A paradox: Insulin inhibits expression and secretion of resistin which induces insulin resistance

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Abstract

AIM: To confirm whether insulin regulates resistin expression and secretion during differentiation of 3T3-L1 preadipocytes and the relationship of resistin with insulin resistance both in vivo and in vitro.

METHODS: Supernatant resistin was measured during differentiation of 3T3-L1 preadipocytes. L6 rat myoblasts and hepatoma cell line H4IIE were used to confirm the cellular function of resistin. Diet-induced obese rats were used as an insulin resistance model to study the relationship of resistin with insulin resistance.

RESULTS: Resistin expression and secretion were enhanced during differentiation 3T3-L1 preadipocytes. This cellular differentiation stimulated resistin expression and secretion, but was suppressed by insulin. Resistin also induced insulin resistance in H4IIE hepatocytes and L6 myoblasts. In diet-induced obese rats, serum resistin levels were negatively correlated with insulin sensitivity, but not with serum insulin.

CONCLUSION: Insulin can inhibit resistin expression and secretion in vitro, but insulin is not a major regulator of resistin in vivo. Fat tissue mass affects insulin sensitivity by altering the expression and secretion of resistin.

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Key words: Resistin; Insulin; Insulin resistance

INTRODUCTION

Obesity is a worldwide health problem directly linked to several disease processes such as hypertension and type 2 diabetes mellitus[1]. Adipose tissue is not only an organ for passive energy reserve, but also an active endocrine organ secreting a wide range of hormones and other protein factors called adipokines[2]. Among the adipokines, resistin is involved in insulin sensitivity and glucose tolerance[3,4] while others are involved in hemostasis, inflammatory and stress responses, and energy balance[5,6].

Resistin, initially identified in screening for adipocyte-specific transcripts down-regulated by treatment with thiazolidinedione (TZDs), belongs to a novel family of cysteine-rich proteins, each with a unique tissue distribution[7,8]. In rodents, resistin predominantly expressed in white adipose tissue[9] reduces insulin sensitivity in adipocytes and skeletal muscles by impairing insulin-mediated glucose transport and inducing the expression of suppressor of cytokine signaling 3 (SOCS3)[10,11], and regulates fasting blood glucose by increasing hepatic glucose release[10]. Therefore, resistin might provide a link between obesity and diabetes mellitus.

Initial studies on the regulation of resistin indicate that resistin expression is reduced by fasting and increases rapidly on refeeding[3]. Circulating resistin levels are elevated in genetically obese (ob/ob, db/db) mice, and obese is induced by a high-fat diet[9]. Insulin inhibits resistin mRNA expression in 3T3-L1 preadipocytes[11,12]. However, these data do not support a role of resistin in muscle[9]. If resistin is mainly regulated by insulin, the major function of resistin is to induce insulin resistance, forming an insulin-resistant insulin sensitivity positive feedback loop that cannot exist in vivo.

In the present study, resistin expression and secretion were elevated during 3T3-differentiation of L1...
preadipocytes. This cellular differentiation-stimulated resistin expression and secretion were suppressed by insulin. Resistin also induced insulin resistance in H4IIIE hepatocytes and L6 myoblasts. In diet-induced obese rats, serum resistin levels were negatively correlated with the insulin sensitivity index (ISI). No negative correlation was found between the levels of fasting serum insulin and resistin, suggesting that insulin is not the major regulator of resistin in rodents.

MATERIALS AND METHODS

Cell culture
3T3-L1 preadipocytes were cultured at 37°C in an atmosphere containing 50 mL/L CO₂ and 950 mL/L air. The cells were maintained in growth medium consisting of Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, USA), 45 mmol/L glucose, 10% heat-inactivated fetal bovine serum (FBS, Gibco BRL, USA), 2 mmol/L L-glutamine, and 50 U/mL penicillin and 50 ng/mL streptomycin (Sigma, USA). Induction of adipocytic differentiation of 3T3-L1 cells was performed as described elsewhere[13]. Briefly, 3T3-L1 cells were grown in DMEM supplemented with 10% FBS until confluence. Two days after complete confluence (d 0), cells were cultured in DMEM supplemented with 10% FBS and 0.5 mmol/L 1-methyl-3-isobutylxanthine (Sigma, USA). 0.25 μmol/L dexamethasone (Sigma, USA) and 100 nmol/L insulin (Sigma, USA) for 48 h. From d 2 to 4, the full medium was supplemented with 100 nmol/L insulin only. The cells were then switched back to DMEM containing only 10% FBS for the remaining days. Cultures were replenished every 2 d.

