Humoral Regulation of Resistin Expression in 3T3-L1 and Mouse Adipose Cells

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Resistin is a hormone secreted by adipocytes that acts on skeletal muscle myocytes, hepatocytes, and adipocytes themselves, reducing their sensitivity to insulin. In the present study, we investigated how the expression of resistin is affected by glucose and by mediators known to affect insulin sensitivity, including insulin, dexamethasone, tumor necrosis factor-α (TNF-α), epinephrine, and somatropin. We found that resistin expression in 3T3-L1 adipocytes was significantly upregulated by high glucose concentrations and was suppressed by insulin. Dexamethasone increased expression of both resistin mRNA and protein 2.5- to 3.5-fold in 3T3-L1 adipocytes and by ~70% in white adipose tissue from mice. In contrast, treatment with troglitazone, a thiazolidinedione antihyperglycemic agent, or TNF-α suppressed resistin expression by ~80%. Epinephrine and somatropin were both moderately inhibitory, reducing expression of both the transcript and the protein by 30–50% in 3T3-L1 adipocytes. Taken together, these data make it clear that resistin expression is regulated by a variety of hormones and that cytokines are related to glucose metabolism. Furthermore, they suggest that these factors affect insulin sensitivity and fat tissue mass in part by altering the expression and eventual secretion of resistin from adipose cells. *Diabetes* 51: 1737–1744, 2002

The protein hormone resistin is secreted from adipocytes and antagonizes insulin-stimulated glucose metabolism in skeletal muscle myocytes, hepatocytes, and adipocytes themselves. As such, administration of resistin to mice diminishes glucose tolerance (1). Resistin also has an inhibitory effect on adipose differentiation (2), suggesting that in addition to being an important regulator of insulin sensitivity, resistin may also modulate adipogenesis.

Insulin resistance, which is the hallmark of type 2 diabetes (3–6), is induced by mostly unidentified genetic elements combined with factors such as obesity, high-fat diet, insufficient exercise, inflammation, and abnormal plasma levels of various hormones (7–9). In that regard, serum resistin levels are markedly elevated in insulin-resistant mice fed a high-fat diet, indicating that resistin is likely crucially involved in the insulin resistance associated with obesity and a high-fat diet (1). On the other hand, the degree to which resistin is involved in the pathogenesis of insulin resistance associated with factors other than obesity remains unclear. We therefore examined the effect on resistin expression of various hormones and cytokines known to affect insulin sensitivity.

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DMEM, Dulbecco's modified Eagle's medium; PPAR, peroxisome proliferator–activated receptor; TNF-α, tumor necrosis factor α.

RESEARCH DESIGN AND METHODS

Materials. Dexamethasone was purchased from Sigma, somatropin (rDNA origin) (Genotropin) was from Pharmacia Upjohn, recombinant murine tumor necrosis factor α (TNF-α) was from Genzyme, troglitazone was from Sankyo, and 3-isobutyl-1-methylxanthine was from Wako Bioproducts. The enhanced chemiluminescence detection system was from Amersham Pharmacia Biotech. All other reagents from commercial sources were of analytical grade.

Antibody. The antiresistin antibody used in this study was raised in rabbits against recombinant resistin protein produced in Sf-9 cells using a baculovirus system as previously described (10). The antibody was affinity purified on Affigel-10 (BioRad, Hercules, CA) columns, to which the recombinant resistin had been coupled, and was then extensively dialyzed against PBS. The antibody against mouse β-actin was purchased from Sigma.

Cell culture. 3T3-L1 fibroblasts were initially maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% donor calf serum (Life Technologies) under an atmosphere of 90% air/10% CO_2 at 37°C. Differentiation was induced 2 days after the cultures reached confluence by incubating the cells for 48 h in DMEM supplemented with 0.5 mmol/l of 3-isobutyl-1-methylxanthine, 4 μg/ml dexamethasone, and 10% fetal bovine serum (Life Technologies). Thereafter, the cells were maintained for an additional 4–10 days in DMEM supplemented with 10% fetal bovine serum, and the medium was changed every other day. With this protocol, >90% of the cells expressed the adipocyte phenotype.

Gene transduction. To obtain recombinant adenoviruses, the expression cosmid cassette was ligated to a cDNA encoding resistin with a COOH-terminal Flag epitope, after which homologous recombination of the recombinant cosmid cassette with its parental virus genome was carried out as previously described (10). As a control, we also constructed an adenoviral vector into which LacZ cDNA was subcloned. Confluent cultures of COS-7 cells, maintained in DMEM supplemented with 10% fetal bovine serum, were then infected with the indicated adenovirus for 3 days. Thereafter, the medium was collected, and Western blot analysis was carried out using an anti-Flag or antiresistin antibody as a probe.

Animals. Mice were purchased from Tokyo Experimental Animals and fed a standard rodent diet (60% carbohydrate, 24.5% lipid, and 15.5% protein). Some mice received intramuscular injections of dexamethasone (10 mg/kg) once a day for 5 consecutive days. Food was withdrawn 12–14 h before experimentation, at which time the mice were killed by decapitation. White epididymal adipose tissue was then removed and immediately homogenized for 30 s in 10 volumes of Isogen RNA isolation kit using a polytron operated at maximum speed.

