Resistin-like Molecule β Activates MAPKs, Suppresses Insulin Signaling in Hepatocytes, and Induces Diabetes, Hyperlipidemia, and Fatty Liver in Transgenic Mice on a High Fat Diet*

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Resistin and resistin-like molecules (RELMs) are a family of proteins reportedly related to insulin resistance and inflammation. Because the serum concentration and intestinal expression level of RELMβ were elevated in insulin-resistant rodent models, in this study we investigated the effect of RELMβ on insulin signaling and metabolism using transgenic mice and primary cultured hepatocytes. First, transgenic mice with hepatic RELMβ overexpression were shown to exhibit significant hyperglycemia, hyperlipidemia, fatty liver, and pancreatic islet enlargement when fed a high fat diet. Hyperinsulinemic glucose clamp showed a decreased glucose infusion rate due to increased hepatic glucose production. In addition, the expression levels of IRS-1 and IRS-2 proteins as well as the degrees of insulin-induced phosphatidylinositol 3-kinase and Akt activations were attenuated in RELMβ transgenic mice. Similar down-regulations of IRS-1 and IRS-2 proteins were observed in primary cultured hepatocytes chronically treated (for 24 h) with RELMβ, suggesting the insulin resistance-inducing effect of RELMβ to be direct. Furthermore, it was shown that RELMβ acutely and markedly activates ERK and p38, while weakly activating JNK, in primary cultured hepatocytes. This increased basal p38 phosphorylation level was also observed in the livers of RELMβ transgenic mice. In conclusion, RELMβ, a gut-derived hormone, impairs insulin signaling probably via the activations of classic MAPKs, and increased expression of RELMβ may be involved in the pathogenesis of glucose intolerance and hyperlipidemia in some insulin-resistant models. Thus, RELMβ is a potentially useful marker for assessing insulin resistance and may also be a target for future novel anti-diabetic agents.

Insulin resistance is a major cause of type 2 diabetes, and recent studies have revealed many independent mechanisms regulating insulin sensitivity. Among them, much attention has been paid to the roles of secreted proteins in insulin resistance. Resistin (also known as FIZZ3 ADSF, mXCP4, or hXCP1) was identified as a factor that is secreted by adipocytes and causes insulin resistance (1). This finding was supported by not only in vitro experiments using cultured cells but also animal experiments, i.e. mice with adenoviral resistin expression, infusion of recombinant resistin, the use of neutralizing antibody, or by generating resistin gene knock-out mice (2–6). However, some clinical studies have failed to demonstrate a close relationship between obesity or insulin resistance and the serum resistin concentration in humans (7, 8). Thus, while resistin apparently induces insulin resistance, the involvement of resistin in the pathogenesis of human diabetes and obesity remains unclear.

On the other hand, there are three resistin-related proteins, termed RELMα (resistin-like molecule, FIZZ1, or mXCP2), β (FIZZ2, mXCP3, or hXCP2), and γ (mXCP1) (9). RELMα is expressed in white adipose tissue, the lung, tongue and bone marrow, whereas the expression of RELMβ is strictly limited to the small and large intestines, especially goblet cells. Although RELMγ is expressed in mouse spleen, bone marrow, intestines, and a variety of other tissues, corresponding to each developmental stage depending on CAAT/enhancer-binding protein-ε (10), its human homolog has not been identified. These proteins contain a highly conserved cysteine-rich C terminus (Cys-X(10)-Cys-X(8)-Cys-X(10)-Cys-X(8)-Cys-X(10)-Cys-X(8)-Cys) and a signal peptide sequence at their N termini, as observed in resistin. Assuming that they have biological activities similar to that of resistin, these RELMs and resistin are considered to be equally important for the regulation of insulin sensitivity. Indeed, it was reported that injecting mice with either resistin or RELMβ induced insulin resistance (11). In addition, we found the intestinal expression and serum concentration of RELMβ to be increased in insulin resistant models such as high fat fed and db/db mice (12). Thus, we speculated that the inflammatory state of the intestine, overeating, bowel movements, and/or nutrient absorption might regulate the intestinal expression and serum level of RELMβ and thereby regulate whole-body insulin sensitivity.