The rat hepatoma cell line H4IIIE was cultured at 37°C in an atmosphere containing 50 mL/L CO₂ and 950 mL/L air, and maintained in DMEM containing 1 g/L glucose and 10% FBS. The cells were incubated in serum-free DMEM (1 g/L glucose) overnight before assay. Glucose levels were adjusted to 4.5 g/L and H4IIIE cells were treated with resistin (50 ng/mL) (Alexis, USA) for 2 h prior to insulin (100 nmol/L) stimulation for 2 h. Glycogen synthesis was then assayed as previously described[14].

L6 rat myoblasts were maintained in DMEM supplemented with 10% FBS and differentiated into myotubes by exposure to DMEM supplemented with 2% FBS. Myogenic differentiation to myotubes was confirmed morphologically and biochemically as previously described[16].

Resistin secretion
The supernatants of 3T3-L1 preadipocytes were collected on d 0, 4, 6, and 8 after differentiation and centrifuged to remove cells that might have detached from the culture flasks. The supernatants were kept at -20°C until assayed for resistin content by enzyme immunoassay (ADL, USA).

RNA preparation and amplification by RT-PCR
Total RNA was isolated from cultured 3T3-L1 cells using the TRIZOL method (Invitrogen, USA). Single strand cDNA synthesis was performed. In brief, the reverse transcription mixture contained 1 μg total RNA, 0.5 μg of oligo d(T) primer, 4 μL of 5 × RT buffer, 0.5 mmol/L deoxynucleotides, 50 U of RNase inhibitor, and 200 U of reverse transcriptase (Promega, USA) in a total volume of 20 μL, the reaction was carried out at 42°C for 1 h followed by heat inactivation at 95°C for 5 min. The number of cycles and reaction temperatures used in the PCR assay were optimized to provide a linear relationship between the amount of input template and the amount of PCR product[14]. The primers used for amplification, together with their specific optimum cycling conditions, were as follows:

Mouse resistin (a 415 bp product): [sense primer: 5'-CAA ACAAGACTTCAACTCC-3', antisense primer: 5'-ACA CACACCTTCTCCACTA-3', annealing temperature (TA) 58°C, 33 cycles].

β-actin (a 240 bp product): [sense primer: 5'-TAA AGA CCTCTATGCCCACAGT-3', antisense primer: 5'-CAG GATGGAGGGGCGGACTCATC-3' annealing temperature (TA) 57°C, 25 cycles].

Glycogen detection
H4IIIE cells were serum starved overnight in DMEM containing 0.2% FBS prior to resistin and/or insulin treatment in all experiments. Cells were lysed with 30% KOH and the vials were kept at 100°C for 20 min. After addition of anhydrous ethanol, the vials were centrifuged at 4000 × g for 15 min with the supernatants discarded. Distilled water (0.5 mL) and 1 mL of 0.2% anhydride (0.2 g of anhydride in 100 mL of 98% H₂SO₄ (g/mL), prepared freshly within 1 h) were added, and the vials were placed into boiling water for 20 min. The optical density at 620 nm of the solution in vials was determined by photometry. This method could detect 1.6 μg of glucose per mL, which is equivalent to 1.44 μg of glycogen per mL[17].

2-Deoxyglucose uptake assay
Myotubes were serum starved overnight in DMEM containing 0.2% FBS prior to resistin and/or insulin (10 nmol/L 15 min) treatment in all experiments. Uptake of 2-deoxy-D-[3H] glucose (CIC, China) was assayed for 10 min as previously described[14]. Briefly, the cells were washed with ice-cold phosphate-buffered saline, and then 200 μL NaOH (1 mol/L) was added to each well. Aliquots of the cell lysate were transferred to the scintillation vials for radioactivity counting and the remainder were used for protein assay. Non-specific uptake was determined in the presence of cytochalasin B (10 μmol/L) and subtracted from all values.

Animals
Forty-eight weaned male Sprague-Dawley rats, supplied by the Animal Center of Jiangsu Province, were acclimated to 22°C in a 12 h light/12 h dark cycle with free access to a standard chow diet for at least a week before grouping. High energy diet contained 10% milk powder, 10% glucose, 10% egg, 10% oil, and 60% standard feed[19]. Animals received this diet for 7 wk. Insulin sensitivity was defined by a value of ISI {ISI = Ln [1/(fasting plasma insulin*glucose)]}[20].
Statistical analysis
The data were presented as mean ± SE. Statistical analysis was undertaken using one-way ANOVA or the paired Student’s t-test where appropriate. Serum resistin levels in diet-induced obese rats were compared with the insulin sensitivity index and levels using the Bivariate correlation. Differences between groups were considered statistically significant when \( P < 0.05 \).

