Expression of DGAT2 in White Adipose Tissue Is Regulated by Central Leptin Action*

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Acyl-CoA:diacylglycerol acyltransferase (DGAT) enzymes catalyze the final step in mammalian triglyceride synthesis, and their functions are considered to be involved in the mechanisms of obesity, insulin resistance, and leptin resistance. Insulin receptor substrate-2 (IRS-2)-deficient mice exhibit obesity-associated with hypertrophic adipocytes and leptin resistance. Screening for transcripts of genes involved in fatty acid and triglyceride synthesis to investigate the mechanism of the hypertrophic change in the adipocytes showed that expression of DGAT2 mRNA was up-regulated in the white adipose tissue (WAT) of Irs2−/− mice, whereas that of DGAT1 was down-regulated. This reciprocal expression of DGAT1 and DGAT2 was also observed in WAT of leptin-deficient ob/ob mice. A high fat diet also resulted in increased DGAT2 expression in WAT of leptin-deficient ob/ob mice. A high fat diet also resulted in increased DGAT2 expression in WAT of leptin-deficient ob/ob mice. Interestingly, Dgat2−/− mice exhibit increased insulin sensitivity and a leptin-sensitive phenotype associated with decreased tissue triglyceride content, suggesting that DGAT1 is somehow involved in insulin and leptin action throughout the body (7, 8). Recently, Dgat2−/− mice have been reported to exhibit marked reduction of triglyceride and fatty acids in the body, suggesting a critical role of DGAT2 in lipogenesis; however, the physiological roles of DGAT2 in adult mice are still unknown because Dgat2−/− mice die soon after birth (9).

The insulin receptor substrate (IRS) proteins play a key role in signal transduction from the insulin receptor (10, 11) and are major intracellular phosphorylation targets of activated insulin receptor tyrosine kinase. Irs2−/− mice develop diabetes because of inadequate β-cell proliferation combined with insulin resistance (12–14). Another noteworthy feature of Irs2−/− mice is that they exhibit increased adiposity associated with leptin resistance (15, 16). We previously reported that increased expression of sterol regulatory element binding protein-1 (SREBP-1) mRNA in the liver of Irs2−/− mice causes fatty liver, and we attributed its development to leptin resistance (16). We have also reported that the obese phenotype in Irs2−/− mice contributes to their insulin resistance (17).

In this study, we discovered that the adipocytes of Irs2−/− mice are hypertrophic and concluded that fatty acid and triglyceride synthesis must be activated in Irs2−/− mouse adipocytes. Screening for genes involved in the fatty acid and triglyceride synthesis pathways revealed that DGAT2 mRNA was significantly up-regulated in Irs2−/− mouse white adipose tissue (WAT) but that expression of DGAT1 was reduced. This antithetical expression of DGAT1 and DGAT2 was also observed in the WAT of ob/ob mice, however, SREBP-1 mRNA was not increased, suggesting that SREBP-1 is not the major regulator of DGAT expression. Intracerebroventricular administration of leptin reduced DGAT2 mRNA expression in Irs2−/− and ob/ob mice, independently of DGAT1 expression. In contrast in 3T3-L1 cells, leptin did not affect the expression of DGAT, and induction of hypertrophy increased expression of both DGAT1 and DGAT2. The relationship catalyzes the final step in mammalian triglyceride synthesis (2), and two DGAT enzymes have been identified (3–5). Although the genes encoding DGAT1 and DGAT2 belong to different gene families, both genes are ubiquitously expressed and the enzymes they encode have similar substrate specificity (5). Dgat1−/− mice have been reported to exhibit normal growth on a chow diet, and to be resistant to diet-induced obesity (6). Interestingly, Dgat1−/− mice exhibit increased insulin sensitivity and a leptin-sensitive phenotype associated with decreased tissue triglyceride content, suggesting that DGAT1 is somehow involved in insulin and leptin action throughout the body (7, 8).

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between hormonal action and regulation of DGATs in adipose tissue is discussed.