RNA preparation. Total cell RNA was isolated from 3T3-L1 adipocytes using an Isogen RNA isolation kit (Nippon Gene, Tokyo). RNA concentrations were

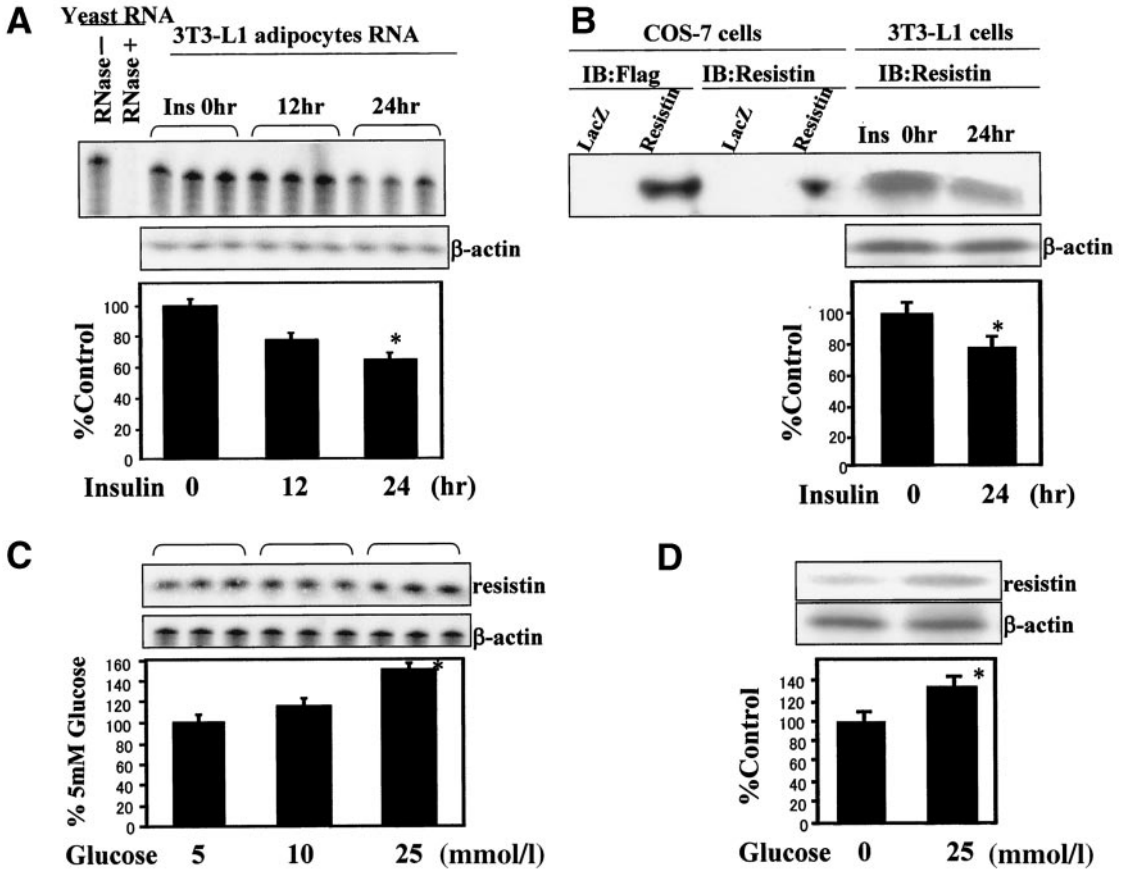

FIG. 1. Effect of insulin and glucose on expression of resistin mRNA and protein in 3T3-L1 adipocytes. *A* and *B*: 3T3-L1 adipocytes were incubated with 100 nmol/l insulin for the indicated periods in DMEM containing 25 mmol/l glucose during the insulin stimulation. Expression of resistin or β-actin mRNA and protein were assessed using an RNase protection assays (*A*) and Western blot analysis (*B*), respectively. *A*: Two RNase protection assays, carried out in tubes containing the same amount of labeled mouse resistin probe plus 10 μg yeast RNA, which does not hybridize the probe, served as controls. Buffer without RNase was added to one of the tubes, which served as a positive control, and buffer with RNase was added to the other tube, which served as a negative control. *$P < 0.005$ vs. control. *B*: LacZ or Flag-tagged resistin in medium conditioned by COS-7 cells transfected with the indicated adenoviral vector was probed using anti-Flag or antiresistin antibody, respectively. Immunoblot analysis with anti–β-actin antibody was conducted for each cell lysates as a control. *$P < 0.005$ vs. 5 mmol/l. *C* and *D*: 3T3-L1 adipocytes were incubated for 24 h with the indicated concentration of glucose, after which expression of resistin RNA (*C*) and protein (*D*) were assessed as in *A* and *B*. The mRNA and protein of β-actin were measured as controls. The intensities of the bands were analyzed with a molecular imager. Bars depict means ± SE from three independent experiments and are shown as percent of control; representative blots are shown in the upper part of the graphs.

estimated based on absorbance at 260 nm, and 10 μg RNA from each sample was used for the RNase protection assay described below.

Preparation of riboprobes. To obtain mouse resistin cDNA, PCR was performed based on reported sequences obtained from mouse cDNA libraries. The amplified fragment, which corresponded to nucleotides 1–345 of mouse resistin cDNA, was subcloned into pBluescript II SK minus, after which the resultant plasmids were then linearized with *Cla*I and used for in vitro expression. The fragment corresponding to nucleotides 1–250 of mouse β-actin was obtained by PCR, subloned into pBluescript II SK minus, and used as a control.