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The abbreviations used are: RELM, resistin-like molecule; MAPK, mitogen-activate protein kinase; IRS, insulin receptor substrate; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; 2DG, 2-deoxy-o-glucose; PI, phosphatidylinositol; SAP, serum amyloid P; PPARα, peroxisome proliferator-activated receptor α.
Herein, to examine chronic effects of the elevated serum RELMβ observed under insulin-resistant conditions such as a high fat diet and in db/db mice, we generated transgenic mice overexpressing RELMβ and analyzed their metabolic profiles relating to insulin resistance and changes in the insulin signaling pathway. Furthermore, to investigate the physiological significance of elevated serum RELMβ, we investigated the effects of RELMβ on insulin signaling using primary cultured hepatocytes. Herein, we show that RELMβ activates three MAPKs and down-regulates IRS-1/2 proteins and suppresses insulin signaling. Our observations suggest that RELMβ may be a useful marker for assessing insulin resistance associated with obesity and may also serve as a target for future novel anti-diabetic agents.

**MATERIALS AND METHODS**

Antibodies—The affinity-purified antibodies against RELMβ, insulin receptor substrate (IRS)-1, IRS-2, tyrosine phosphorylation (4G10), and Akt/protein kinase B were prepared as previously described (13). The antibodies against phospho-Ser473 of Akt, phospho-p44/p42, p44/42, Akt/protein kinase B were prepared as previously described (13). The receptor substrate (IRS)-1, IRS-2, tyrosine phosphorylation (4G10), and p42/p44 antibodies were purchased from Cell Signaling Technology.

Preparation of Recombinant RELMβ—Adenoviruses expressing RELMβ were constructed using an Ad Easy kit (Quantum Biotechnology). HEK293 T cells transfected with RELMβ adenovirus produced 50–100 mg/liter RELMβ protein in the medium. Serum-free medium CD293 (Invitrogen) was used to collect the secreted protein from confluent HEK293 T cells for 2 days. The medium was harvested and spun down to remove cells. The medium was then purified and concentrated using a high 5 column and Biologic LP system (Bio-Rad) as described previously (4). Greater than 95% purity was confirmed by silver staining with a Silver Staining Kit (BEXEL Biotechnology), and quantities were determined by Western blotting, using commercially available recombinant RELMβ (Peprotech) as the standard. The medium of HEK293T cells transfected with β-galactosidase expressing adenovirus was used to prepare a control solution. The pH values of RELMβ and control solution were adjusted to 7.4.

Construction of RELMβ and Generation of Transgenic Mice—The open reading frame of RELMβ was obtained employing PCR based on the previously reported sequence (9). This RELMβ cDNA was cloned into a pAT15-3 vector containing the SAP promoter and rat β-globin intron (Fig. 1a), which was used for the generation of RELMβ transgenic mice.

Animals and High Fat Diet—The C57/B6 line was used to generate RELMβ transgenic mice. All animal studies were conducted according to the Japanese guidelines for the care and use of experimental animals. All animal experiments were performed after 4-week high fat diet loading, unless otherwise indicated. The high fat diet had, basically, the previously described composition (14), except that the skim was added to the formulation. Food intakes were determined daily for 5 consecutive days. Food was withdrawn 12 h before each experiment.

Immunoblotting of RELMβ—Serum RELMβ concentrations in transgenic mice and their littermates were determined by immunoblotting. Two microliters of serum was boiled in Laemmli sample buffer containing 100 mM dithiothreitol. Samples were subjected to SDS-PAGE, transferred to 0.1-μm pore nitrocellulose, and immunoblotted using anti-RELMβ antibody (1:1000). Proteins were visualized with enhanced chemiluminescence (ECL or ECL plus) and exposed to ECL film (Amersham Biosciences).

Serum Glucose, Lipids, and Hepatic Triglyceride and Glycogen—Blood glucose was measured with a portable blood glucose monitor, Freestyle Kissei (Kissei Pharmaceutical, Japan). The plasma insulin level was determined with an enzymatic immunoassay kit (Amersham Biosciences). Serum triglyceride, cholesterol, and free fatty acids were assayed with Triglyceride E test Wako, Cholesterol E test Wako, and NEFA C test Wako (Wako Chemicals, Japan), respectively. Serum adiponectin was assayed with an adiponectin measurement kit (Otsuka Pharmaceuticals, Japan). Hepatic total lipid was extracted and assayed using the Folch method, as described previously (15). The triglyceride content was assayed as described above. Hepatic glycogen content was measured as previously described (16).

Tolerance Test—Glucose (2 g/kg glucose load), insulin (0.75 unit/kg insulin), and pyruvate (2 g/kg pyruvate) tolerance tests were performed as previously described (17).

