Insulin Resistance in SHR/NDmc-cp Rats Correlates with Enlarged Perivascular Adipocytes and Endothelial Cell Dysfunction in Skeletal Muscle

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Summary  Ectopic adipose tissue in skeletal muscle is implicated in the development of insulin resistance, which is frequently induced by abnormal dietary habits such as excessive eating and a high-fat diet. However, the characteristics of ectopic adipocytes are unknown. In this study, we investigated the characteristics of ectopic adipocytes in the skeletal muscle of spontaneously hypertensive corpulent congenic (SHR/NDmc-cp) rats as a model of insulin resistance from excessive eating. SHR/NDmc-cp rats displayed overt insulin resistance with high plasma glucose, insulin, and triacylglycerol concentrations relative to control Wistar-Kyoto (WKY) rats. In contrast, streptozotocin (STZ)-treated WKY rats had high glucose but low insulin concentrations. Ectopic adipocytes were found around blood vessels in the gastrocnemius in SHR/NDmc-cp rats. Areas of perivascular adipocytes and protein expression of resistin were greater in SHR/NDmc-cp rats than in control and STZ-treated WKY rats. The level of the phosphorylated (active) form of endothelial nitric oxide synthase in the gastrocnemius was lower in SHR/NDmc-cp rats than in the other groups. Insulin-resistant SHR/NDmc-cp rats showed enlarged perivascular adipocytes and greater endothelial cell dysfunction in the gastrocnemius.

Key Words  resistin, ectopic adipocytes, eNOS, insulin resistance

Abnormal dietary habits such as excessive eating and a high-fat diet induce insulin resistance and related diseases such as type-2 diabetes, hypertension and metabolic syndrome. A major tissue responsible for development of insulin resistance is skeletal muscle because skeletal muscle stores glucose as glycogen and is responsible for approximately 75% of insulin-dependent glucose incorporation. A reduction in skeletal muscle glucose incorporation following the development of insulin resistance leads to hyperglycemia during postprandial and fasting periods, and the subsequent development of type-2 diabetes (1). Therefore, maintaining insulin sensitivity in skeletal muscle by controlling dietary habits is important to prevent the onset and progression of type-2 diabetes. Glucose is taken up by skeletal muscle via glucose transporter 4 (GLUT4), which is translocated from intracellular storage vesicles to the plasma membrane in an insulin-dependent manner (2). Insulin-induced translocation of GLUT4 is signaled by the insulin recep-
tissue, particularly visceral adipose tissue (5). Insulin-resistant adipose tissue, particularly visceral adipose tissue, secretes several adipocytokines, including tumor necrosis factor (TNF), interleukin (IL)-6, and resistin. Circulating concentrations of these adipocytokines were closely and positively associated with the development and progression of insulin resistance and type-2 diabetes in humans (6, 7). Furthermore, cell-based studies showed that IL-6, for example, induces insulin resistance at concentrations of 20–100 ng/mL in adipocytes (8, 9), and 20 ng/mL in hepatocytes (10). The circulating concentration in human blood is typically <40 pg/mL (11–15), although its concentrations may reach 2–10 ng/mL in adipose tissue (14, 15). Therefore, it seems likely that cytokines secreted near target peripheral tissues, rather than from visceral fat, are responsible for the induction of insulin resistance in target peripheral tissues. Several studies have demonstrated that adipose tissue depots are also located around metabolic tissues. These fats are called “ectopic” fats. In particular, ectopic adipose tissue deposits have been found around the heart, coronary artery, and other arteries in human and animal models (16–20). The absence of adipose tissue around the aorta may promote the development of atherosclerosis. Additionally, the presence of dysfunctional adipose tissue, exhibiting insulin resistance, around the aorta may also result in the development of atherosclerosis via the secretion of pro-insulin-resistant adipocytokines, such as monocyte chemoattractant protein-1, and inadequate adiponectin secretion (18, 21). These results suggest that ectopic insulin-resistant adipose tissue, particularly in skeletal muscle, could induce insulin resistance in nearby tissues. In skeletal muscle, adipose tissue may accumulate in the interstitium of the muscle bundle and in myocytes in humans, and the level of accumulation is positively associated with insulin resistance (22–24). However, it is not yet clear whether perivascular adipocytokines in skeletal muscle are involved in the development of insulin resistance in humans or animal models.