RNase protection assay. RNase protection assays were carried out according to the manufacturer's instructions (RPA III; Ambion, Austin, TX). Pooled 10-μg samples of total RNA from adipocytes were hybridized with the riboprobes for resistin. After treatment with RNase, the protected fragments were resolved on 5% polyacrylamide-urea gels and subjected to autoradiography. The intensities of the resultant bands were then determined using a Molecular Imager GS-525.

Immunoprecipitation and immunoblotting. 3T3-L1 adipocytes and mouse adipose tissue, prepared as described above, were lysed in PBS containing 1% Triton, 0.35 mg/ml PMSF (phenylmethylsulfonyl fluoride), and 100 mmol/l sodium vanadate, after which the lysates were centrifuged for 10 min at 15,000*g* and 4°C to remove insoluble materials. The supernatants were then immunoprecipitated with 5 μg antiresistin antibody. The resultant immunoprecipitates were boiled in Laemmli sample buffer containing 100 mmol/l dithiothreitol, after which SDS-PAGE and immunoblotting were carried out using antiresistin antibody as a probe (10). The intensities of the resultant bands were then determined using a Molecular Imager GS-525.

TNF-α measurement. Serum was collected from mice immediately after decapitation. Serum TNF-α levels were then measured using an ELISA (enzyme-linked immunosorbent assay) system (Amershan Pharmacia Biotech) according to the manufacturer's instructions.

Statistical analysis. Results are expressed as means ± SE. Comparisons were made using unpaired Student's *t* test. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Effect of insulin and glucose on the expression of resistin. The effects of 100 nmol/l insulin on expression of resistin mRNA and protein in cultured 3T3-L1 adipocytes were assessed using RNase protection assays (Fig. 1*A*) and Western blot analysis, respectively, with antiresistin and anti-Flag antibodies as probes (Fig. 1*B*). We found that insulin diminished expression of the mRNA and protein by 37 and 30% ($P < 0.005$), respectively, within 24 h after its addition to the cell cultures. During the same period, resistin expression was unchanged in cells incubated without the added insulin (data not shown). Moreover, control assays using yeast RNA, which does not hybridize the resistin probe, confirmed that RNA from 3T3-L1 adipocytes hybridized and protected the probe RNA from

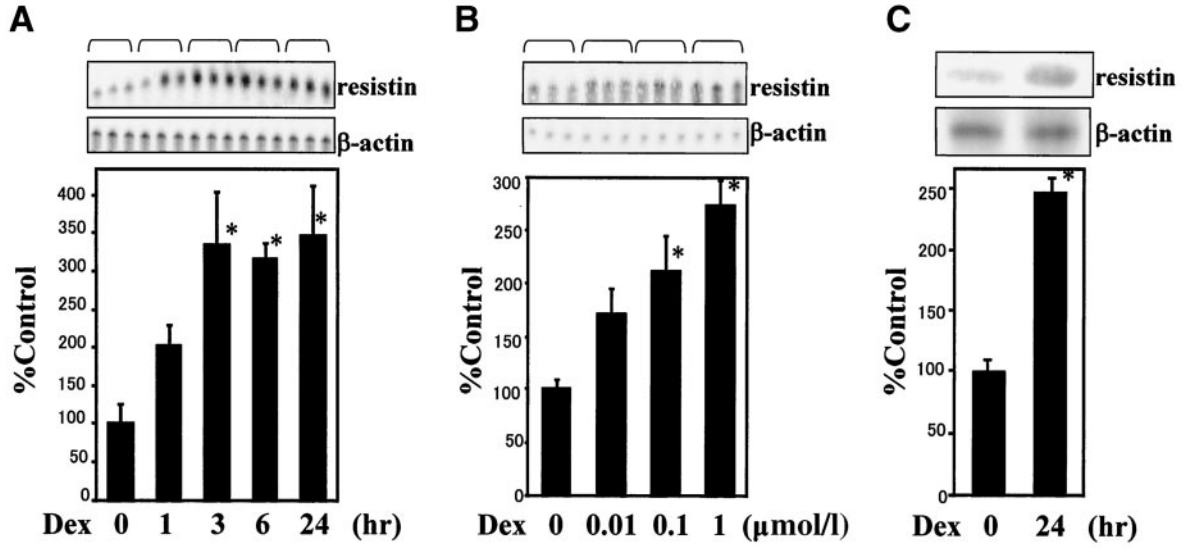

FIG. 2. Effect of dexamethasone on expression of resistin mRNA and protein in 3T3-L1 adipocytes. *A* and *B*: Cells were incubated with 1 μmol/l dexamethasone for the indicated periods up to 24 h (*A*) or incubated with the indicated concentrations of dexamethasone for 24 h (*B*). Thereafter, resistin mRNA was measured using an RNase protection assay. *C*: Cells were incubated for 24 h, with or without 1 μmol/l dexamethasone, after which resistin protein expression was assessed by immunoblotting with antiresistin antibody. The mRNA and protein of β-actin were assessed as controls. Bars indicate means ± SE from three independent experiments and are shown as percent of control; representative blots are shown in the upper part of the graphs. *$P < 0.005$ vs. control.

digestion (Fig. 1*A*), and no proteins were recognized in medium conditioned by cells overexpressing LacZ, confirming the specificity of our antiresistin antibody (Fig. 1*B*). In contrast, mRNA and protein levels of β-actin were not affected by insulin treatments.