Glucose Clamp Study—The glucose clamp study was performed, as previously described (18–21), with some modifications. In brief, mice were implanted with catheters that were exteriorized at the back of the neck and encased in silastic tubing. Four days after surgery, the animals were fasted for 3 h and used for the experiments. $[\text{d}-\text{3H}]$Glucose (Amersham Biosciences) was injected (bolus 10 μCi, 0.1 μCi/min, for 240 min) intravenously. After a 90-min basal period, a blood sample was collected for determination of glucose-specific activity and the blood glucose level. At time 0, hyperinsulinemic-euglycemic clamps were started, and 10 milliunits/kg/min human insulin (Novolin R, Novo Nordisk) was continuously infused for 150 min. The blood glucose concentration was clamped at 90–120 mg/dl, for at least 60 min, by estimating the blood glucose concentration at 5-min intervals and adjusting the rate of glucose solution infusion. Blood samples were taken to determine blood glucose, insulin, and plasma $[\text{d}-\text{3H}]$glucose every 30 min for 120 min. Then, 12 μCi of $[\text{14C}]$2-DG (Amersham Biosciences) was injected, and blood samples were taken at 122, 125, 130, and 150 min to determine blood glucose and plasma $[\text{14C}]$2DG. At 150 min, the gastrocnemius (type IIB fibers) muscle, soleus (type I and type IIA fibers) muscles, and epididymal fat were immediately excised and frozen in liquid nitrogen, then stored at –80 °C until future tissue analysis.

Plasma and Tissue Assays in Glucose Clamp Study—After deproteinization with barium hydroxide (Ba(OH)₂, 0.3 N) and zinc sulfate (ZnSO₄, 0.3 N), $[\text{d}-\text{3H}]$glucose and $[\text{14C}]$2DG radioactivities of plasma were determined by dual channel liquid scintillation counting. Hepatic glucose production and the glucose disposal rate were calculated for the basal period and the steady-state portion of the glucose clamp as previously described (21). Muscle and fat samples were weighed and homogenized in 0.5% perchloric acid. Homogenates were centrifuged and neutralized with NaHCO₃. The sample was then separated into two aliquots. One was counted directly to determine $[\text{14C}]$2DG and $[\text{14C}]$2DG-6-phosphate (C-2DG) radioactivities. The other was treated with Ba(OH)₂ and ZnSO₄ to remove $[\text{14C}]$2DG and any tracer incorporated into glycogen and then counted to determine $[\text{14C}]$2DG radioactivity. $[\text{14C}]$2DG is the difference between the two aliquots. Tissue glucose uptake was calculated as described (20).

In Vivo Insulin Stimulation—In vivo insulin stimulation was performed, as previously described (22), with some modifications. In brief, mice were anesthetized with pentobarbital sodium. The portal vein was exposed, and 0.4 ml of normal saline (0.9% NaCl) with or without insulin (25 milliunits/g body wt) was injected. The livers were removed 30 s later, and hind limb skeletal muscles were removed 90 s thereafter and immediately homogenized with a Polytron homogenizer in 6 volumes of solubilization buffer. Both extracts were centrifuged at 15,000 × g for 30 min at 4 °C, and the supernatants were used as samples for immunoprecipitation, immunoblotting, or kinase assay of PI 3-kinase and Akt/protein kinase B.
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**FIGURE 1.** a, schematic drawing of the construct for RELMB gene overexpression, b, heptic expressions and elevated serum concentrations of RELMB in the two lines of RELMB transgenic mice. Immunoblotting revealed line 1 and line 2 transgenic mice to have high and low levels of RELMB overexpression, respectively. c, quantification using recombinant RELMB as the standard. Western blotting was performed by transferring samples, using standards for the same membrane. Values are presented as means ± S.E. In line 2 mice, statistical significance is indicated by an asterisk (*, p < 0.05 for RELMB transgenic mice versus control age-matched mice. NC, normal chow; HF, high fat.

Immunoprecipitation and Immunoblotting—Supernatants containing equal amounts of protein (8 mg) were incubated with anti-IRS-1 and anti-IRS-2 antibodies (5 mg/ml each) and then incubated with 100 µl of protein A- and G-Sepharose. The samples were washed and then boiled in Laemmli sample buffer containing 100 mM dithiothreitol. Total lysates were also boiled to allow detection of Akt, AktSer73 phosphorylation, the three MAPKs, and their phosphorylations. Total lysates or immunoprecipitants were subjected to Western blotting, blotted with the antibodies of another 10% G10 antibody. Band intensities were quantified with Image J (National Institutes of Health).