In the present study, we used spontaneously hypertensive corpulent congenic (SHR/NDmc-cp) rats, an animal model of metabolic syndrome with obesity, hypertension, hyperlipidemia and hyperglycemia based on insulin resistance accompanied by excessive eating: normal model Wistar-Kyoto (WKY) rats; and streptozotocin (STZ)-treated WKY rats, which is a model with hyperglycemia from deficiency of insulin secretion from pancreatic β-cells. Using these models, we examined: (1) whether ectopic adipose tissue is present in the gastrointestinal; (2) whether adipocytes are enlarged; and (3) whether ectopic adipocytes exhibit insulin resistance in SHR/NDmc-cp rats compared with the other models. We also examined whether adipocytokine protein levels secreted from ectopic adipose tissue and endothelial cell function (eNOS activation) in skeletal muscle differed among the models.

**MATERIALS AND METHODS**

**Animals.** Five-week-old male SHR/NDmc-cp rats and Wistar-Kyoto (WKY) rats were purchased from Japan SLC, Inc. (Shizuoka, Japan) and maintained in cages under controlled conditions (temperature 23±2˚C; humidity 55±5%; 12-h light/dark cycle). Rats were fed a laboratory diet (MF: Oriental Yeast Co., Ltd., Tokyo, Japan) and had free access to food and water throughout the study. At 16 wk of age, WKY rats were divided into two groups, a control group and a STZ-treated group. Rats in the STZ group were injected with 40 mg/kg body weight of STZ dissolved in 0.9% (w/v) NaCl solution into the tail vain. Rats in the control and SHR/NDmc-cp groups were injected with vehicle alone. Because tail vein blood glucose concentrations did not exhibit significant hyperglycemia at 5 d after STZ injection, rats in the STZ group received an additional dose of 60 mg/kg body weight STZ, while the other groups were injected with vehicle alone. Blood glucose levels in the STZ group showed significant hyperglycemia at 5 d after the additional STZ injection. Non-fasting plasma samples at 18 wk of age were collected from the tail vein using a capillary tube containing heparin-lithium (Terumo, Co., Ltd., Tokyo, Japan) between 0930 h and 1000 h. Rats were then killed by decapitation between 1000 h and 1200 h, and gastrocnemius muscles were collected. The experimental procedures used in the study conformed to the guidelines of the Animal Usage Committee of the University of Shizuoka.

**Biochemical analysis.** Plasma glucose and triacylglycerol concentrations were measured using commercial assay kits (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Plasma insulin concentrations were determined using a high-sensitivity rat insulin enzyme-linked immunosorbent assay (Morinaga, Yokohama, Japan). Gastroc nemius tissues were fixed, embedded in paraffin, and cut into 3–4 μm widths. After deparaffinization, slides were washed with water and stained with Carazzi’s hematoxylin solution (Muto Pure Chemicals Co., Ltd., Tokyo, Japan) for 20 min and then washed with running water. Slides were then incubated with 1% hydrochloric acid alcohol for 30 s and washed with running water, then incubated with 0.05% lithium carbonate for 30 s and washed with running water, and lastly dipped in 70% ethanol, and stained with 1% eosin Y solution (Muto Pure Chemicals Co., Ltd.) for 5 min. After incubating with 100% ethanol, slides were mounted in Malinol mounting medium (Muto Pure Chemicals Co., Ltd.). These tasks and photographing were performed by Histo Science Laboratory Co., Ltd. (Tokyo, Japan).

