Impaired glucose tolerance is accompanied by decreased insulin sensitivity in tissues of mice implanted with cells that overexpress resistin

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Abstract

Aim/hypothesis. Resistin, the expression of which is suppressed by thiazolidinedione treatment in adipocytes, is one of the key molecules for the tight link between adiposity and insulin resistance. Here, we show the *in vivo* effects of resistin on insulin sensitivity in mature mice using a cell implantation method.

Methods. Resistin cDNA was transfected into 3T3-L1 pre-adipocytes, which were then implanted into subcutaneous areas of nude mice. Metabolic analyses were performed 4 or 6 weeks after implantation.

Results. The mice implanted with 3T3-L1 cells overexpressing resistin (R-mice) showed significantly ($p < 0.05$) increased plasma resistin levels. After a glucose load plasma insulin levels were significantly greater in R-mice than in mice implanted with mock-transfected cells (M-mice). The AUC of insulin after glucose loading was positively correlated with circulating resistin levels. Significantly decreased glucose

responses after insulin injection were observed in R-mice, compared to M-mice. The insulin-induced phosphorylation level of IRS-1 was significantly lower in muscles of R-mice than M-mice. The expression of TNF- α mRNA in intra-peritoneal fat tissues was significantly greater in R-mice than in M-mice, but there was no difference between the two groups with regard to subcutaneous fat tissues. The concentration of TNF- α in plasma was positively correlated with resistin levels in R-mice.

Conclusions/interpretation. Resistin, when actually secreted from cells in mature mice, causes disturbed glucose metabolism, possibly based on decreased insulin sensitivity in muscle. The *in vivo* effects of resistin on insulin sensitivity might be in part mediated by increased TNF- α expression in visceral fat tissues.

Keywords Adipocytes · Implantation · Insulin sensitivity · Resistin · TNF- α

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Abbreviations: cDNA, complementary DNA · FBS, fetal bovine serum · HOMA, homeostasis model assessment for insulin resistance · M-mice, mice transfected with mock cells · R-mice, mice transfected with resistin cDNA · TZD, thiazolidinedione

Introduction

Adipose cells secrete bioactive peptides such as leptin, TNF α and adiponectin, which are known to modulate glucose and lipid metabolism [1, 2]. The disturbance of these adipose-derived factors is thought to lead to metabolic diseases accompanied by obesity. One underlying cause of the disturbance is well related to visceral fat accumulation [3, 4]. The reduction of accumulated fat in visceral areas is actually accompanied by improved glucose and lipid metabolism [5]. In the course of visceral fat mass reduction, the levels of circulating bioactive peptides are also decreased [5].

We have recently established a cell implantation method using established cultured pre-adipocytes or

cells transfected with complementary DNA (cDNA) for the analysis, in mature mice, of glucose and lipid metabolism, both of which are closely related to visceral fat accumulation [6, 7]. The cells implanted into visceral, and not subcutaneous, areas express TNF- α mRNA, and the bioactive peptide secreted causes decreased insulin sensitivity in muscle [6]. Furthermore, TNF- α -induced insulin resistance in muscle is reversed by treatment with thiazolidinedione (TZD) [7]. This unique model using cell implantation made it possible to identify the physiological and pathological effects of bioactive peptides in mature mice.

Resistin was originally identified as a murine gene whose expression is suppressed by TZD treatment in adipocytes [8]. An agent which causes insulin resistance, TNF- α , negatively regulated the expression and secretion of resistin in cultured adipocytes [9, 10]. The bioactive action of resistin is mediated by the regulation of other adipocyte-derived factors and/or their direct competitive or co-ordinate interaction in target tissues [11].

To identify the effect of resistin on insulin sensitivity in vivo, particularly on TNF- α expression in visceral fat, we implanted cells transfected with resistin cDNA into subcutaneous areas of mature mice.

Materials and methods

Materials, cells and animals. DMEM containing 25 mmol/l glucose and PBS were from Sigma (St Louis, Mo., USA). The mammalian expression vector pcDNA3.1/Hygro(-) was supplied by Invitrogen (Carlsbad, Calif., USA). Hygromycin B was from Wako (Osaka, Japan). Human insulin was provided by Eli Lilly (Kobe, Japan). Antibodies against IRS-1 and phosphotyrosine were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). The established pre-adipocyte cell line, 3T3-L1, was obtained from the American Type Culture Collection (Manassas, Va., USA) and cultured in DMEM containing 10% fetal bovine serum (FBS) at 37 °C in a 5% CO₂ incubator. Male ICR nude mice were purchased from Charles River (Yokohama, Japan). The mice had free access to standard rodent chow (MF; Oriental Yeast, Tokyo, Japan) and water.