**Measurement of PI 3-Kinase and Akt/Protein Kinase B Activity**—For PI 3-kinase assay, the supernatants containing equal amounts of protein were immunoprecipitated for 2 h at 4°C with anti-IRS-1, anti-IRS-2 or 4G10 antibody and protein A- or G-Sepharose. PI 3-kinase activities in the immunoprecipitants were assayed as previously described (23). For the Akt kinase assay, an Akt kinase assay kit (Cell signaling) was used according to the manufacturer's instructions.

**Glucose Uptake by Isolated Soleus Muscle in Vitro**—Insulin-stimulated glucose uptake by the soleus muscle was measured as described previously (24). Mouse soleus muscles were isolated and incubated for 30 min in KHB buffer, with or without human insulin (2 milliunits/ml). The muscles were then rinsed for 10 min at 29°C and incubated for 20 min at 29°C in KHB buffer containing 8 mM 2-deoxy-D-[1,2-3H]glucose (2-DG) (2.25 µCi/ml) and 32 mM [14C]mannitol (0.3 µCi/ml). After incubation, the muscles were rapidly solubilized. Radioactivity in the resultant samples was counted, and 2-DG uptake rates were corrected for extracellular trapping with mannitol counts.

Effects of RELMB on Insulin Action in Primary Hepatocytes—Hepatocytes were isolated from fasted C57Bl6 mice by collagenase perfusion, as described previously (25). To determine the effects of RELMB on insulin signaling, the dishes were split into two groups corresponding the presence and absence of RELMB stimulation for 24 h. The RELMB concentration was 1 µg/ml. The cells were then serum-starved for 3 h and stimulated with 10^{-8} M insulin for 5 min at 37°C. IRS-1 and IRS-2 protein contents were evaluated by immunoblotting as described above. Insulin-induced IRS-1 and IRS-2 phosphorylations were evaluated by 4G10 immunoprecipitation and immunoblotting as described above.

**Effect of RELMB on Phosphorylations of MAPKs**—To determine the effects of RELMB on MAPK phosphorylations with their time course and dose dependence, primary hepatocytes were stimulated with 1 µg/ml RELMB for 10 or 30 min, and with 0.01, 0.1, or 1 µg/ml RELMB for 15 min. Phosphorylations of p44/p42 (ERK1/2), p38 MAPK, and p54/p66 (SAPK/JNK) were evaluated by immunoblotting as described previously (26).

**Tissue Hematoxylin-Eosin Staining**—The liver and pancreas were removed from transgenic mice and their littermates and formalin-fixed. Samples were routinely embedded in paraffin. Approximately 5-µm-thick slices obtained from these samples were stained with hematoxylin and eosin. Mean pancreatic islet area was histomorphometrically analyzed as previously described (27), using Image J (National Institutes of Health).

**Ribonuclease Protection Assay**—Riboprobes of enzymes were amplified from mouse embryonic cDNA using PCR primers and subcloned as already described (13). Total RNA from the liver and primary cultured hepatocytes was isolated using TRizol reagent (Isogen, Nippon Gene, Japan). A 10-µg RNA sample was used for each assay. RNase protection assays were carried out according to the manufacturer's instructions (RPA III kit, Ambion, Austin, TX). Intensities of the resultant bands were determined using BAS2000 (Fuji film, Japan).

**Statistical Analysis**—Results are expressed as means ± S.E., and significance was assessed using unpaired Student’s t tests, unless otherwise indicated.

**RESULTS**

**Generating RELMB Transgenic Mice**—We generated transgenic mice, the livers of which express RELMB, by inserting RELMB cDNA downstream from the serum amyloid P (SAP) promoter. As expected, liver-specific expression in these transgenic mice was confirmed (data not shown), and two lines were established. In the livers of line 1 mice, RELMB was highly overexpressed, and the serum RELMB concentration exceeded that of the non-transgenic mice by 10-fold (Fig. 1c). In line 2 mice, RELMB was moderately overexpressed, and the resulting elevation of serum RELMB was approximately double that in non-transgenic mice (Fig. 1c). Hepatic RELMB expression was reportedly increased in response to high fat feeding. Thus, increases in serum RELMB with a high fat diet were observed in wild-type and line 2 mice, whereas there was no marked increase in the line 1 mice in which hepatic RELMB overexpression was high. These observations indicate that a high fat diet increases endogenous RELMB expression but not transgene-derived RELMB expression.

No significant difference was observed in terms of growth or adolescence between RELMB transgenic mice and their littermates. At the time of sacrifice (age 16 weeks, after 4 weeks of being fed a high fat diet) and within the observation period, there was no significant difference in body weight or food intake.