**Western blot analysis.** Total protein fractions were extracted from the gastroc nemius using radio-immunoprecipitation assay buffer (1% Nonidet P-40, 0.1% sodium lauryl sulfate, 0.1% sodium deoxycholate, 20 mM Tris-HCl pH 8.0, 5 mM ethenalamine, 0.1% sodium deoxycholate, 150 mM NaCl, protease inhibitor tablet [Roche Molecular Biochemicals, Mannheim, Germany]/10 mL, and phosphatase inhibitors [1 mM NaMoO₄, 50 mM NaF and 1 mM NaVO₄]). Lysates were centrifuged at 17,750 × g for 30 min at 25˚C. The soluble supernatants were normalized for their total protein concen-
trations using the Lowry method (25), and stored at −20°C until analysis. Extracts were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and transferred to Immobilon membranes (Merck Millipore, Billerica, MA) at 80 mA for 90 min in Tris/glycine/methanol transfer buffer. Membranes were blocked for 60 min at room temperature in 5% skimmed milk (Megmilk Snow Brand Co., Ltd., Tokyo, Japan) in phosphate-buffered saline (PBS; pH 7.4) containing 0.05% Tween 20 and 0.5 m NaCl (PBS-Tween) or Blocking one-P (Nacalai Tesque, Inc., Kyoto, Japan). Membranes were then incubated with a primary anti-

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<th>WKY</th>
<th>SHR/NDmc-cp</th>
<th>STZ</th>
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<tr>
<td>Body weight (g)</td>
<td>402±3.5b</td>
<td>603±9.4c</td>
<td>346±5.0a</td>
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<td>Mesenteric adipose tissue</td>
<td>1.66±0.05b</td>
<td>2.04±0.08c</td>
<td>0.86±0.05a</td>
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<tr>
<td>weight (g/100 g body weight)</td>
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<tr>
<td>Plasma glucose (mg/dL)</td>
<td>172.5±7.9a</td>
<td>326.0±40.8b</td>
<td>532.1±21.8c</td>
</tr>
<tr>
<td>Plasma triacylglycerol (mg/dL)</td>
<td>63.7±6.4a</td>
<td>946.5±94.0c</td>
<td>174.5±29.3b</td>
</tr>
<tr>
<td>Plasma insulin (ng/mL)</td>
<td>2.10±0.34b</td>
<td>14.01±0.12c</td>
<td>0.61±0.04a</td>
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Values are expressed as means±SE for 5–6 animals. a,b,c Values not sharing a common superscript are significantly different (p<0.05) from one another.

Fig. 1. Adipocyte morphology, adipocyte area, and triacylglycerol content in the gastrocnemius. (A) Tissue sections stained with hematoxylin and eosin. Perivascular adipocytes are labeled with arrows. #, blood vessels. (B) Area of perivascular adipocytes. (C) Intramuscular triacylglycerol levels. Values in B and C are means±SE (n=5–6). Values not sharing a common superscript are significantly different from one another (p<0.05).
body diluted in 5% skimmed milk in PBS-Tween or Blocking one-P at 4˚C for 7 h. The primary antibodies were resistin (Cell Signaling Technology, MA), α-tubulin (Sigma-Aldrich, MO), endothelial NO synthase (eNOS; Santa Cruz Biotechnology, CA), phosphorylated eNOS (p-eNOS; Ser1177; Santa Cruz Biotechnology), α-subunit of AMPK (AMPKα; Cell Signaling Technology) and phosphorylated AMPKα (p-AMPKα; Thr172; Cell Signaling Technology). After washing with PBS-Tween, membranes were incubated with biotinylated anti-rabbit IgG or mouse IgG (GE Healthcare, Little Chalfont, United Kingdom), or horseradish peroxidase (HRP)-conjugated anti-goat IgG (Santa Cruz Biotechnology) diluted in 3% skimmed milk in PBS-Tween at 4˚C for 7 h. After washing with PBS-Tween, membranes incubated with biotinylated anti-rabbit or anti-mouse IgG were washed with PBS-Tween and incubated with an anti-biotin HRP-conjugated antibody (Cell Signaling Technology) at 4˚C for 7 h. Membranes were then washed with PBS-Tween, and positive signals were detected by chemiluminescence (Western Lightning ECL Pro; PerkinElmer, MA).

