ↓ Egfl13, ↓ Rp6ka2, ↓ Rela, ↓ Mapk12, ↓ Flnb, ↓ Pak1, ↓ Fgfl7, ↓ Crk, ↓ Chepl1, ↓ Jun, ↓ Tp53, ↓ Map2k6, ↓ Chek1, ↓ Tab1, ↓ Prkaa, ↓ Plag2g6, ↓ Egfr, ↓ Il1b, ↓ Akg, ↓ Mapk8, ↓ Tgb2, ↓ Pppl3c, ↓ Rp6ka5, ↓ Fgfr4, ↓ Nkbb2</td>
</tr>
<tr>
<td>Calcium signaling</td>
<td>0.02</td>
<td>↑ Cdc38, ↓ Pik3, ↓ Adrb3, ↓ P2rx5, ↓ Myl2b, ↓ Slc8a3, ↓ Ryr1, ↓ Hrb2, ↓ Tnc2, ↓ Trpc1, ↓ Adcy4, ↓ Ap2a3, ↓ Pkh1a, ↓ Gnal, ↓ Slc25a4, ↓ Tnc1, ↓ Chp1, ↓ Cysl1, ↓ Camk2a, ↓ Grpr, ↓ F2r, ↓ Camk2b, ↓ Ap2b3, ↓ Edhrb, ↓ Adcy3, ↓ P2rx6, ↓ Pld1, ↓ Adcy2, ↓ Erbb4, ↓ Adrb1, ↓ Prkca, ↓ Pnip, ↓ F2rx7, ↓ Dnd5, ↓ Ip1r, ↓ Nos2, ↑ Egfr, ↓ Avr3b, ↓ Trhr, ↓ Ppp3cb</td>
</tr>
</tbody>
</table>

↑ and ↓ denote up- and downregulated, respectively, in SHR-Retn transgenic rats vs. SHR controls. SPIA, signaling pathway impact analysis; GSEA, gene set enrichment analysis; BAT, brown adipose tissue; FWER, familywise error rate; FDR, false discovery rate.

Rela, Prkca, Nkbbib), GSEA detected deregulated expression of genes in insulin signaling, MAPK signaling, and calcium signaling pathways (Table 2).

DISCUSSION

Resistin was originally discovered as a circulating adipokine and a potential link between obesity, insulin resistance, and Type 2 diabetes in mice (29). However, the exact mechanisms whereby resistin induces reduced insulin action in rodents are not fully understood. In the present study, we found that 1) SHR-Retn rats express a nonsecreted form of the mouse Retn transgene in BAT, 2) the expression of Retn transgene is associated with a significantly lower weight of BAT, 3) reduced palmitate incorporation into BAT lipids, and 4) decreased palmitate and glucose oxidation in BAT. These results thus provide evidence that autocrine effects of transgenic resistin attenuate physiological differentiation of BAT and result in reduction of BAT depot size and impairment of lipid and glucose metabolism in BAT. In our previous studies in SHR-Retn transgenic rats, we observed increased serum FFA, an ectopic fat accumulation in skeletal muscles, and a significantly reduced sensitivity to insulin action (22, 24). The results of the present study suggest that increased circulating free fatty acid levels and ectopic fat accumulation might also be a consequence of reduced palmitate oxidation in BAT in transgenic SHR-Retn rats. Autocrine effects of resistin in BAT might thus represent a new mechanism linking obesity with insulin resistance through reduced dissipation of energy though direct evidence for causal role of resistin in BAT in the pathogenesis of systemic insulin resistance remains to be established.
In the current study, we observed the same rate of carnitine-dependent oxidation of endogenous fatty acids by BAT mitochondria isolated from SHR-Retn and SHR rats. Correspondingly, there were no significant differences in oxidation of added palmitate, suggesting that there is an equal capacity of mitochondria to oxidize FFA. Also, equal rates of respiration by isolated mitochondria utilizing glycerol-3-phosphate as a substrate in BAT of SHR-Retn and SHR suggest that the specific capacity of the mitochondrial oxidative phosphorylation apparatus per gram of tissue in BAT is equal between SHR-Retn and SHR. Hence the downregulation of fatty acid oxidation by intact BAT observed in SHR-Retn transgenic rats must originate from upstream events of palmitate utilization in mitochondria, possibly uptake by BAT cells and/or incorporation and release from tissue triglycerides. This may be in line with the observed downregulation of palmitate incorporation into triglycerides in BAT from SHR-Retn vs. SHR rats.