**Glucose and Lipid Metabolic Profiles of RELMB Transgenic Mice**—The body weight, fasting serum glucose, serum insulin, and lipid concentrations of RELMB transgenic mice did not differ from those of control mice when both were fed normal chow, at the age of 16 weeks.
Furthermore, glucose, insulin, and pyruvate tolerance tests showed no significant differences between 16-week-old RELMβ-overexpressing mice and their littermates (Fig. 2, a–d, and TABLE ONE). However, the serum glucose concentration was significantly more elevated in both lines of transgenic mice (n = 25, p < 0.01) when fed a high fat diet (TABLE TWO), than those in the control mice. Under these conditions, hyperinsulinemia (n = 6, p < 0.05), hyperlipidemia (n = 6, p < 0.05), and increased hepatic triglyceride content (n = 8, p < 0.05) were observed in the transgenic mice.

Starting at the age of 16 weeks (line 1), or 20 weeks (line 2), mice were given a high fat diet for 4–6 weeks, and glucose, insulin, and pyruvate tolerance tests were then performed (Fig. 3). The glucose tolerance test confirmed an elevated fasting serum glucose concentration (Fig. 3, a and d) and revealed glucose intolerance in the transgenic mice. The insulin tolerance test revealed insulin resistance in the transgenic mice (Fig. 3, b and e). Finally, the pyruvate tolerance test showed greater elevation of the serum glucose concentration in the transgenic mice, as compared with the control mice (Fig. 3, c and f), indicating insufficient suppression of hepatic gluconeogenesis in the transgenic mice. Taking these results together, RELMβ transgenic mice are insulin-resistant and glucose-intolerant, particularly in the liver. These tendencies were more evident in the highly RELMβ-overexpressing line. In terms of adipocytokines,
the levels of serum free fatty acid and adiponectin were not altered ($n = 8–10$).

**Histological Analysis of Liver and Pancreas**—Histology showed fatty liver and islet hyperplasia in transgenic mice as compared with control mice ($n = 3$, age 16 weeks, after 4 weeks on a high fat diet, Fig. 4). Quantitatively, the mean islet area in RELMβ-overexpressing mice was significantly increased, as compared with that in control mice, by approximately 2.5-fold (TABLE TWO). In contrast, no significant difference in adipocyte size or mass was seen in epididymal or subcutaneous fat at the time of sacrifice (data not shown).

**Glucose Clamp Study and Glucose Uptake in Vivo and in Vitro**—Six-month-old mice fed a high fat diet for 4 weeks were used for the glucose clamp study. In the basal state, glucose levels were $113.3 \pm 2.0$ versus $158 \pm 9.5$ mg/dl (control versus transgenic), and insulin levels were $5.0 \pm 1.2$ versus $12.1 \pm 5.4$ ng/ml. There was no significant difference in either glucose utilization or hepatic glucose output (Fig. 5, b and c). In the hyperinsulinemic euglycemic clamp study, glucose levels were $96.0 \pm 5.3$ versus $100.8 \pm 5.3$ mg/dl, and insulin levels were $20.3 \pm 5.2$ versus $22.4 \pm 2.8$ ng/ml. RELMβ transgenic mice showed a 35% lower glucose infusion rate (Fig. 5a) due to markedly insufficient suppression of hepatic glucose output, (about one-fifth of the expected suppression, Fig. 5c), whereas hyperinsulinemia had no effect on the glucose disappearance rate (Fig. 5b).

We found no significant difference in glucose uptake in vivo during the clamp by either muscles or fat (Fig. 5d), nor in insulin stimulated glucose uptake in vitro by isolated soleus muscle between RELMβ-overexpressing mice and their littermates, at 3 months of age after 4 weeks on a high fat diet, i.e. immediately before the experiment (Fig. 5e).

**Insulin Signaling in RELMβ Transgenic Mice**—To reveal the mechanism of glucose intolerance in the RELMβ transgenic mice, we investi-
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**FIGURE 5.** a–d, glucose clamp study of RELM β overexpressing line 1 at 6 months of age, following 4 weeks of being fed a high fat diet. Blood samples were collected every 30 min to determine the glucose specific activity and blood glucose and plasma insulin concentration during 90-min basal period and 150-min clamp period. At the end of the study, tissues were sampled for determination of tissue glucose uptake rates during the clamp period. a, glucose infusion rate in the hyperinsulinemic euglycemic state. b, glucose disposal rate in basal and hyperinsulinemic states. c, glucose production rate in basal and hyperinsulinemic states. d, glucose uptake by soleus muscle, gastrocnemius muscle, and epididymal fat in vivo. e, glucose uptake by isolated soleus muscle in vitro. −, without insulin stimulation; +, with insulin stimulation. Values are presented as means ± S.E. In b–e, statistical analysis was performed using two-way analysis of variance. Statistical significance is indicated by an asterisk: *,