Other measurements. Body weight was measured every 2–3 d. Adiponectin concentrations in the gastrocnemius were determined using a rat adiponectin enzyme-linked immunosorbent assay (CircuLex, Nagano, Japan), using the normalized protein extracts. The areas of perivascular adipocytes within the gastrocnemius were determined. The area of each adipocyte was calculated as follows: area=\((\text{major axis}/2)\times(\text{minor axis}/2)\times3.14\).

Statistics. Results are expressed as means±standard error of the mean (SE). The significance of difference among groups was determined by analysis of variance with Fisher’s least significant difference post-hoc tests. Values of \(p<0.05\) were considered to indicate statistical significance.

RESULTS

Physical and biochemical parameters in experimental animals

Body weight, mesenteric adipose tissue weight, and plasma triacylglycerol and insulin concentrations were much higher in SHR/NDmc-cp rats than in control or STZ-treated WKY rats. Plasma glucose levels were highest in STZ-treated WKY rats, followed by SHR/NDmc-cp rats and control WKY rats (Table 1).

Presence of ectopic adipocytes in the gastrocnemius

Adipocytes were found in the perivascular area, but not in the interstitium of muscle bundles in the gastrocnemius in all three groups. The perivascular adipocyte area was greater in SHR/NDmc-cp rats than in the other groups. Muscle triacylglycerol concentrations were higher in SHR/NDmc-cp rats than in the other groups (Fig. 1).

Characteristics of ectopic adipocytes in the gastrocnemius

To investigate the characteristics of ectopic adipocytes in the gastrocnemius, we determined the protein expression levels of adipokines associated with insulin resistance (resistin) and insulin sensitivity (adiponectin). Protein levels of resistin were markedly higher in SHR/NDmc-cp rats than in the other groups, whereas protein levels of adiponectin did not differ among the three groups (Fig. 2).

Levels of proteins related to insulin sensitivity in endothelial cells in the gastrocnemius

Next, we determined the expression levels of proteins associated with insulin sensitivity of skeletal muscle
cells and endothelial cells in the gastrocnemius. The protein levels of AMPKα, which is a regulator of insulin sensitivity in skeletal muscle, were not significantly different among the three groups. The protein levels of p-AMPKα, the active form of AMPKα, were not significantly different between SHR/NDmc-cp and control WKY rats, but were higher in STZ-treated WKY rats than in the other groups. Protein levels of eNOS, which is specifically expressed in endothelial cells, were not significantly different between SHR/NDmc-cp and control WKY rats, but were higher in STZ-treated WKY rats than in the other groups. Protein levels of p-eNOS, the active form of eNOS, were much lower in SHR/NDmc-cp rats than in the other groups (Fig. 3).

**DISCUSSION**

In this study, we detected adipocytes around blood vessels in the gastrocnemius of SHR/NDmc-cp rats, as well as in STZ-treated and control WKY rats. In particular, perivascular adipocytes were enlarged in SHR/NDmc-cp rats, but not in STZ-treated or control WKY rats. Additionally, triacylglycerol concentrations in the gastrocnemius and plasma triacylglycerol and insulin concentrations were higher in SHR/NDmc-cp rats than in the other groups. Considering that the perivascular adipocytes in the gastrocnemius were mostly atrophied in STZ-treated WKY rats, excess insulin secretion was likely responsible for excess fat accumulation in the perivascular adipocytes in SHR/NDmc-cp rats. Interestingly, expression of the resistin, an adipocytokine that inhibits insulin signaling by associating with insulin receptor (26–28), was much higher in SHR/NDmc-cp rats than in the other groups, whereas no significant differences were found for adiponectin, an adipocytokine associated with insulin sensitivity. Resistin is involved in inactivation of endothelial function (29), and in impaired glucose metabolism in myocytes and adipocytes (30, 31). Moreover, resistin impairs insulin-stimulated vasodilatation in an insulin dose-dependent manner (26). Thus, it seems likely that resistin secreted from perivascular adipocytes in skeletal muscle is involved in the development of insulin resistance in skeletal muscle.

