

Functional Analysis of Tumor Necrosis Factor (TNF) Receptors in TNF- α -Mediated Insulin Resistance in Genetic Obesity*

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ABSTRACT

Although obesity has become the most common metabolic disorder in the developed world and is highly associated with insulin resistance and noninsulin-dependent diabetes mellitus, the molecular mechanisms underlying these disorders are not clearly understood. Tumor necrosis factor- α (TNF- α) is overexpressed in obesity and is a candidate mediator of obesity-induced insulin resistance. Complete lack of TNF- α function through targeted mutations in TNF- α gene or both of its receptors results in significant improvement of insulin sensitivity in dietary, chemical, or genetic models of rodent obesity. In this study,

we have analyzed the *in vivo* role of TNF signaling from p55 [TNF receptor (TNFR) 1] and p75 (TNFR 2) TNFR in the development of insulin resistance by generating genetically obese mice (*ob/ob*) lacking p55 or p75 TNFRs. In the *ob/ob* mice, the absence of p55 caused a significant improvement in insulin sensitivity. p75 deficiency alone did not affect insulin sensitivity but might potentiate the effects of p55 deficiency in animals lacking both TNFRs. These results indicate that TNF- α is a component of insulin resistance in the *ob/ob* model of murine obesity and p55 TNFR is the predominant receptor mediating its actions. (*Endocrinology* 139: 4832–4838, 1998)

OBESITY, with an increasing prevalence, has become the most common metabolic disorder in the developed world (1). Currently, 54.4% of U.S. adults are overweight [body mass index (BMI) >25] and 22.5% are obese (BMI >30 kg/m²), representing an 8% increase over 1980 levels (2). Furthermore, obesity is a major risk factor for insulin resistance and noninsulin-dependent diabetes mellitus (NIDDM) as well as hypertension, dyslipidemias, and cardiovascular disease (3). In the United States, approximately 17% of obese and 11% of overweight adults suffer from NIDDM associated with obesity (2, 4). Although it has long been recognized that obesity-induced insulin resistance is one of the critical components of NIDDM (3), the molecular mechanisms linking obesity and insulin resistance have remained elusive.

One molecule with a potential role in obesity-related insulin resistance is tumor necrosis factor- α (TNF- α) (5). TNF- α is overexpressed in adipose tissue in many rodent models of obesity and affects insulin sensitivity (6–11). Complete absence of TNF- α gene or both of its receptors results in significant improvement in insulin sensitivity in mice with dietary, hypothalamic, or genetic obesity (11, 12). In humans, elevated TNF- α expression in adipose and muscle tissue is positively correlated with the degree of obesity and the level of hyperinsulinemia, and negatively related to the adipose tissue lipoprotein lipase activity (13–15). Several recent studies also show that circulating TNF- α levels are increased in obese patients with NIDDM (16, 17) and positively correlate

with serum leptin concentrations, an adipocyte-derived protein of energy homeostasis. Finally, people carrying a *NcoI* polymorphism of the TNF- α gene, which leads to a higher rate of transcription of TNF- α , have higher basal serum insulin levels, decreased insulin sensitivity, and increased percent body fat and serum leptin concentrations (18). Another polymorphism in the 5' region of TNF- α gene has also been shown to correlate with adiposity in the Pima Indian population (19).

The molecular mechanism of TNF- α -induced insulin resistance primarily involves the inhibition of the insulin receptor tyrosine kinase activity in cultured adipocytes, hepatoma cells, fibroblasts, and myeloid 32D cells (10, 20–22) and in whole animals (11, 23). In addition to its direct actions on insulin-sensitive tissues, TNF- α regulates leptin secretion (24–26) and free fatty acid (FFA) release from adipocytes (27). In the absence of TNF- α , there is significant reduction in obesity-induced hyperlipidemia and hyperleptinemia, both of which could contribute to changes in insulin sensitivity (11, 24). In cultured adipocytes, TNF- α could also down-regulate the expression of the insulin-sensitive glucose transporter-4 (GLUT4) and other molecules involved in insulin action (9, 28). However, the *in vivo* relevance of this mechanism in regard to TNF- α -induced insulin resistance is not clear in obese animal models (11).