**FIGURE 6.** The protein contents and phosphorylations of IRS-1/2 in liver (a–d) and muscle (e–h) from 3-month-old RELM β-overexpressing line 1 mice, which had been fed a high fat diet for 4 weeks. Values are presented as means ± S.E. for RELM β transgenic mice versus control age-matched mice.

 gated protein contents, phosphorylations, and insulin signaling cascade activity using the highly RELM β-overexpressing (line 1, Fig. 1b) line 1 3-month old mice, which had been fed a high fat diet for 4 weeks. We found that IRS-1 and IRS-2 protein contents were significantly decreased in the livers and muscles of transgenic mice (IRS-1: 34% decrease in liver, 40% in muscle, p < 0.05; IRS-2: 64% in liver and 36% in muscle, p < 0.05) (Fig. 6, a, c, e, and g). The insulin-induced tyrosine phosphorylations of IRS-1 and IRS-2 were also decreased in the livers and muscles of transgenic mice (Fig. 6, b, d, f, and h). In the liver, the decrease in IRS-2 phosphorylation (p < 0.05) was more evident than that of IRS-1, whereas the IRS-1 decrease was more evident in muscle. Similarly, the PI 3-kinase activities associated with IRS-1 and IRS-2 in response to insulin stimulation were significantly suppressed, by ~40%, in transgenic mice (Fig. 7, a–e). Finally, Akt kinase activity in the presence of insulin stimulation was also decreased in both liver (40%) and muscle (35%) in transgenic mice (Fig. 8, a–e), whereas Akt protein contents were not significantly altered.

RELMB Activates ERK, p38, and JNK while Suppressing Insulin Signaling in Primary Cultured Hepatocytes—To investigate the effects of RELMB on insulin signaling in the liver, primary cultured hepatocytes were prepared. The treatment of hepatocytes with 1 μg/ml of recombinant RELMB for 24 h induced apparent decreases in both IRS-1 (70% p < 0.005) and IRS-2 (49%, p < 0.005) protein contents (Fig. 9, a and b). Similarly, insulin-stimulated phosphorylations of IRS-1 and IRS-2 were also suppressed by treatment with RELMB (Fig. 9, c and d). Subsequently, mRNA levels of gluconeogenic enzymes, glucose-6-phosphatase, and phosphoenolpyruvate carboxylase kinase were elevated (Fig. 9, e and f). This suggested RELMB to directly produce insulin resistance in the liver.

Next, to reveal the molecular mechanism underlying the suppressed expression and phosphorylation of IRS-1 and IRS-2, we studied the effects of RELMB on the three MAPKs, ERK, p38, and SAPK/JNK, all of which can reportedly induce insulin resistance. Hepatocytes were treated with 1 μg/ml RELMB or 10 ng/ml tumor necrosis factor α for 10 or 30 min, and the phosphorylations of ERK,
p38, and JNK were assessed by immunoblotting with the antibodies to detect phosphorylation of p44/p42 (ERK1/2), p38 MAPK, and p54/p46 (SAPK/JNK). RELMβ was revealed to markedly phosphorylate ERK and p38, but JNK only weakly (left side of Fig. 10, a and c). The phosphorylations induced by RELMβ were more intense 30 min after the initiation of stimulation than at 10 min, whereas tumor necrosis factor α-induced phosphorylations of MAPKs were shown to peak at ~10 min (right side of Fig. 10a). The effect of RELMβ was concentration-dependent, and as little as 10 ng/ml RELMβ stimulated all three MAPKs (Fig. 10, b and d). In addition, increased phosphorylation of p38 was observed in both the liver and muscle of RELMβ transgenic mice (Fig 11) in the basal, i.e. fasted, state, although there were no significant changes in the phosphorylations of ERK and JNK.

Key Enzymes for Lipid Metabolism Showed Modestly Altered Transcriptional Levels in RELMβ Transgenic Mice—To investigate the mechanisms of hyperlipidemia in RELMβ transgenic mice, mRNA levels of key lipid metabolic enzymes were evaluated using a RELMβ-overexpressing line (line 1) i.e. 3-month-old mice that had been fed a high fat diet for 4 weeks. A lipogenic enzyme (fatty acid synthase, 60%) was increased and lipolytic enzymes (carnitine palmitoyltransferase-1, 32%, PPARα: peroxisome proliferator activated receptor-α, 33%) were decreased (TABLE THREE). These changes probably contribute to the mechanisms underlying the moderate hyperlipidemia in RELMβ-overexpressing mice.