EXPERIMENTAL PROCEDURES

Animals—Irs2−/− mice were generated as described previously (14). Irs2−/− mice were originally maintained on the C57BL/6 and CBA hybrid background and backcrossed with the C57BL/6 strain for at least three generations. Wild-type (WT) mice and Irs2−/− mice were used for the following experiments when they had reached 14–18 weeks of age. ob/ob mice on a C57BL/6 background were purchased from the Jackson Laboratory (Bar Harbor, ME). C57BL/6 mice were purchased from Oriental Yeast Co., Ltd. (Osaka, Japan). All other materials were purchased from Oriental Yeast Co., Ltd.

Histological Analysis—An epididymal fat pad was removed from each animal, fixed in 10% formaldehyde/phosphate-buffered saline, and maintained at 4 °C until used. Fixed specimens were dehydrated and maintained at 4 °C until used. Fixed specimens were dehydrated and embedded in paraffin. The fat pad was then cut into 6-μm sections, and they were mounted on silanized slides. After deparaffinization, the sections were stained with hematoxylin and eosin.

RNA Preparation and Northern Blot Analysis—Mice were sacrificed after fasting for 24 h except the case especially indicated, and the epididymal fat pad was excised to obtain WAT. Total RNA was prepared from WAT or 3T3-L1 cells by using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Northern blot analysis was performed on 10 μg of total RNA according to the standard protocol. The total RNA was loaded onto a 1.0% agarose gel then transferred to a nylon membrane (Hybond N⁺; Amersham Biosciences). The cDNA probes for mouse DGAT1 and DGAT2 were prepared by cloning reverse transcriptase-polymerase chain reaction products from mouse WAT RNA into TA cloning vectors (Invitrogen). The polymerase chain reaction primers used to generate these probes were: DGAT1, forward primer, 5′-TGAAGGACGGACCTGGTCGACAGC-3′; reverse primer, 5′-GGGCTTCTATGGAGTCTGAGAT-3′; and DGAT2, forward primer, 5′-GGGGGTCGTCGATGGGGCACT-3′, reverse primer, 5′-CGACGGTGGTGATGGCGTTGAGT-3′, and DGAT2, 5′-GAGCGGTCTGGCGATGGGGCACT-3′. The corresponding bands were quantified by exposure of BAS 2000 to the filters and analysis of the images with BASStation software. n = 4–5 male mice per group. Values are means ± S.E. *p < 0.05.

3T3-L1 Cell Culture, Induction of Adipocyte Differentiation, and RNA Interference—3T3-L1 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum, and induction of adipogenic differentiation was carried out according to methods described previously (20). After induction for 8 days, the cells were starved for 8 h and then treated with murine recombinant leptin (PeproTech EC Ltd., London, UK) or human insulin (Humulin R; Lilly) at the concentration indicated. The cells were lysed with TRIzol reagent 12 h later. In the hypertrophy-induction experiments, 3T3-L1 cells were cultured on gelatin-coated cell culture dishes (21). siRNAs were chemically synthesized, annealed, and transfected into 3T3-L1 cells at day 5 after induction with Lipofectamine (Invitrogen) and Plus Reagent (Invitrogen). The sequences of the sense siRNAs were: DGAT1, 5′-GAUUCUUUGUUACGUCUGACTT-3′; DGAT2, 5′-GACAUUUUCUCGACUCCUGTT-3′. Forty-eight hours after transfection, the cells were lysed for total RNA extraction.

Real-time Quantitative PCR—Total RNA was extracted from 3T3-L1 cells with TRIzol reagent according to the manufacturer's instructions.
RESULTS

Increased SREBP-1 Expression in the Hypertrophic Adipocytes of Irs2−/− Mice—We previously reported that Irs2−/− mice exhibit obesity, fatty change in the liver, and up-regulation of SREBP-1 mRNA expression, all of which are consequences of their phenotype of leptin resistance (16, 23). Impaired leptin action causes adipocyte hypertrophy as shown in obese mouse models, e.g. ob/ob mice, db/db mice, and KKAy mice. Histological study revealed that Irs2−/− mice have enlarged adipocytes (Fig. 1a), and we examined the expression of genes involved in fatty acid and triglyceride synthesis pathway by Northern blot analyses as a means of investigating the mechanism of the hypertrophy of Irs2−/− mouse adipocytes.