TNF- α is shown to function through its two transmembrane receptors, p55 and p75 (29, 30). These receptors undergo ligand-induced multimerization and associate with a distinct set of signaling molecules such as TNF receptor-associated death domain protein (TRADD) and TNF receptor-associated factors (TRAFs) (31–35). Although the ligand binding domains of both TNF receptors (TNFR) show a high degree of homology, each have distinct intracellular domains indicating different biological functions. To date, p55 recep-

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tor signaling is demonstrated to be the dominant effector in TNF- α biology including apoptosis, tissue necrosis, and non-specific immunity (29, 33, 36–40). So far, p75 receptor has been implicated in few activities such as thymocyte growth, T cell cytotoxicity, and granulocyte colony-stimulating factor expression (40, 41).

Very little is known about TNF- α signaling in adipocytes, primarily because of the limitations of current experimental systems. *In vitro* studies suggest that both p75 and p55 receptors might mediate TNF- α action on insulin signaling in murine (42) and human (43) adipocytes. Although these studies demonstrated a dominant role for p55, p75 function was also necessary for the inhibition of insulin receptor signaling by TNF- α , especially in human adipocytes (43). Although it has been previously shown that the lack of TNF- α function improves insulin sensitivity in obese animals, no study has demonstrated which TNFR(s) mediates this function *in vivo*. To address this question, we generated lean and obese (*ob/ob*) mice with targeted null mutations in each TNFR. TNF- α signaling through either p55 or p75 has been completely abolished in these animals (44, 45). Analysis of these animals demonstrated that TNF- α action on insulin sensitivity in obesity is mediated predominantly through the p55 TNFR.

Materials and Methods

Generation of *ob/ob*-TNFR-deficient mice

Animals with targeted null mutations at both TNFR1 and TNFR2 loci ($p55^{-/-}$, $p75^{-/-}$) were generated by crossing $p75^{-/-}$ mice (C57BL/6 and 129 mixed background back-crossed into C57BL/6 for two generations) with $p55^{-/-}$ (C57BL/6 background) (44, 45). Obese (*ob/ob*) mice deficient in each TNFR were generated by crossing mice with targeted null mutations at both TNFR1 and TNFR2 loci ($p55^{-/-}$, $p75^{-/-}$) with *Ob/ob* mice (C57BL/6 background), to produce animals heterozygous at the TNFR1, TNFR2, and *ob* loci ($p55^{+/-}$, $p75^{+/-}$, *Ob/ob*). The resulting triple heterozygotes were then crossed with each other to produce *OB/OB* and *ob/ob* littermates with mutations at one or both TNFRs. This same cross also generated *OB/OB* and *ob/ob* wild-type littermates as controls (11). The genetic background of the final progeny of this cross is estimated to be at least 90% C57BL/6 (46). The phenotype of the double mutant animals was previously reported elsewhere (11). All animals that are heterozygous (mutant) at the *ob* locus remained lean throughout the study compared with the wild-type littermates. We therefore, refer to the animals that are not homozygous for the *ob* mutation as lean in the text. All mice that are homozygous for the *ob* mutation (*ob/ob*) developed obesity and hence are referred to as obese in the text. All animal experimentation was carried out in accordance with the NIH guidelines for the care and use of laboratory animals and was approved by an institutional review board.

Analysis of gene expression

Total RNA was extracted from tissue samples by cesium chloride extraction protocol (47), and 30 μ g RNA was used for Northern blot analysis, as described previously (9). Complementary DNA probes for TNF- α , p55, p75, and the 36B4 (gift of Dr. Kevin Claffey, Harvard Medical School, Boston, MA) were radioactively labeled to specific activities of at least 10^9 dpm/ μ g with [32 P] α -deoxycytidine triphosphate (6000 Ci/mmol) by the random priming method, as described previously (9). Differences in loading were adjusted to 18S ribosomal marker 36B4 expression.

Body composition analysis

Whole body composition was determined by digesting mice in alcoholic potassium hydroxide at 60 C until complete saponification (48).

Carcasses were then analyzed for total protein by biuret assay and total glycerol content (GPO-Trinder, Sigma Chemical Co., St. Louis, MO). Fat body mass per lean body mass was calculated as the arbitrary ratio of whole body glycerol (millimoles per liter) over protein (grams per liter). Epididymal fat pads from 20-week-old mice were excised bilaterally and weighed.