DISCUSSION

Although the gut is the site of nutrient absorption, the gastrointestinal tract secretes several hormones such as glucagon-like peptide-1, gastric inhibitory polypeptide, and ghrelin. These hormones reportedly influence metabolic conditions by regulating insulin secretion and appetite (28–31). Recently, two RELMs were also identified as hormones secreted from the mouse small intestine and colon (9, 10), and we previously reported that these gut-derived RELMs are apparently up-
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regulated in insulin-resistant rodent models. Thus, we speculated that the gastrointestinal tract might regulate insulin sensitivity by secreting these RELMs into the circulation in response to intestinal circumstances, i.e., inflammation, the food intake amount, and/or the nutritional contents of the food. Based on this hypothesis, this study was designed to answer questions as to whether or not the increased expression of gut-derived RELMβ actually induces insulin resistance, and if so, what molecular mechanisms underlie this regulation. To answer these questions, we generated transgenic mice with hepatic RELMβ overexpression, because overexpression in the liver is a commonly used method of investigating the chronic functions of several hormones, including leptin, growth hormone, and so on.

We speculate that RELMβ might possess dual roles; one in the circulation and the other in the gut. RELMβ functioning in the gut would be excreted into the stool. We consider hepatic RELMβ-overexpressing mice to be useful for investigating the role of RELMβ in the circulation, because overexpressing RELMβ in the liver, assuming a high focal concentration, would mimic the physiological pattern of RELMβ concentrations, because RELMβ probably initially flows into the liver via the portal vein.

We first demonstrated both line 1 and line 2 RELMβ transgenic mice, on a high fat diet, to phenotypically show hyperglycemia, hyperlipidemia, and hyperinsulinemia. Taking the data for line 2 into consideration, it seems that a relatively small increase in RELMβ expression can induce significant insulin resistance, which suggests that the increased serum RELMβ concentrations observed in the insulin resistant rodent models are physiologically significant. The consequent islet hyperplasia was considered to be due to prolonged insulin resistance. However, on the other hand, it should be noted that, when the mice were fed a normal diet, these metabolic abnormalities did not become overt even in the highly RELMβ-overexpressing line (line 1). This finding suggests that RELMβ itself induces a limited degree of insulin resistance that is not sufficient to cause overt diabetes or hyperlipidemia.

Some additional factor(s) causing insulin resistance (in the case of our experiments, the high fat diet feeding) in addition to the increased serum RELMβ concentration would be necessary to induce diabetes or hyperlipidemia. It is also reasonable to regard increased RELMβ expression as just one of several independent molecular mechanisms underlying high fat diet insulin resistance.

Next, we demonstrated the presence of hepatic insulin resistance in...
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FIGURE 11. MAPK phosphorylations and protein levels from 3-month-old RELMβ-overexpressing line 1 mice that had been fed a high fat diet, after an overnight fast. Western blots for phospho-p44/p42(ERK1/2), phospho-p38, p38, phospho-p54/p46(SAPK/JNK), and p54/p46 were performed. Each condition was examined in triplicate. The scale represents the percentage of control. Statistical significance is indicated by an asterisk: *, p < 0.05 for control mice versus transgenic mice.

TABLE THREE

| mRNA levels of key lipid metabolic enzymes in the liver, measured using ribonuclease protection assay (RPA) using RELMβ overexpressing line 1 mice, at the age of 3 months, which had been fed a high fat diet for 4 weeks |
|---------------------------------------------------------|
| Control                   | RELMβ transgenic |
|---------------------------|------------------|
| LDLR                      | 1.00 ± 0.09      | 1.02 ± 0.06 |
| PPARβX                    | 1.00 ± 0.13      | 0.72 ± 0.03 |
| CPT1                      | 1.00 ± 0.07      | 0.77 ± 0.07 |
| SREBP-1a                  | 1.00 ± 0.08      | 0.98 ± 0.08 |
| SREBP-1c                  | 1.00 ± 0.17      | 0.92 ± 0.08 |
| ACC                       | 1.00 ± 0.04      | 1.09 ± 0.01 |
| FAS                       | 1.00 ± 0.09      | 1.60 ± 0.34 |
| SCD1                      | 1.00 ± 0.20      | 1.03 ± 0.15 |

* p < 0.05.