To assess the effect of glucose on resistin expression, cells were analyzed after incubating them for 24 h with 5, 10, or 25 mmol/l D-glucose (Figs. 1*C* and *D*). At a concentration of 25 mmol/l, D-glucose increased expression of resistin mRNA and protein by 50 and 35%, respectively, as compared with that seen at a concentration of 5 mmol/l ($P < 0.005$). β-actin expression was not altered. Thus, in 3T3-L1 adipocytes, resistin gene expression is upregulated and protein content is increased by a high concentration of glucose and is downregulated by insulin.

Effect of dexamethasone on the expression of resistin in 3T3-L1 adipocytes and mouse epididymal white adipose tissue. When 3T3-L1 adipocytes were cultured in the presence or absence of 1 μmol/l dexamethasone, expression of resistin mRNA was found to be significantly elevated only 1 h after initiating dexamethasone stimulation (Fig. 2). This upregulation became maximal within 3 h, at which time expression of resistin mRNA was 3.3-fold greater than control ($P < 0.005$). Thereafter, levels of resistin expression remained elevated for up to 24 h. During the same period, resistin expression was un-

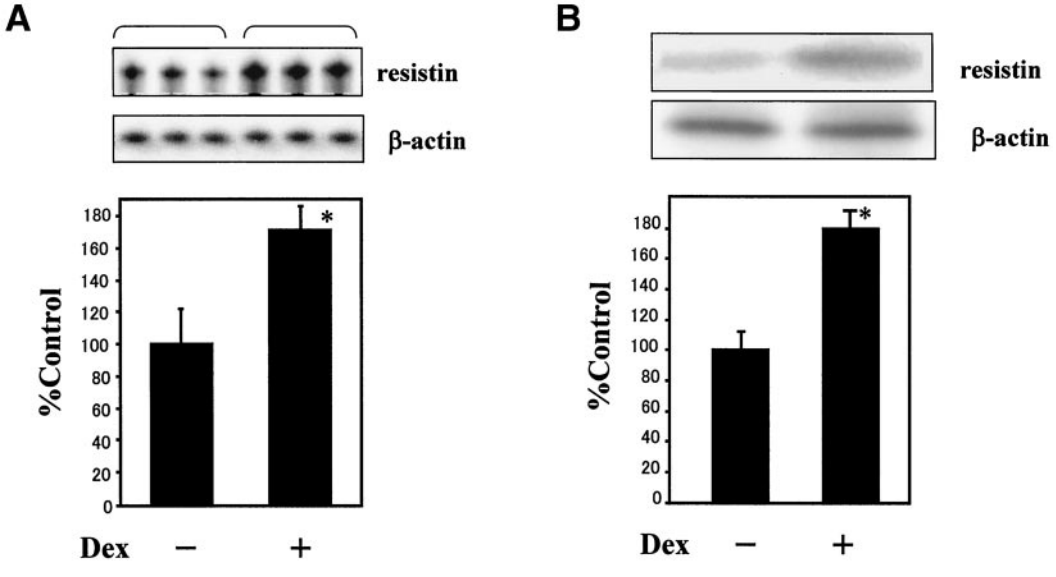

FIG. 3. Effect of dexamethasone on expression of resistin mRNA and protein in epididymal white adipose tissue from mice. After intramuscular injection with 10 mg/kg dexamethasone once a day for 5 consecutive days, total RNA was prepared from epididymal white adipose tissue, and expression of resistin mRNA (*A*) and protein (*B*) was assessed as in Fig. 1. The mRNA and protein of β-actin were assessed as controls. Bars depict means ± SE from three independent experiments and are shown as percent of control; representative blots are shown in the upper part of the graphs. *$P < 0.005$ vs. control.