Generation of 3T3-L1 cells overexpressing mouse resistin. Mouse resistin cDNA was obtained by PCR from a mouse cDNA library (Clontech, West Lafayette, Ind., USA) using specific oligonucleotide primers. The cDNA fragment was subcloned into pcDNA3.1/Hygro(-), and the amplified sequence was confirmed on both sides. The plasmids were transfected into 3T3-L1 cells using FuGENE 6 transfection reagent (Roche, Tokyo, Japan). The cells stably expressing resistin were selected with Hygromycin B (95 mmol/l) and subcloned. Mock-transfected cells were prepared by the transfection of pcDNA3.1/Hygro(-) without resistin cDNA. The 3T3-L1 cells overexpressing resistin and the mock clones were cultured in DMEM supplemented with 10% FBS.

Cell implantation in mice. Both the established 3T3-L1 cells overexpressing resistin, and the mock cells were grown to near confluence, trypsinised and suspended in DMEM with 10%

FBS. After centrifugation, cell pellets were re-suspended in PBS. Eight-week-old ICR nude mice were injected subcutaneously with either 1×10⁷ cells transfected with resistin cDNA (R-mice) or with 1×10⁷ mock cells (M-mice). The injection through 21G needles into subcutaneous areas of nude mice was performed under anaesthetisation, as described [6, 7]. Mice were housed in micro-isolator cages under specific pathogen-free conditions throughout the experiments. Every 2 weeks after injection, body weight was measured. Blood samples were collected in the fasting condition every 2 weeks after implantation. We did a glucose tolerance test 4 weeks after cell implantation, and an insulin tolerance test 6 weeks after cell implantation. Then the mice were killed by cervical dislocation, and the implanted subcutaneous adipose tissue areas collected.

Blood samples. Blood samples were obtained from retro-orbital venous plexus of the mice, which had fasted for more than 17 h. Plasma samples were immediately prepared by centrifugation at 1500 g for 15 minutes at 4 °C. Plasma insulin, resistin and TNF- α were detected by ELISA kits from the following suppliers respectively: Morinaga, Yokohama, Japan; Phoenix Pharmaceuticals, Calif., USA; and BioSource International, Camarillo, USA (Human TNF- α UltraSensitive Kit). Blood glucose levels were determined by Advantage II (Roche Diagnostics, Tokyo, Japan).

Tolerance tests. Glucose tolerance tests were performed on conscious mice after a 17-h fast. The test was conducted by oral administration of glucose (2 mg/g body weight) and measurement of blood glucose and plasma insulin at 15, 30 and 60 minutes after glucose load. The insulin tolerance test was performed on conscious mice that had been fed a normal chow diet. The test was conducted by intra-peritoneal injection of human insulin (0.75 U/kg body weight), with blood being drawn from the tail vein at 0, 15, 30, 45 and 60 minutes after the injection and the corresponding blood glucose levels measured.

Protein analyses. We did the immunoblot analysis for resistin using conditioned medium incubated for 24 h subsequent to undergoing 10-fold concentration by means of a Centricon-100 (Millipore, Bedford, Mass., USA). Rabbit polyclonal antibody against mouse resistin (AB3708, Chemicon, Temecula, Calif., USA) was used for immunodetection as described [8]. To determine tyrosine phosphorylation levels of IRS-1 in muscle, the mice were fasted overnight (15 h) before receiving an intra-peritoneal injection of insulin at 2 U/kg body weight. After 2 min, the mice were anaesthetised and the hindlimb muscles removed. Protein preparation was as described [8]. Prepared protein samples were precipitated with antibodies against IRS-1 (1:100 dilution) at 4 °C for 2 h, followed by incubation with 15 μ l of Protein G-Sepharose 4FF (Amersham Bioscience, Piscataway, N.J., USA) at 4 °C for 12 h. The beads were washed three times and heated at 100 °C for 5 min. The immunocomplexes were separated with 7.5% SDS-PAGE and transferred to polyvinylidene fluoride membranes (NEN Life Science, Boston, Mass., USA), which were first incubated with 1% gelatine at room temperature for 1 h, and then with anti-phosphotyrosine antibody (1:2000 dilution) or anti-IRS-1 antibody (1:1000 dilution) at room temperature for 3 h. Finally the membranes were incubated with horseradish peroxidase-linked antibody against mouse immunoglobulin (1:5000 dilution), and the blots were detected with enhanced chemiluminescence western blotting detection reagents (Amersham). The blots were quantified with the imaging analyser LAS-1000 (Fuji Photo Film, Tokyo, Japan). The relative amount of each signal

was determined by densitometric scanning using NIH Image software version 1.59 for Macintosh.