RELMB transgenic mice, maintained on a high fat diet, by hyperinsulinemic glucose clamp test, which showed markedly increased hepatic glucose output while glucose utilization was intact. The pyruvate tolerance test, which reflects hepatic gluconeogenesis, yielded complementary results, because pyruvate is a substrate for gluconeogenesis (17). In contrast, we did not find changes in insulin-induced glucose transport activity in either peripheral tissues in vivo, or in isolated skeletal muscles in vitro from RELMB transgenic as compared with control mice. Indeed, histochemical analysis revealed fatty livers in the RELMB transgenic mice, although there was no obvious obesity at the time of sacrifice. Thus, it seems that glucose intolerance and hyperlipidemia in RELMB transgenic mice are attributable mainly to hepatic insulin resistance. There is a curious discrepancy between the apparent insulin resistance in the insulin signaling pathway (e.g. insulin-induced PI 3-kinase activity) and unchanged insulin-induced glucose uptake by muscle, suggesting one or more compensatory mechanisms involving insulin-induced glucose transport, although this remains entirely speculative. In view of organ cross-talk, primary hepatic insulin resistance might affect glucose uptake in other peripheral tissues.

Subsequently, to elucidate whether or not the contribution of RELMB to hepatic insulin resistance is direct, and what changes in signal transduction are induced by RELMB, we performed experiments using primary cultured hepatocytes. Importantly, the expressions of IRS-1 and IRS-2 were demonstrated to be down-regulated in RELMB-treated hepatocytes as well as liver and muscle tissues from RELMB transgenic mice. In good agreement with the decreased IRS-1 and IRS-2 contents, PI 3-kinase activities associated with IRS-1 and IRS-2 and Akt activation, which are reportedly essential for insulin-induced metabolic actions, were also attenuated. These findings suggest that RELMB functions directly in insulin-sensitive cells such as hepatocytes to suppress insulin signaling rather than as a consequence of hepatic lipid accumulation or of altered functions of other tissues such as adipose. The pattern of change in IRS protein contents is similar to that of ob/ob mice (32). Given the elevated serum concentration of RELMB in the ob/ob mice, it may be reasonable to consider the elevated serum RELMB concentration to at least contribute to the decreased IRS-1 and IRS-2 contents.

The next question concerns which type of signal transduction induced by RELMB is involved in suppressing insulin signaling. Several previous studies have confirmed that the activation of ERK-1/2 or p38 MAPK leads to down-regulation of IRS-1 and IRS-2 (26). The activated JNK reportedly phosphorylates the serine residue in IRS-1 and suppresses insulin-induced PI 3-kinase activation. Thus, we investigated the effect of RELMB on MAPK signaling using primary cultured hepatocytes. We found that ERK1/2, p38, and SAPK/JNK are phosphorylated in response to RELMB. It was also recently reported that resistin phosphorylates and activates ERK-1/2 and p38 in smooth muscle cells (33). Thus, resistin and RELMB are likely to have the same function. In addition, a significant increase in p38, but not ERK or JNK, phosphorylation was observed in both liver and muscle tissues of RELMB transgenic mice. Because stimulation with RELMB is constitutive in transgenic mice, we speculated that the effect of RELMB might be difficult to detect but our results were consistent with those obtained using primary cultured hepatocytes.

Lipid metabolism remains an important issue. Some hepatic lipolytic and lipogenic enzymes were altered to promote lipid accumulation in the liver. These modest changes apparently reflect the moderate hyperlipidemia and liver steatosis of RELMB transgenic mice. However, it is noteworthy that the key transcriptional factor, SREBP-1c, regulating lipid synthesis was not altered. The activation of p38 and other MAPKs
by RELMβ may be involved in regulating lipid metabolism as previously reported (34–36).

Our results suggest that chronic stimulation by RELMβ causes glucose intolerance and hyperlipidemia associated with impaired insulin signaling, and the activations of three MAPKs are probably involved in this insulin signaling suppression. Although our findings suggest the importance of increased RELMβ expression in the pathogenesis of diabetes and hyperlipidemia, there are still limitations in interpreting the physiological role of RELMβ in the pathogenesis of insulin resistance, because glucose homeostasis is modestly impaired in RELMβ knock-out mice on a high fat diet only. Thus, further studies are necessary to elucidate how much RELMβ actually contributes to insulin resistance caused by various factors. For example, the percentages of RELMβ present in circulating blood as monomers, dimers, and hexamers and the biological activities of each form, should be determined. Identification of the specific RELMβ receptor as well as the relationship between serum RELMβ and insulin sensitivity in humans are important. Further studies may reveal that RELMβ is a potentially useful marker for assessing insulin resistance associated with obesity and may serve as a target for novel anti-diabetic agents.

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