Since the Northern blots revealed increased (3.4-fold) expression of SREBP-1 mRNA in Irs2−/− mouse adipose tissue (Fig. 1b), we analyzed expression of its downstream target genes. The results showed increased expression of FAS, malic enzyme, and GPAT, all of which are downstream targets of SREBP-1, suggesting that increased expression of SREBP-1 contributed to fatty acid synthesis in enlarging Irs2−/− mouse adipocytes (Fig. 1b).

Decreased DGAT1 and Increased DGAT2 Expression in the WAT of Irs2−/− and ob/ob mice—Next, we studied triglyceride synthesis pathway in an attempt to identify the genes responsible for enlarging the adipocytes of Irs2−/− mice. Two enzymes that catalyze the final step of triglyceride synthesis, have been identified and named DGAT1 and DGAT2 (5, 24). Surprisingly, expression of DGAT1 was found to be significantly reduced (0.62-fold) in Irs2−/− mouse adipose tissue, whereas expression of DGAT2 was clearly increased (3.3-fold, Fig. 2, a–c). We
examined expression of the genes in WAT of another model of obesity, ob/ob mice. Both Irs2/−/− mice and ob/ob mice are considered to show obesity because of attenuated leptin action. A similar reciprocal relationship between DGAT1 and DGAT2 expression has also been observed in ob/ob mice (DGAT1: 0.50-fold, DGAT2: 4.7-fold), indicating that adipocyte hypertrophy associated with attenuated leptin action may be responsible for the changes in expression, not the lack of IRS-2 per se (Fig. 2, a–c). The fact that SREBP-1 expression was not elevated in the WAT of ob/ob mice (Fig. 2a) is an important finding, because it is consistent with the findings in previous reports (25, 26) and suggests that SREBP-1 may not play a major role in the regulation of DGAT1 and DGAT2 expression in the WAT of obese animals.

A High Fat Diet Reduced DGAT1 and Increased DGAT2 Expression in the White Adipose Tissue of C57BL/6 Mice—A high fat diet is known to induce leptin resistance and obesity in rodents (27, 28). We examined the effect on WAT of obesity and leptin resistance acquired as a result of consuming a high fat diet in C57BL/6 inbred mice. Seven-week-old C57BL/6 mice were fed a high fat diet or a regular chow diet for 4 weeks, and a high fat diet induced significantly more weight gain of the mice than a regular chow diet (8.5 ± 0.3 g versus 3.5 ± 0.3 g). Plasma leptin concentrations clearly increased in the mice fed a high fat diet (22.5 ± 3.2 ng/ml versus 2.6 ± 0.2 ng/ml). Plasma non-esterified fatty acid (NEFA) concentrations were reduced in high fat diet group (0.74 ± 0.05 mEq/liter versus 1.4 ± 0.1 mEq/liter). Northern blot analysis showed that DGAT2 expression increased (1.9-fold) but DGAT1 decreased (0.73-fold) in WAT of the diet-induced obese mice (Fig. 3, a–c). These changes are essentially the same as observed in genetically obese mice, such as Irs2−/− mice and ob/ob mice (Fig. 2, a–c). To inspect the possibility that change of plasma fatty acid might directly regulate DGAT expression, we intravenously infused lipid (20% w/v triglyceride emulsion, 5 ml/kg/h) and heparin (6 units/h) simultaneously for 5 h in C57BL/6 mice. Plasma NEFA concentrations were significantly increased by lipid plus heparin infusion (lipid + heparin group: 4.3 ± 0.2 mEq/liter, saline + heparin group: 1.1 ± 0.1 mEq/liter), however, DGAT1 and DGAT2 mRNA expression in WAT was not altered (data not shown).