Recent studies have demonstrated that the transfer of insulin from blood to the interstitium of skeletal muscle is the rate-limiting step in insulin-dependent glucose uptake (32). Insulin induces NO-dependent vasodilatation in skeletal muscle (33) by activating IRS-2 through the phosphorylation of eNOS at Ser1177 in endothelial cells (34). It is reported that IRS-2 deficiency in mice results in eNOS inactivation, which impairs vasodilatation and glucose uptake in skeletal muscle (4). Therefore, insulin sensitivity of endothelium in skeletal muscle is an important component of insulin-dependent glucose uptake in skeletal muscle. Interestingly, we found that protein levels of p-eNOS in the gastrocnemius were markedly lower in SHR/NDmc-cp rats than in the other groups. It has been reported that resistin reduces insulin-evoked eNOS phosphorylation in endothelial cells (26). In addition, resistin injection into rats induces vascular endothelium dysfunction by reducing eNOS protein and NO levels (35). Thus, adipocyte enlargement and increased protein levels of resistin may be involved with inactivation of eNOS and insulin resistance of skeletal muscle in SHR/NDmc-cp rats. It should be noted that insulin resistance-inducible adipocytokines, including resistin, are also secreted from other adipose tissues, particularly in visceral fat (36). However, the circulating concentrations of these adipocytokines in human blood are typically <40 pg/mL (11–15), although concentrations may reach 2–10 ng/mL in the adipose tissue (14, 15). It seems likely that resistin secreted from perivascular adipocytes in skeletal muscle, rather than from other adipose tissues, leads to inactivation of eNOS and the development of insulin resistance in skeletal muscle. These insights are supported by the results of a study in which TNF-α, another adipocytokine, was found to be secreted from perivascular adipocytes and induced macrovascular dysfunction, leading to the development of cardiovascular diseases associated with arteriosclerosis (37). Another possibility is that other cytokines and free-fatty acid secreted by perivascular adipocytes are related to insulin resistance development in skeletal muscle. However, direct evidence of associations between perivascular adipocytes and the functions of vascular endothelium in skeletal muscle are still unclear. Research is required to determine whether the concentrations of cytokines, including resistin, are higher in perivascular adipocytes in skeletal muscle, and in skeletal muscle close to perivascular adipocytes, than in circulating concentrations. Research is also required to determine whether concentrations in perivascular adipocytes in skeletal muscle, and in skeletal muscle close to perivascular adipocytes, but not circulating concentrations, suppress vascular endothelium function, including eNOS phosphorylation, in vascular endothelial cells.

It should be noted that ectopic adipose tissue in human skeletal muscle is predominantly localized in the interstitium among muscle bundles, and lipid accumulation, as measured by computed tomography, is greater in obese subjects than in healthy subjects (22, 23). In this study, we did not find ectopic adipose tissue or fat accumulation in the interstitium among muscle bundles in rats. However, this may be a specific characteristic of SHR/NDmc-cp rats, so this should be investigated in other animal models. Nevertheless, we did find marked enlargement of perivascular adipocytes, and this was closely and positively associated with insulin resistance and endothelial cell dysfunction. In humans, it was reported that skeletal muscle insulin resistance is more strongly associated with intramyocellular lipid than with extramyocellular lipid (38). It was also reported that intramyocellular lipid content was higher in endurance-trained athletes than in lean and obese subjects, and insulin sensitivity was similar in athletes and lean subjects (24). So far, no human studies have examined whether perivascular adipose tissue in skeletal muscle is associated with skeletal muscle insulin resistance. Therefore, human studies using skeletal muscle biopsies are needed to examine whether insulin resistance is associated with the area of perivascular adipocytes and...
protein levels of resistin.