RESULTS

Increased resistin secretion during 3T3-L1 preadipocyte differentiation inhibited by insulin
Resistin secretion was enhanced during 3T3-L1 preadipocyte differentiation (\( P < 0.05 \), Figure 1A). Resistin secretion was about 3-fold higher in matured 3T3-L1 adipocytes (d 8) than in 3T3-L1 preadipocyte (d 0) (\( P < 0.05 \)). The effect of insulin (100 nmol/L) on resistin secretion was then assessed in cultured 3T3-L1 adipocytes on d 6 and d 8. Six days after induction of differentiation, 100 nmol/L insulin reduced secretion of resistin by 13% (\( P < 0.05 \), Figure 1B). Eight days after induction of differentiation, 100 nmol/L insulin reduced secretion of resistin by 20% (\( P < 0.05 \), Figure 1B).

Upregulation of resistin mRNA level during 3T3-L1 preadipocyte differentiation inhibited by insulin
Resistin mRNA was not detectable in undifferentiated 3T3-L1 cells, but was evident by d 4 after the induction of differentiation into adipocytes (Figure 2A). Resistin mRNA was up-regulated during 3T3-L1 preadipocyte differentiation (Figure 2A), and 100 nmol/L insulin decreased resistin mRNA 6 and 8 d after differentiation (Figure 2B).

Induction of cellular insulin resistance by resistin
Since insulin could inhibit resistin expression and secretion <i>in vitro</i>, additional studies were initiated to determine whether resistin plays a role in insulin resistance. Hepatocytes and myotubes are two important targets of insulin<sup>21</sup>. Glycogen synthesis in insulin-stimulated hepatocytes is the most important marker of hepatocyte insulin sensitivity<sup>22</sup>. After treatment with resistin for 2 h, basal glycogen synthesis decreased about 15% and insulin-stimulated glycogen synthesis decreased about 25% in H4IIE cells (\( P < 0.05 \), Figure 3A). After treatment with resistin for 2 h, basal 2-deoxyglucose uptake decreased about 50% and insulin-stimulated 2-deoxyglucose uptake decreased about 60% in myotubes (\( P < 0.05 \), Figure 3B), suggesting that resistin could induce cellular insulin resistance, and both hepatocytes and myotubes are important targets of resistin.

Negative correlation of serum resistin with insulin sensitivity but not with serum insulin
Serum resistin levels correlated with rat ISI (\( r = -0.662, P = 0.005 \)) in both control and diet-induced obese rats (Figure 4A). Resistin was positively correlated with insulin (\( r = 0.592, P = 0.016 \), Figure 4B), suggesting that insulin could not inhibit resistin secretion <i>in vivo</i>.

DISCUSSION
Obesity is associated with insulin resistance and type 2 diabetes, implying that adipose tissue plays a role in the development of such disorders<sup>23</sup>. Besides storing fat, adipose tissue is also an active regulation centre, providing signals to guide metabolism by secreting adipokines<sup>1-2</sup>. Resistin is a newly discovered adipokine that is believed
to provide a link between obesity and diabetes. Under conditions of insulin resistance and type 2 diabetes, fat tissue is subjected to increased levels of insulin, which may have a major impact on adipokine levels. Studies have verified that circulating levels of insulin are correlated with specific adipokines in rodents and humans, with interleukin-6 and leptin levels showing a consistently positive association with insulin levels. However, the association of resistin with insulin remains contradictory.

Resistin is one of the adipocytokines secreted by adipose tissue and has been shown to modulate both glucose and lipid metabolism in vivo and in vitro. In L6 rat skeletal muscle cells, it has been shown that resistin does not alter insulin receptor signaling but affects insulin-stimulated glucose uptake, presumably by decreasing the intrinsic activity of cell surface glucose transporters. In mature 3T3-L1 adipocytes, resistin reduces insulin-stimulated glucose uptake by activating SOCS3, which is an inhibitor of insulin signaling. In addition, it was reported that resistin takes part in insulin resistance in resistin fat-specific transgenic rats by releasing free fatty acids (FFA) from adipose tissue.