Reciprocal Expression of DGATs Was Not Observed in Hypertrophic 3T3-L1 Cells—If the hypertrophic change per se is the major repressor of DGAT1 in the adipocytes, the reciprocal expression of DGAT1 and DGAT2 would be mimicked also in cultured cells. Hypertrophic change was induced in 3T3-L1 cells by culturing on gelatin-coated culture dishes for a few weeks after induction of differentiation, and the cells contained higher triglyceride levels and exhibited alterations of gene expression, such as increased resistin expression and decreased adiponectin expression, which was reminiscent of in vivo adipocyte hypertrophy (21). At day 17 of differentiation, hypertrophic 3T3-L1 cells showed significantly increased (5.1-fold) triglyceride content and increased DGAT1 and DGAT2 expression, compared with the fully differentiated cells at day 10 (DGAT1: 2.8-fold, DGAT2: 2.8-fold, Fig. 4, a–c), suggesting
that adipocyte non-autonomous mechanism in vivo should be required for the reciprocal regulation of DGAT1 and DGAT2.

Expression of DGATs Was Unaffected by Leptin or Inhibition of Either Isozyme in 3T3-L1 Cells—We hypothesized that systemic interaction among the organs involved with obesity and leptin action in vivo is required for the antithetical regulation of DGAT1 and DGAT2. This hypothesis includes the possibility that leptin may directly increase DGAT1 and suppress DGAT2 expression. However, DGAT1 and DGAT2 showed similar patterns of expression in WAT either from Irs2/H1102/H1102 mice with hyperleptinemia (16) or from ob/ob mice lacking secretion of leptin, which led us to conclude that the reciprocal expression of DGATs is not caused by a direct effect of leptin on white adipocytes. In fact, exposure to leptin did not affect either DGAT1 or DGAT2 expression in 3T3-L1 cells, but 1 nM insulin significantly increased DGAT2 expression. Cyclophilin was used as an internal control. n is 6 per group. c–f, results of RNA interference experiments. DGAT1 (c and e) or DGAT2 (d and f) mRNA was knocked down by siRNA oligonucleotides in 3T3-L1 cells as described under “Experimental Procedures.” Luciferase siRNA was used as an unrelated control. Expression of DGAT1 and DGAT2 was measured by TaqMan real-time PCR. n is 8 per group. Values are means ± S.E. *, p < 0.05. N.S., difference not significant.

Intracerebroventricular Infusion of Leptin Reduced DGAT2 Expression in WAT Independently of DGAT1 Expression—Finally, we hypothesized that DGAT1 and DGAT2 expression in WAT is regulated by leptin action throughout the body. To verify the hypothesis, we investigated the gene expression in the WAT of Irs2/H1102/H1102 mice and ob/ob mice after continuous intracerebroventricular infusion of leptin. Leptin infusion at a rate of 300 ng/h yielded lean Irs2/H1102/H1102 mice and overcame their leptin resistance as previously reported (17), and it reduced DGAT2 mRNA expression by about 60% in the WAT of Irs2/H1102/H1102 mice whereas it significantly increased DGAT1 expression (Fig. 6, a–c). Smaller amount of leptin infusion (10 ng/h) was enough to reduce body weight of ob/ob mice, and it clearly reduced DGAT2 expression by about 40% (saline group:leptin group 1:0.59) whereas DGAT1 expression was unaltered (saline group:leptin group = 1:0.96) (Fig. 6d). These results suggested the hypothesis that central leptin action in vivo is responsible for the expression of DGAT2 in WAT.

DISCUSSION

We have found that adipocyte size is inversely correlated with systemic insulin sensitivity: that larger adipocytes are
associated with insulin resistance, and smaller adipocytes are associated with insulin sensitivity (1, 20). We reported that obesity associated with leptin resistance contributes to insulin resistance of \( I_{rs2} \)/H11002/H11002 mice as well as insulin signaling defects in the liver (17). Because histological analysis revealed larger adipocytes in leptin-resistant \( I_{rs2} \)/H11002/H11002 mice, we concluded that attenuated leptin action may be the link between insulin resistance and adipocyte hypertrophy.

The present study was undertaken to clarify the molecular mechanism of the adipocyte enlargement in leptin-resistant \( I_{rs2} \)/H11002/H11002 mice. We suspected that triglyceride synthesis was increased in \( I_{rs2} \)/H11002/H11002 mice as well as insulin signaling defects in the liver (17). Because histological analysis revealed larger adipocytes in leptin-resistant \( I_{rs2} \)/H11002/H11002 mice, we concluded that attenuated leptin action may be the link between insulin resistance and adipocyte hypertrophy.