RT-PCR. Total RNA was extracted from fat tissues using the Isogen kit (Nippon Gene, Tokyo, Japan). Single-stranded cDNA was synthesised and amplified using GeneAmp Gold RNA PCR kit (Applied Biosystems, Tokyo, Japan). The sequences of the mouse TNF- α primers were: 5' sense (5'-GGCAGGTCTACTTTGGAGTCATTGC-3') and 3' anti-sense (5'-ACATTCGAGGCTCCAGTGAATTCGG-3') (GenBank code: NM008509). Primers for β -actin were 5'-TGGAATCCTGTGGCATCCATGAAAC-3' and 5'-TAAACGCGAGCTCAGTAACAGTCCG-3' (GenBank code: NM007393). The amplification was performed under conditions of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min, and extension at 72 °C for 1 min for 35 cycles. The amplified products were subjected to 2% agarose gel electrophoresis, and visualised after ethidium bromide staining.

Statistical analysis. Values are reported as means $\pm$ SD. Statistical analysis was performed using a two-tailed *t*-test, with differences considered significant at a *p* value of less than 0.05.

Results

Implantation of cells transfected with resistin cDNA into the subcutaneous area in mice. In order to establish the resistin-overexpressing cells, human resistin cDNA in the mammalian expression vector was transfected into 3T3-L1 pre-adipocytes. As shown in Figure 1, a selected cell line showed a 3- to 4-fold increase of human resistin secretion in the incubated medium over a period of 24 h, compared to cells transfected with mock plasmid.

We then implanted the resistin-overexpressing cells into the subcutaneous area as described previously [6, 7]. The plasma concentration of resistin increased gradually in the mice implanted with these cells (R-mice), and this rose to 68.4 ± 27.4 ng/ml ($n=6$) at four weeks (Fig. 2a). However, the mice implanted with mock-transfected cells (M-mice) did not show any significant difference from those of PBS-injected mice (33.1 ± 8.8 ng/ml, $n=6$, vs 26.5 ± 7.9 ng/ml, $n=5$). Body weight during experimental periods did not differ between R-mice and M-mice (27.0 ± 0.5 g vs 27.5 ± 0.5 g).

Figure 2b shows the HOMA-IR values in mice 4 weeks after implantation. R-mice showed significantly increased values compared to M-mice ($p < 0.05$). The increased HOMA-IR value in R-mice suggests that circulating resistin, which is secreted from the implanted cells, disturbs glucose metabolism.

Glucose tolerance test in mice implanted with the resistin-overexpressing cells. We therefore analysed glucose and insulin levels for 60 minutes after an oral glucose load. There was no difference in fasting glucose concentrations between R-mice and M-mice, and this remained so for subsequent, non-fasting glucose levels during the 60 minutes after glucose intake

Fig. 1. Resistin secreted from 3T3-L1 pre-adipocytes transfected with resistin cDNA (lane 1) or mock plasmid (lane 2). Media of cells conditioned for 24 h were concentrated to 1/10 using Centricon-100 and subjected to gel electrophoresis. This was then used for immunoblotting using rabbit anti-mouse resistin antibody (see Materials and methods). Lane 3 shows an immunodetectable signal of 9.8 M_r recombinant resistin (100 ng)

Fig. 2. Circulating resistin levels (a) and HOMA-IR (b) of mice implanted with cells transfected with resistin cDNA (R-mice) or mice implanted with cells transfected with mock plasmid (M-mice) respectively. The measurements were performed 4 weeks after implantation. Bars show means $\pm$ SD ($n=6$). * $p < 0.05$

(Fig. 3a). Although fasting plasma insulin levels were not different between these mice, R-mice had significantly higher levels than M-mice ($p < 0.05$) at 15 minutes after glucose intake (Fig. 3b). Accordingly, the level of insulin-AUC in R-mice was 1.6 fold higher than that in M-mice (Fig. 3d). Figure 3e shows the relationship between plasma resistin levels and the insulin-AUC in these implanted mice. The level of insulin-AUC was positively correlated with the plasma resistin level ($p < 0.05$). These results suggest that the increased resistin level in plasma caused the increased insulin response after a glucose load in mice.