To search for molecular mechanisms of transgenic resistin effects, we measured gene expression profiles in BAT isolated from SHR-Retn transgenic vs. SHR control rats. We found that transgenic expression of Retn was associated with deregulated expression of genes involved in skeletal muscle development, inflammatory pathways, connective tissue development, MAPK signaling, insulin signaling, and calcium signaling. Brown adipocytes are derived from the myogenic lineage. Myogenic and skeletal muscle developmental genes were reported to be downregulated during brown adipocyte differentiation (35) but were upregulated in adult mice after 2–4 wk of a high-fat diet (15). In the present study, we observed increased expression of skeletal muscle genes in SHR-Retn animals, which suggests that transgenic resistin may be involved in regulation of developmental processes in BAT. In addition, expression of transgenic resistin was associated with activation of genes from complement and coagulation cascades and deregulated expression of genes from overlapping Toll-like receptor (TLR) signaling and interleukin 1 signaling pathways, which were associated with deregulation of genes from p38 MAPK signaling (Dusp10, Map3k1, Mapk12, Map2k6), C-Jun NH2-terminus kinases MAPK signaling (Dusp10, Dusp8, Map3k1, Mapk8), and nuclear factor kappa B (NF-κB) signaling pathways (Pik3r1, Rela, Prkca, Nfkbia, Nfkbia2). It has been demonstrated that resistin binds to TLR4 in humans and thus plays an important role in inflammatory cascade (33). Activation of TLR4 by resistin can induce not only inflammatory response but also insulin resistance in adipocytes (28). Intra-cellular signals induced by resistin involves NF-κB and MAP kinase signaling. This was confirmed by our results (Table 2 and Results), which show deregulated genes from Toll-like, NF-κB, and MAP kinase signaling pathways. These results also support the possibility that, in rat, TLR4 serves as a receptor for resistin and mediates its effects on BAT. Furthermore, SHR-Retn transgenic vs. SHR control rats exhibited increased expression of connective tissue development genes, which suggests possible predisposition to fibrosis in BAT. Together these findings suggest that autocrine effects of transgenic resistin predispose BAT to inflammation and fibrosis as well as to a resistance to insulin action.

Recently, BAT has also been discovered in adult humans. Since the activity and amount of BAT decrease with age it can be speculated that this process might be important for development of obesity and insulin resistance, which prevail in aging populations. However, in humans, resistin is not expressed in adipocytes of WAT or BAT but rather in macrophages and it has been demonstrated that transgenic expression of human resistin in macrophages in mice with knocked-out endogenous resistin exacerbated adipose tissue inflammation and an insulin resistance (25). While aP2 is also expressed in macrophages and aP2 promoter may therefore drive transgenic resistin expression in rat macrophages, we did not observe significant Retn expression in macrophages of SHR-Retn animals (24). Furthermore, the role of macrophages in BAT is largely unknown, and it remains to be investigated whether macrophage-derived resistin plays any role in BAT regulation in humans (18). Nevertheless, even if resistin in humans does not induce insulin resistance through its direct role on WAT and BAT, a better understanding of its contribution in WAT and BAT in rodent models might help to distinguish the WAT/BAT-specific functions (unique to rodents) from contributions of macrophage-specific resistin that are potentially conserved between humans and rodent models.

In summary, we have analyzed autocrine effects of transgenic resistin on BAT glucose and lipid metabolism and mitochondrial function in the SHR-Retn vs. nontransgenic SHR controls. We observed that autocrine effects of transgenic resistin are associated with reduced relative weight of interscapular BAT, significantly reduced basal and insulin-stimulated incorporation of palmitate into BAT lipids, and significantly decreased palmitate and glucose oxidation. Gene expression profiles in BAT identified differentially expressed genes involved in skeletal muscle and connective tissue development, inflammation, and MAPK and insulin signaling. Together with previously published results showing prodiabetic effects in old SHR-Retn transgenic rats (24), these results provide compelling evidence that autocrine effects of resistin in BAT might play an important role in the pathogenesis of insulin resistance in the rat.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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