The present study was undertaken to clarify the molecular mechanism of the adipocyte enlargement in leptin-resistant \( I_{rs2} \)/H11002/H11002 mice. We suspected that triglyceride synthesis was increased in \( I_{rs2} \)/H11002/H11002 mice, and screening for expression of enzymes involved in the triglyceride synthesis pathway revealed increased expression of DGAT2 in \( I_{rs2} \)/H11002/H11002 mice adipocytes. We further demonstrated that DGAT2 expression was increased when leptin action was attenuated, and that the expression decreased when leptin was administered intracerebroventricularly to downsize the adipocytes, suggesting that DGAT2 is involved in the physiological process of enlarging adipocytes in adult animals. The lipoatrophic phenotypes of \( D_{gat2} \)/H11002/H11002 mice are striking whereas those of \( D_{gat1} \)/H11002/H11002 mice are relatively mild (6, 9). Surprisingly, the lack of DGAT2 causes marked reduction of fatty acid, which is an essential component of triglyceride synthesis (9). Major lipogenic enzymes involved in fatty acid synthesis are localized on endoplasmic reticulum (ER). Human DGAT2 is reported to be expressed on ER (29) and overexpression of mouse DGAT2 induces large cytosolic lipid droplets accumulation whereas overexpression of mouse DGAT1 results in small lipid droplets around the cell periphery (9). In fact, an online program to predict subcellular localization of protein by its amino acid sequence (PSORT II Prediction, psort.nibb.ac.jp/form2.html) calculated the probabilities of mouse DGAT1 and DGAT2 localization as: DGAT1, plasma membrane 52.2%, ER 17.4%, vacuolar 13.0%; DGAT2, ER
55.6%, mitochondrial 11.1%, Golgi 11.1%, nuclear 11.1%, cytoplasmic 11.1%. Proximity of DGAT2 to lipogenic enzymes on ER may influence its accessibility to fatty acid, and increase of DGAT2 expression in enlarged adipocytes may be advantageous to store fatty acid de novo synthesized around ER when leptin action is attenuated.

We previously demonstrated that the SREBP-1 gene and its downstream target genes expression is up-regulated in the liver of Irs2−/− mice and results in fatty liver (16). We attributed this to leptin resistance, which is consistent with the down-stream target genes expression is up-regulated in the leptin-deficient ob/ob mice, suggesting that SREBP-1 does not contribute much to the adipocyte enlargement in ob/ob mice. Recent studies have shown a strong correlation between increased tissue triglyceride content and insulin resistance. Since both leptin resistance in Irs2−/− mice and leptin deficiency in ob/ob mice result in fatty liver in an SREBP-1-dependent manner and adipocyte hypertrophy in a DGAT2-dependent manner, loss of leptin action is considered to be a common mechanism of increased tissue triglyceride content, causing insulin resistance.

Our hypothesis is that leptin resistance provides a link between larger adipocytes and insulin resistance. In fact, mice with various levels of leptin activity can be arrayed according to adipocyte size, e.g. ob/ob mice, diet-induced obese (DIO) mice, WT mice, and transgenic skinny mice overexpressing leptin (30). As leptin activity increases, adipocyte size, which is large in leptin-deficient ob/ob mice, gradually becomes smaller, and ultimately it becomes too small to be seen, as in the leptin transgenic mice. As adipocyte size decreases in association with increased leptin activity, insulin resistance improves. Leptin transgenic skinny mice show insulin-sensitive phenotypes, quite unlike other insulin-resistant lipoatrophic mice with insufficient leptin activity (30). Thus leptin action provides a link between adipocyte size and insulin resistance.

The results of the present study suggest that DGAT2 may provide a molecular link between the loss of leptin action and larger adipocytes in adult mammals. Loss of leptin action or leptin resistance in human obesity may also explain the increased tissue triglyceride contents, as exemplified by fatty liver and larger adipocytes, that is frequently associated with type 2 diabetes.

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