Insulin sensitivity in mice implanted with resistin-overexpressing cells. In order to know whether the increased insulin level after glucose loading is related to insulin sensitivity in tissues, the implanted mice were intraperitoneally loaded with insulin (Fig. 4a). Plasma glucose levels decreased gradually after insulin injection for 60 minutes in M-mice. The decreased levels in R-mice at 30 and 45 minutes were significantly lower than those in M-mice. These results, together with the above results in Figure 3, indicate that the higher insulin response after a glucose load in R-mice is probably accompanied by decreased insulin sensitivity in tissues. We therefore examined insulin sensitivity in muscle, because the muscle is known to regulate insulin sensitivity in mice. The phosphorylation signal of IRS-1 in the presence of insulin was signifi-

Fig. 3. Oral glucose tolerance test in the implanted mice. Plasma glucose (a) and insulin (b) levels at 15, 30 and 60 minutes after oral glucose administration were measured in mice implanted with cells that had been transfected with resistin cDNA (R-mice, closed circles), or in mice implanted with mock plasmid (M-mice, open circles) respectively. * $p < 0.05$ vs M-mice. The AUCs of glucose and insulin (c, d respectively) were assessed 4 weeks after implantation. Each value is means $\pm$ SD ($n=6$), * $p < 0.05$; † $p = \text{NS}$. e. The correlation between plasma resistin levels and the AUC of insulin in the implanted mice ($n=12$); $p < 0.05$; $r^2=0.41$

cantly lower in R-mice than in M-mice (Fig. 4b). The decreased level of phosphorylated IRS-1 in muscle shows that the decreased insulin sensitivity identified above was probably partly based on decreased insulin action in muscles.

Induction of TNF- α expression in mice implanted with resistin-overexpressing cells. We have previously shown that 3T3-L1 cells implanted into the mesenteric area induce expression of TNF- α in mice, and thus that the resulting increase in TNF- α in plasma decreases the phosphorylation signal of IRS-1 in the

presence of insulin in muscles [6]. To discover why increased resistin concentrations in plasma accompany decreased insulin sensitivity in muscles, followed by disturbed glucose response after insulin loading, we analysed TNF- α expression in visceral fat tissues and its concentration in plasma of the implanted mice. Figure 5a shows RT-PCR analysis of TNF- α mRNA expression in subcutaneous and mesenteric fat tissues of the implanted mice. The TNF- α transcript level was clearly increased in mesenteric fat tissues, compared to subcutaneous fat tissues, of R-mice. There was no obvious difference between mesenteric and subcutaneous fat tissues in M-mice. The average signal intensity for amplified products was significantly higher in R-mice than in M-mice.

Finally we analysed the relationship between resistin and TNF- α levels in plasma, to know whether the increased circulating resistin levels affected circulating TNF- α levels in the implanted mice. As shown in Fig. 5b, plasma levels of TNF- α were significantly positively correlated with those of resistin. Thus, the induced TNF- α expression in mesenteric fat and its increased concentration in plasma may contribute to the decreased insulin sensitivity in muscles, which was accompanied by disturbed glucose response after

Fig. 4. Insulin sensitivity in tissues of implanted mice. Plasma glucose levels (**a**) at 15, 30, 45 and 60 minutes after intraperitoneal injection of insulin was measured in mice implanted with cells transfected with resistin cDNA (R-mice, closed circles), or in mice implanted with mock plasmid (M-mice, open circles). Measurements were done 6 weeks after implantation. Values are means $\pm$ SD ($n=6$). * $p<0.05$ vs M-mice. **b.** Tyrosine phosphorylation and immunoreactive protein levels of IRS-1 in muscles from R-mice or M-mice respectively. Measurements were performed 6 weeks after implantation. The mice were fasted for 15 h before intraperitoneal injection of insulin at 2 U/kg body weight. After 2 minutes, tissues were isolated, equal protein amounts of tissue lysates were precipitated with anti-IRS-1 antibody and immunoprecipitates were subjected to immunoblot analysis with anti-phosphotyrosine (PY) or anti-IRS-1 antibody. The relative amount of each signal compared to control was determined by densitometric scanning. Data are expressed as intensity ratio of phosphotyrosine and IRS-1 ($n=3$). Photos are representative of three experiments. * $p<0.05$

insulin loading in mice implanted with resistin-over-expressing cells.