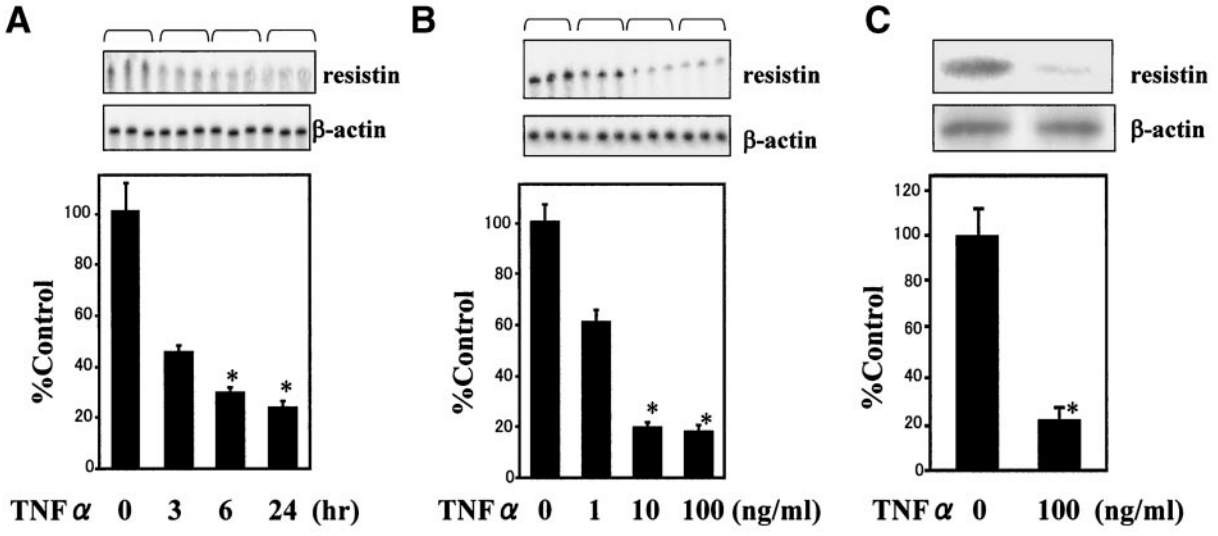

FIG. 4. Effect of TNF-α on expression of resistin mRNA and protein in 3T3-L1 adipocytes. *A* and *B*: Cells were incubated with 100 ng/ml TNF-α for the indicated periods up to 24 h (*A*) or incubated for 24 h with or without 1, 10, or 100 ng/ml TNF-α (*B*). Thereafter, resistin mRNA was measured using an RNase protection assay. *C*: Cells were incubated for 24 h with or without 100 ng/ml TNF-α, after which resistin protein expression was assessed by immunoblotting with antiresistin antibody. The mRNA and protein of β-actin were assessed as controls. Bars depict means ± SE from three independent experiments and are shown as percent of control; representative blots are shown in the upper part of the graphs. *$P < 0.005$ vs. control.

changed in cells incubated without the added dexamethasone (data not shown). The effect of incubating cells with dexamethasone for 24 h was concentration dependent, with significant ($P < 0.005$) elevations in resistin expression obtained at concentrations as low as 10 nmol/l (Fig. 2*B*). Furthermore, expression of resistin protein in cells incubated for 24 h with 1 μmol/l dexamethasone was 150% higher ($P < 0.005$) than that seen in untreated cells (Fig. 2*C*), indicating that increased mRNA levels led to increased expression of the protein. In contrast, the amounts of mRNA and protein of β-actin were unchanged by the treatment with dexamethasone.

To test whether dexamethasone also upregulates resistin expression in vivo, mice were administered 10 mg/kg dexamethasone once a day for 5 consecutive days. This protocol resulted in a 3.8-fold increase in fasting serum insulin levels over that seen in vehicle-treated animals (dexamethasone 2.9 ± 1.0 μU/dl and control 0.8 ± 0.1 μU/dl), though there was no significant change in fasting blood glucose level and a 26% decline in body weight (dexamethasone 22.7 ± 0.8 g and control 30.6 ± 0.7 g). Likewise, TNF-α levels were not significantly affected by dexamethasone administration (dexamethasone 4.08–2.98 pg/ml and control 3.62–1.18 pg/ml). The elevated serum insulin was considered to reflect the presence of insulin resistance. At the same time, resistin mRNA and protein expression in white adipose tissue was increased by ~70 and 80%, respectively ($P < 0.005$) (Figs. 3*A* and *B*).

TNF-α and troglitazone suppress expression of resistin. TNF-α strongly suppressed resistin expression in

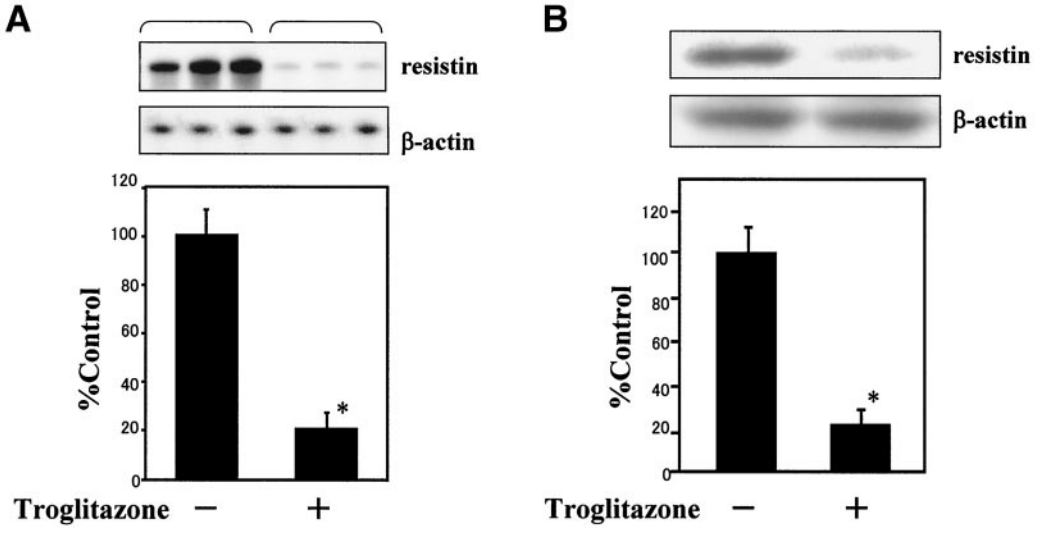

FIG. 5. Effect of troglitazone on expression of resistin mRNA and protein in 3T3-L1 adipocytes. Cells were incubated with 10 μmol/l troglitazone for 24 h, after which expression of resistin mRNA (*A*) and protein (*B*) was assessed as in Fig. 1. The mRNA and protein of β-actin were assessed as controls. Bars depict means ± SE from three independent experiments and are shown as percent of control; representative blots are shown in the upper part of the graphs. *$P < 0.005$ vs. control.