In the present study, resistin expression and secretion were increased during 3T3-L1 preadipocyte differentiation and resistin mRNA was undetectable in undifferentiated 3T3-L1 cells but was evident by d 4 after the induction of differentiation into adipocytes. The highest expression of resistin mRNA was detected on d 8 after induction of differentiation. Insulin had a marked suppressive effect on resistin mRNA levels in 3T3-L1 adipocytes and inhibited resistin secretion 6 and 8 d after induction of differentiation, suggesting that resistin does not play a role in insulin resistance.

Then, we investigated whether resistin impairs insulin sensitivity in vitro, showing that resistin could induce cellular insulin resistance in hepatocytes and myotubes. That is a paradox, because resistin is not regulated by insulin but induces insulin resistance. If both are correct, they will form a deadly insulin-resistin-insulin sensitivity positive feedback loop.

To confirm which one plays the primary role in vivo, we analyzed the relationship between serum resistin and...
insulin and their sensitivity in diet-induced obese rats. Diet-induced insulin resistance is a relevant model for the most common form of insulin resistance in humans.\(^{29}\)

In our study, serum resistin strongly correlated with rat insulin sensitivity and resistin was positively correlated with insulin, suggesting that insulin could not inhibit resistin secretion in vitro. A number of factors can regulate resistin secretion, such as glucose, epinephrine, and somatropin.\(^{27,30}\). Therefore, insulin may regulate resistin although it is not the major regulator.

In summary, insulin inhibits resistin secretion while resistin induces insulin resistance. Serum resistin correlates with rat insulin sensitivity, meaning that insulin is not the major regulator of resistin. Resistin may play a role in diet-induced insulin resistance by inducing insulin resistance in hepatocytes and myocytes.

**COMMENTS**

**Background**

Type 2 diabetes mellitus is closely associated with obesity. Resistin is a recently identified adipokine involved in insulin sensitivity and glucose tolerance. So the investigation of insulin and resistin interaction seems to be important.

**Research frontiers**

In this article, resistin biological function and interaction of resistin to insulin were studied. We also demonstrated the secretion levels of resistin in vitro and in vitro.

**Innovations and breakthroughs**

Resistin induces insulin resistance in hepatocytes, but insulin inhibits resistin secretion in vitro. Since serum resistin correlates with rat insulin sensitivity, insulin is not the major regulator of resistin and resistin may play a role in diet-induced insulin resistance by inducing insulin resistance in hepatocytes and myocytes.

**Applications**

Resistin is a newly identified hormone secreted by adipocytes. Resistin induces insulin resistance both in vivo and in vitro. This establishes a new field in the pathogenesis of type 2 diabetes.

**Peer review**

This paper discusses the regulatory mechanisms of resistin and insulin. The study is well designed and the paper is well written. The topic is of scientific value.