Discussion

In this study, we used a cell implantation method into mature mice to clarify the modulating action of resistin on insulin sensitivity in tissues. The mice implanted with resistin-overexpressing 3T3-L1 cells

Fig. 5. Expression in fat tissues and plasma concentration of TNF- α in mice implanted with cells transfected with resistin cDNA. **a.** Total RNA prepared from subcutaneous (Sub.) and mesenteric (Mesen.) fat tissues of mice implanted with cells transfected with resistin cDNA (R-mice), or mice implanted with mock plasmid (M-mice) respectively was used for cDNA synthesis (see Materials and methods). RT-PCR analysis for 35 cycles was performed using specific primers for TNF- α or β -actin. One half (TNF- α : 300 bp), or one 10th (β -actin: 349 bp) of amplified fragments were used for electrophoresis on a 2.0% agarose gel. The relative amount of each signal for TNF- α compared to that for β -actin as control was determined by densitometric scanning ($n=3$). Photos and data are representative of three experiments. * $p<0.05$; † $p=NS$. **b.** Correlation between plasma resistin and TNF- α levels in the implanted mice ($n=20$). * $p<0.05$; $r^2=0.32$

showed increased plasma insulin levels after glucose loading, although glucose levels were not significantly changed in comparison with the mice implanted with mock-transfected cells. An insulin tolerance test showed that the higher plasma insulin level is probably the result of decreased insulin sensitivity in tissues in mice implanted with resistin-overexpressing 3T3-L1 cells. In accordance with these results, the insulin-induced phosphorylation level of IRS-1 was decreased in muscles of mice implanted with resistin-over-

expressing 3T3-L1 cells. These results indicate that resistin, when actually secreted from the implanted cells, causes decreased insulin sensitivity in tissues of mature mice.

Resistin was originally identified as a murine gene, whose expression is suppressed by TZD treatment in adipocytes, and as a key modulator of insulin resistance in tissues that has been linked to adipocytes [8]. However, based on the detection of circulating resistin and its expression in fat tissues in humans and in animal models, conflicting results have been reported with regard to the effects of resistin on insulin sensitivity in tissues [12, 13, 14, 15, 16, 17, 18, 19]. One reason for this might be the interactive actions of resistin and other bioactive peptides to maintain insulin sensitivity in vivo. An in vitro study showed that recombinant resistin reduces glucose uptake in differentiated pre-adipocytes [20]. Another study using recombinant resistin showed that it activated endothelial cell, possibly linking it to cardiovascular disease in metabolic syndrome [21]. Accordingly, recent studies using animal models showed physiological actions of resistin on insulin sensitivity and glucose metabolism in vivo. Rajala and co-workers, using infusion of recombinant protein that resulted in a 2- to 15-fold increase in circulating resistin levels, reported that resistin impairs insulin action on glucose production in liver [21]. Pravenec and colleagues showed that in resistin-transgenic rats impaired glucose metabolism and glucose intolerance were present in the skeletal muscle, despite the absence of any changes in plasma resistin concentrations compared to control rats [11]. Finally, mice lacking resistin exhibit low blood glucose levels after fasting, due to reduced hepatic glucose production [23]. Thus, in addition to the decrease in insulin sensitivity in muscle, resistin seems to have several targets as a metabolic modulator linked to obesity and diabetes. Our study showed that resistin secreted from implanted cells causes decreased insulin sensitivity, partly in muscle, in mature mice. However, in the light of the recent studies cited above, resistin might also regulate glucose production in liver in implanted mice.

It has not been molecularly identified how resistin modifies insulin sensitivity in tissues. In this context, we have recently shown that TNF- α secretion from implanted cells causes insulin resistance in mice [8]. In our implantation model, as shown in Figure 5, the expression of TNF- α transcript in mesenteric fat was increased in mice implanted with resistin-overexpressing cells. Furthermore, plasma TNF- α levels were positively correlated with circulating resistin levels in the implanted mice. The relationships observed between resistin and TNF- α in plasma suggest that resistin causes decreased sensitivity in tissues, possibly mediated by the regulation of another bioactive factor, TNF- α , in addition to its direct interaction with tissues and cells, or by another interaction with other active

molecules. We have recently identified that adipose cells in the visceral area actively produce and secrete vascular endothelial growth factor, in addition to TNF- α [24]. In these secretion processes in visceral fat, the interactive signals among the bioactive peptides might be involved in their regulation machinery. Resistin might play a central role in the regulatory signals for the secretion of such bioactive peptides. Further characterisation of the murine model implanted with cells expressing resistin could help elucidate the in vivo functions of bioactive peptides for the formation of insulin resistance in humans.

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