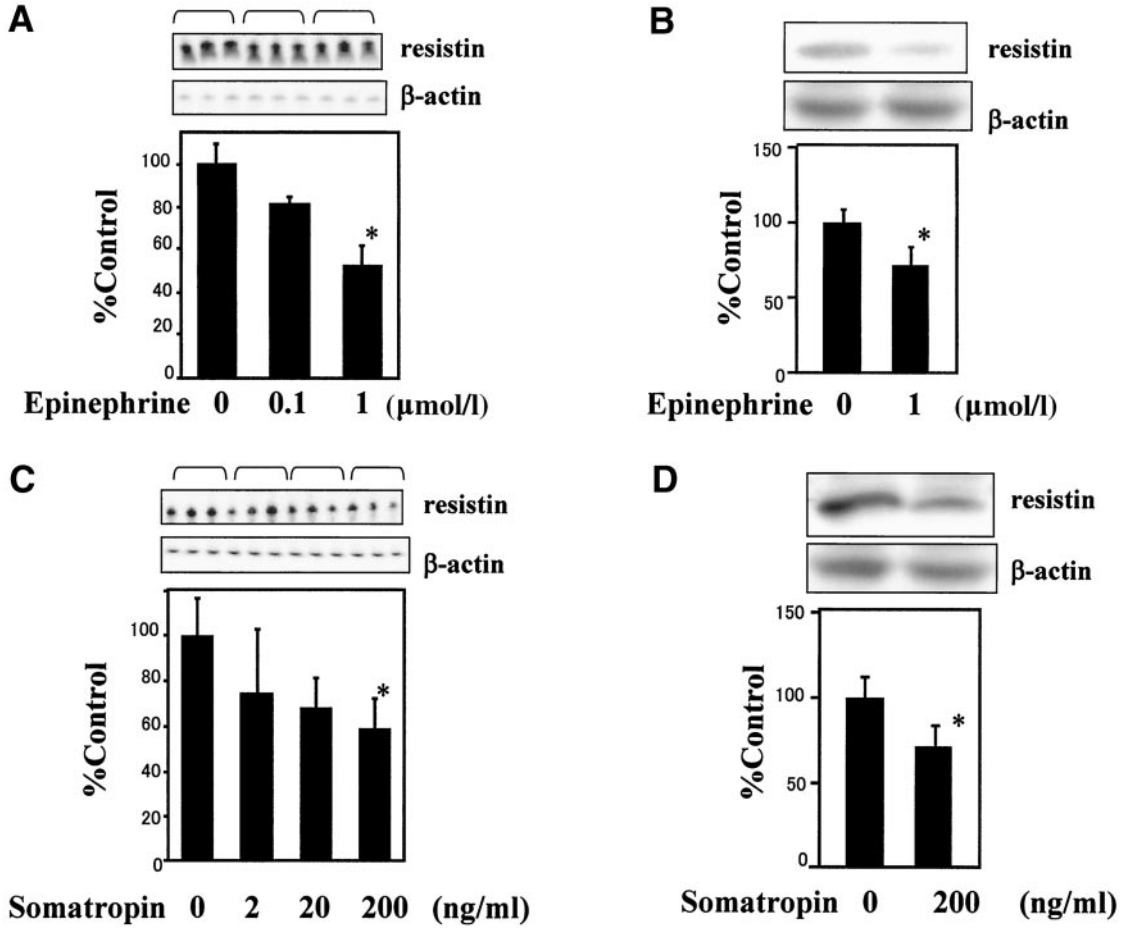

FIG. 6. Effect of epinephrine and somatropin on expression of resistin mRNA and protein in 3T3-L1 adipocytes. Cells were incubated for 24 h with the indicated concentrations of epinephrine or somatropin, after which expression of resistin mRNA (*A* and *C*, respectively) and protein (*B* and *D*, respectively) were assessed as in Fig. 1. The mRNA and protein of β-actin were assessed as controls. Bars depict means ± SE from three independent experiments and are shown as percent of control; representative blots are shown in the upper part of the graphs. *$P < 0.05$ vs. control.

3T3-L1 adipocytes (Fig. 4). Treating cells with 100 ng/ml TNF-α significantly ($P < 0.005$) diminished expression of resistin mRNA within 3 h, and within 24 h, expression was reduced by ~77% (Fig. 4*A*). When cells were exposed for 24 h, as little as 1 ng/ml TNF-α significantly ($P < 0.005$) diminished expression of resistin mRNA (Fig. 4*B*), with both 10 and 100 ng/ml TNF-α reducing expression by ~80% compared with control. Similarly, expression of resistin protein was decreased by ~80% in cells incubated with 100 ng/ml TNF-α for 24 h (Fig. 4*C*).

In a similar fashion, exposing 3T3-L1 adipocytes for 24 h to 10 μmol/l troglitazone, a thiazolidinedione antihyperglycemic agent, diminished expression of both resistin mRNA (Fig. 5*A*) and protein (Fig. 5*B*) by ~80%, which is consistent with earlier findings (1). In contrast, the amounts of mRNA and protein of β-actin were unchanged by these treatments.

The effect of epinephrine and somatropin on resistin expression. Epinephrine had a moderately inhibitory effect on resistin expression. Incubating 3T3-L1 adipocytes with 0.1 or 1 μmol/l epinephrine for 24 h reduced expression of resistin mRNA by 19% (P = NS) and 48% ($P < 0.05$), respectively (Fig. 6*A*). The higher concentration also reduced expression of resistin protein by 38% ($P < 0.05$) (Fig. 6*B*). In addition, at a concentration of 200 ng/ml, somatropin decreased expression of resistin mRNA and protein by 42 and 29%, respectively ($P < 0.05$) (Figs. 6*C* and *D*). The effect of somatropin on resistin expression was dose dependent, reaching statistical significance at 200 ng/ml (Fig. 6*C* and *D*).