**REFERENCES**

1. Kaplan NM. Hypertension and diabetes. J Hum Hypertens 2002; 16 Suppl 1: S56-560
2. Mackall JC, Student AK, Polakis SE, Lane MD. Suppression of preadipocyte differentiation and promotion of adipocyte death by insulin protease inhibitors. J Biol Chem 2001; 276: 51-52
3. Steppan CM, Wang J, Whitelem EL, Birnbaum MJ, Lazar MA. Activation of SOCS-3 by resistin. Mol Cell Biol 2005; 25: 1569-1575
4. Baranerjee RR, Rangwala SM, Shapiro JS, Rich AS, Rhoades B, Qi Y, Wang J, Rajala MW, Pocai A, Scherer PE, Steppan CM, Ahima RS, Obici S, Rossetti L, Lazar MA. Regulation of fasted blood glucose by resistin. Science 2004; 303: 1195-1198
5. Haugen F, Jorgensen A, Drevon CA, Traylor P. Inhibition by insulin of resistin gene expression in 3T3-L1 adipocytes. FEBS Lett 2001; 507: 105-108
6. Shoji M, Sakoda H, Ogihara T, Fujihiro M, Katagiri H, Chin M, Onishi Y, Ono H, Inukai K, Abe M, Fukushima Y, Kikuchi M, Oka Y, Asano T. Humoral regulation of resistin expression in 3T3-L1 and mouse adipose cells. Diabetes 2002; 51: 1737-1744
7. Dowell P, Flexner C, Kiviverovich PO, Lane MD. Suppression of preadipocyte differentiation and promotion of adipocyte death by insulin protease inhibitors. J Biol Chem 2000; 275: 41325-41332
8. Cheng A, Zhang M, Crosson SM, Bao QZ, Saltiel AR. Regulation of the mouse protein targeting to glucagon (PTG) promoter by the FoxA2 forkhead protein and by 3',5'-cyclic adenosine 5'-monophosphate in H411E hepatoma cells. Endocrinology 2006; 147: 3606-3612
9. el-Naggara EA, Kanda F, Okuda S, Maeda N, Nishimoto K, Ishihara H, Chihara K. Direct effects of tumor necrosis factor alpha (TNF-alpha) and insulin on 3T3-L1 myocytes. Kobe J Med Sci 2004; 50: 39-46
10. Wang B, Zhang M, Ni YH, Liu F, Fan HQ, Lei L, Pan QX, Guo M, Chen RH, Guo XR. Identification and characterization of NYGGF4, a novel gene containing a phosphotyrosine-binding (PTB) domain that stimulates 3T3-L1 preadipocytes proliferation. Gene 2006; 379: 132-140
11. Chun Y, Yin ZD. Glycogen assay for diagnosis of female genital Chlamydia trachomatis infection. J Clin Microbiol 1998; 36: 1081-1082
12. Ceddia RB, Somwar R, Maida A, Fang X, Bikopoulos Y, Sweeney G. Global and adiponectin increases GLUT4 translocation and glucose uptake but reduces glycogen synthesis in rat skeletal muscle cells. Diabetologia 2005; 48: 132-139
13. Gong HX, Guo XR, Lei L, Guo M, Liu QQ, Chen RH. Lipolysis and apoptosis of adipocytes induced by neuropeptide Y-Y5 receptor antisense oligodeoxynucleotides in obese rats. Acta Pharmacol Sin 2003; 24: 569-575
14. Liu ML, Xu X, Rang WQ, Li YJ, Song HP. Influence of ovarietomy and 17beta-estradiol treatment on insulin sensitivity, lipid metabolism and post-ischemic cardiac function. Int J Cardiol 2004; 97: 485-493
15. Weigert C, Brobeck K, Staiger H, Kausch C, Machicoa F, Haring HU, Schleicher ED. Palmitate, but not unsaturated fatty acids, induces the expression of interleukin-6 in human myocytes through proteasome-dependent activation of nuclear factor-kappaB. J Biol Chem 2004; 279: 23942-23952
16. Gao Y, Walder K, Sanderland T, Kantham L, Feng HC, Quick M, Bishara N, de Silva A, Augert G, Tenne-Brown J, Collier GR. Elevation in Tanis expression alters glucose metabolism and insulin sensitivity in H411E cells. Diabetes 2003; 52: 929-934
17. Rasouli N, Molavi B, Elbein SC, Kern PA. Ectopic fat accumulation and metabolic syndrome. Diabetes Obes Metab 2007; 9: 1-10
18. Wang Z, Peijie J, Bunschoten A, Bouwman F, Renes J, Mariman E. Insulin modulates the secretion of proteins from mature 3T3-L1 adipocytes: a role for transcriptional regulation of processing. Diabetologia 2006; 49: 2453-2462
19. Faraj M, Lu HL, Gianfalone K. Diabetes, lipids, and adipocyte secretagogues. Biochem Cell Biol 2004; 82: 170-190
20. Cammisotto PG, Gelinas Y, Deshaies Y, Bukowiecki LJ. Regulation of leptin secretion from white adipocytes by...
insulin, glycolytic substrates, and amino acids. *Am J Physiol Endocrinol Metab* 2005; 289: E166-E171

27 **Rajala MW**, Qi Y, Patel HR, Takahashi N, Banerjee R, Pajvani UB, Sinha MK, Gingerich RL, Scherer PE, Ahima RS. Regulation of resistin expression and circulating levels in obesity, diabetes, and fasting. *Diabetes* 2004; 53: 1671-1679

28 **Pravenec M**, Kazdova L, Cahova M, Landa V, Zidek V, Mlejnek P, Simakova M, Wang J, Qi N, Kurtz TW. Fat-specific transgenic expression of resistin in the spontaneously hypertensive rat impairs fatty acid re-esterification. *Int J Obes (Lond)* 2006; 30: 1157-1159

29 **Clore JN**, Helm ST, Blackard WG. Loss of hepatic autoregulation after carbohydrate overfeeding in normal man. *J Clin Invest* 1995; 96: 1967-1972

30 **Lu HL**, Wang HW, Wen Y, Zhang MX, Lin HH. Roles of adipocyte derived hormone adiponectin and resistin in insulin resistance of type 2 diabetes. *World J Gastroenterol* 2006; 12: 1747-1751

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