In this study, we observed that insulin-resistant SHR/NDmc-cp rats showed enlarged perivascular adipocytes, higher insulin levels and lower vascular endothelial function (eNOS activation) in the gastrocnemius of SHR/NDmc-cp rats than in WKY rats. The SHR/NDmc-cp rat is a model with genetic mutation of the leptin receptor (39). It is possible that the genetic mutation of the leptin receptor directly induces enlargement of perivascular adipocytes, resistin levels and vascular endothelial dysfunction. It has been reported that insulin dependent phosphorylation of eNOS in endothelial cells in skeletal muscle was reduced by a high-fat diet in mice (4). Thus, it is very likely that enlarged perivascular adipocytes are induced by excessive eating and a high-fat diet. In addition, it is well known that enlarged adipocytes in visceral fat exhibit insulin resistance, and adipocytes secrete large amounts of resistin (36). These results indicate that excess insulin secretion by excessive eating may induce enlargement of perivascular adipocytes in skeletal muscle and the development of insulin resistance in adipocytes. Enlarged perivascular adipocytes with insulin resistance could secrete high resistin protein levels, and resistin could inhibit eNOS activation in vascular endothelium in skeletal muscle. Studies are needed to examine whether consumption of a high-fat diet and excessive eating in rats induces enlargement of perivascular adipocytes and resistin levels in skeletal muscle. Research is also required to examine whether caloric restriction or supplementation with functional food factors, such as dietary fibers and anti-oxidants, reduces insulin resistance, enlargement of perivascular adipocytes and levels of resistin, and enhances p-eNOS levels in skeletal muscle of animal models with obesity, insulin resistance, type-2 diabetes and metabolic syndrome.

It is possible that resistin secreted by perivascular adipocytes directly induces insulin resistance in skeletal muscle. A previous study has demonstrated that resistin treatment (40), or transfection of the resistin expressing plasmid into cultured myoblasts, reduces glucose incorporation (41). Another study has demonstrated that IRS-2 deficiency in mice results in eNOS inactivation, which impairs vasodilatation and glucose uptake in skeletal muscle (4). It seems likely that resistin induces insulin resistance in both vascular endothelial cells and muscle cells in skeletal muscle. Research is required to determine the contribution of vascular endothelial cells and muscle cells to insulin resistance development in skeletal muscle.

It should be noted that resistin protein expression in gastrocnemius tissue sections was not detected by immunohistochemical staining. It is known that secretary proteins tend to be eluted from tissue sections during some steps of immunohistochemical staining, such as fixation and washing. Another possibility is that resistin antibodies were not reactive in tissue sections. Research is required to examine whether resistin protein is expressed in perivascular adipocytes in the gastrocnemius, and whether protein levels are higher in SHR/NDcp rats than in WKY and STZ-treated WKY rats using other methods, such as laser microdissection of samples, without the steps that can cause removal of secreted proteins, including resistin.

It should be noted that p-AMPKα and eNOS protein levels in the gastrocnemius were higher in STZ-treated WKY rats than in SHR/NDmc-cp and WKY rats. Because STZ-treated WKY rats have lower potency of insulin secretion from pancreatic β-cells, p-AMPKα and eNOS protein levels may be enhanced by decreases in insulin levels in the blood as a compensatory phenomenon.

In conclusion, we observed that obese insulin-resistant SHR/NDmc-cp rats had enlarged perivascular adipocytes, higher levels of resistin, and lower levels of active p-eNOS in the gastrocnemius. These results suggest that insulin-resistant perivascular adipocytes play an important role in vascular endothelial dysfunction in skeletal muscle.

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