The effect of insulin and TNF-α on dexamethasone- and glucose-induced resistin expression. Insulin partially inhibited the stimulatory effect of dexamethasone on resistin expression. Incubating 3T3-L1 adipocytes for 24 h with 1 μmol/l dexamethasone increased expression of resistin mRNA and protein by 186 and 152%, respectively (Fig. 7*A* and *B*). Addition of 100 nmol/l insulin reduced the dexamethasone-induced increase in expression to 102 and 98% over control, respectively ($P < 0.005$ vs. control). TNF-α also inhibited the stimulatory effects of glucose and dexamethasone. Incubating 3T3-L1 adipocytes with 100 ng/ml TNF-α for 24 h, before adding 25 mmol/l glucose or 1 μmol/l dexamethasone, decreased expression of resistin mRNA by 68 and 67% ($P < 0.005$ vs. control), respectively (Fig. 7*C*), and decreased expression of the protein by 73 and 69% ($P < 0.005$ vs. control), respectively (Fig. 7*D*).

DISCUSSION

A variety of factors contribute to the development of insulin resistance, which is the hallmark of type 2 diabetes (8). Among these, obesity is considered to be the most

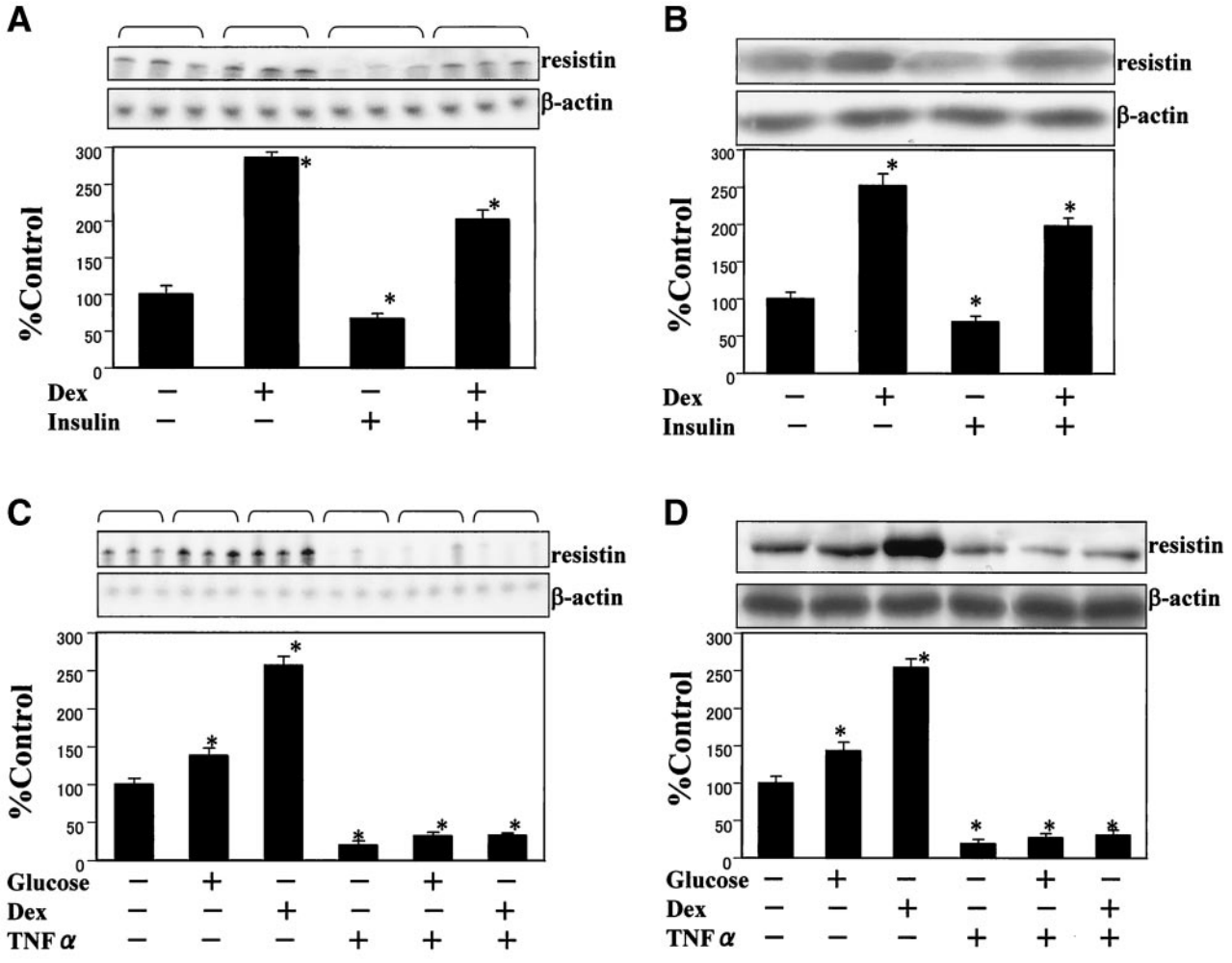

FIG. 7. Effect of insulin and TNF-α on dexamethasone- and glucose-induced resistin expression in 3T3-L1 adipocytes. *A* and *B*: Cells were incubated for 24 h with 100 nmol/l insulin, 1 μmol/l dexamethasone, or both in DMEM containing 5 mmol/l glucose, after which expression of resistin mRNA (*A*) and protein (*B*) were assessed as in Fig. 1. *C* and *D*: Cells were incubated for 24 h with or without 100 ng/ml TNF-α and 40 mmol/l glucose or 25 mmol/l dexamethasone, after which expression of resistin mRNA (*C*) and protein (*D*) were assessed. The mRNA and protein of β-actin were assessed as controls. Bars depict means ± SE from three independent experiments and are shown as percent of control; representative blots are shown in the upper part of the graphs. *$P < 0.005$ vs. control.

common and most important (9). In addition, adipocytes are known to secrete a variety of mediators, including leptin (11,12), free fatty acid (13–15), and adiponectin (16), all of which regulate glucose tolerance. Resistin, whose serum levels are elevated in both genetic and diet-induced models of obesity and insulin resistance (1), was recently added to that list.

Thiazolidinediones are agents developed to reverse insulin resistance. They act by binding to peroxisome proliferator–activated receptor (PPAR)-γ, a ligand-activated transcription factor highly expressed in adipocytes (17–20). The fact that thiazolidinediones act in this fashion substantiates the idea that insulin resistance and its normalization reflect changes in the activity of various intracellular signaling pathways (21–24). Furthermore, the fact that thiazolidinediones suppress expression of resistin and normalize insulin resistance in obese rodents (1) suggests that altered expression of resistin may be a key mechanism underlying obesity-induced insulin resistance as well as the improvement of insulin sensitivity by thiazolidinediones.

We also considered the possibility that other factors known to affect insulin sensitivity also affected the expression of resistin. High levels of glucose significantly enhanced resistin expression, whereas insulin suppressed its expression. In human diabetes, the early prediabetic state is often accompanied by hyperglycemia and hyperinsulinemia (8). Consequently, it is unclear whether resistin is up- or downregulated at this stage, since hyperglycemia and hyperinsulinemia oppositely regulate resistin expression. Alternatively, it is also possible that insulin resistance, as it occurs during the course of type 2 diabetes, reflects the diminished ability of insulin to suppress resistin expression by adipocytes. At more advanced stages of the disease, which are marked by hyperglycemia and hypoinsulinemia, resistin would be expected to be upregulated and thus contribute to hyperglycemia-induced insulin resistance.

We observed that dexamethasone increased resistin expression in both 3T3-L1 adipocytes and white adipose tissue. Glucocorticoids are known to induce insulin resistance, reportedly by affecting insulin binding, insulin receptor substrate-1 phosphorylation, and glucose transporter translocation (25–30). The present findings suggest that glucocor-

ticoid-induced upregulation of resistin expression may also contribute to insulin resistance.

It was of particular interest to us that both troglitazone and TNF-α strongly suppressed resistin expression. This is due to a report by Way et al. (31) showing that, compared with their lean counterparts, resistin expression was significantly lower in the white adipose tissue of several mouse models of genetic and diet-induced obesity and insulin resistance and that this suppression of resistin expression was attenuated by a PPAR-γ agonist. These results apparently contradict the findings of Steppan et al. (1), who showed that serum resistin levels are elevated in such models of obesity and insulin resistance. Our findings may provide a clue that helps explain this apparent discrepancy.

It is known that serum TNF-α levels are elevated in obese rodent models (32,33), which may also contribute to obesity-induced insulin resistance (34–38), and some evidence suggests that thiazolidinediones improve insulin sensitivity by suppressing production of TNF-α in enlarged adipocytes (39). Our findings confirmed that, at least in vitro, thiazolidinediones markedly suppress expression of resistin; however, whether they act in vivo by diminishing circulating TNF-α levels or via PPAR-γ remains unclear. Indeed, thiazolidinediones may suppress or enhance the expression of resistin, depending on TNF-α levels. In mouse models of genetic obesity and insulin resistance, treatment with thiazolidinediones may lower TNF-α levels, which would tend to increase resistin expression. Conversely, expression would tend to be diminished in models in which TNF-α levels were high. In light of our observations, these discussion points represent hypotheses that can now be systematically tested in vivo.

We showed that both epinephrine and somatropin exert a moderately inhibitory effect on resistin expression in vitro. The effect of epinephrine is consistent with an earlier finding that isoproterenol inhibits resistin gene expression in 3T3-L1 adipocytes via a G_s protein–coupled pathway (40). Insulin signal transduction is reportedly modulated by sympathetic nervous system activity, which is in turn modulated by stress, exercise, and fasting (41–43). Nevertheless, whereas epinephrine and growth hormone (44–47) are both known to induce insulin resistance, our results suggest that in contrast to that induced by glucocorticoids, resistin is unlikely to be involved in the insulin resistance induced by epinephrine or somatropin.

The induction of resistin by dexamethasone was partially inhibited by insulin in 3T3-L1 adipocytes. This observation is consistent with the fact that dexamethasone-treated mice are hyperinsulinemic and exhibit upregulated resistin expression. We further observed that TNF-α had a more profound inhibitory effect on glucose- and dexamethasone-induced resistin expression than insulin, suggesting that TNF-α may be a dominant regulator of resistin expression.

It seems apparent from our results that resistin expression is greatly affected by a variety of hormones and environmental factors. Upregulation of resistin expression may be involved in dexamethasone- or hyperglycemia-induced insulin resistance, for instance, although more precise characterization of the physiological activities of resistin will be required to fully understand these processes.

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