Metabolic measurements

Total body weights were measured weekly from age 4–16 weeks. Blood samples were collected after a 6-h daytime fast at 4, 8, and 12 weeks of age. Glucose concentrations in plasma were measured by using glucoanalyzer blood glucose strips (Medisense, Bedford, MA). Serum insulin was measured with a monoclonal antirat insulin RIA (Linco Research, Inc., St. Louis, MO). Glucose and insulin tolerance tests were performed on conscious mice following a 6-h daytime fast. Glucose tolerance tests were done by ip administration of glucose (1.8 mg/g) and measurement of blood glucose at 15, 30, 60, 90, and 120 min from tail blood samples. Insulin tolerance tests were done similarly except for the injection of human insulin (1 IU/kg) (Eli Lilly & Co., Indianapolis, IN) and an additional blood glucose measurement at 45 min.

Results

Generation of TNFR-deficient mice

A total of 1054 progeny were generated from the $p55^{+/-}$, $p75^{+/-}$, *Ob/ob* intercross. The genotype of all animals at all three loci was determined by PCR-based assays. The segregation of alleles in these progeny had a Mendelian distribution (data not shown) demonstrating that the loss of either TNFR resulted in a viable phenotype and did not have any effect on the survival of the *ob/ob* or *OB/OB* mice under laboratory conditions. Analysis of total RNA from the white adipose tissue of obese animals confirmed the lack of TNFR expression in the corresponding mutants (Fig. 1). The absence of one TNFR did not have a significant effect on the expression of the other receptor. The levels of p55 transcript in the obese $p75$ -deficient (*ob/ob*- $p75^{-/-}$) animals were the same as obese wild-type control (*ob/ob*) levels. Likewise, the levels of $p75$ transcript in the obese $p55$ -deficient animals (*ob/ob*- $p55^{-/-}$) were similar to those of the obese wild-type control (*ob/ob*) mice.

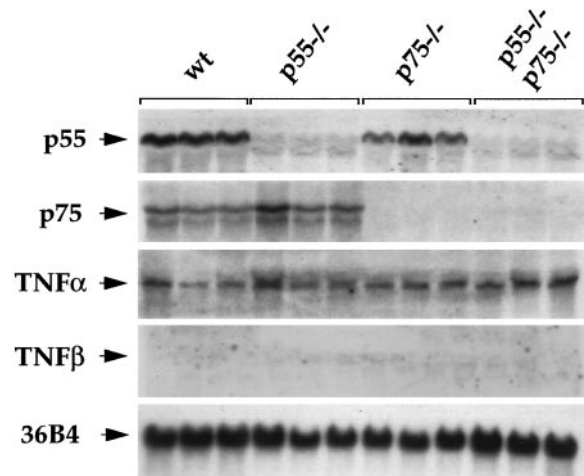


FIG. 1. Northern blot analysis of total RNA from white adipose tissue of *ob/ob*, *ob/ob*- $p55^{-/-}$, *ob/ob*- $p75^{-/-}$, and *ob/ob*- $p55^{-/-}$ $p75^{-/-}$ mice. Blots were hybridized to P^{32} -labeled p55, p75, TNF- α , and 36B4 complementary DNA and were scanned and quantitated by NIH Image 3.01 image analysis program (Bethesda, MD).

Additionally, the GLUT4, peroxisome proliferator-activated receptor- γ , and adiponin transcript levels in the white adipose tissue and GLUT4 transcript levels in the muscle tissue were not altered in obese animals deficient in TNFRs. TNF- β and plasma membrane glycoprotein (PC-1) transcripts in white adipose tissue or TNF- α in muscle tissue were undetectable. All obese animals displayed markedly elevated levels of TNF- α expression in adipose tissue compared with their lean counterparts (data not shown). There was a trend for higher TNF- α messenger RNA levels in all obese receptor-deficient animals, especially in mice lacking both receptors (*ob/ob*-p55^{-/-}-p75^{-/-}) when compared with *ob/ob* animals (Fig. 1).

Growth curves of TNFR-deficient mice

We monitored the growth and total body weight of the entire progeny for 16 weeks (Fig. 2A). No significant difference in body weights was observed between the lean receptor-deficient and wild-type animals throughout the study. In the lean (*OB/OB*) group, the wild-type, p55-deficient (p55^{-/-}), p75-deficient (p75^{-/-}), and p55-p75-deficient (p55^{-/-}-p75^{-/-}) mice weighed 32.1 \pm 0.4, 30.7 \pm 0.7, 30.4 \pm 0.6, and 29.3 \pm 1.0 g, respectively, at week 16. Although the p55^{-/-}-p75^{-/-} lean animals gained less weight than the wild-type controls, this difference was not statistically significant. The intercross between mice heterozygous for both TNFR mutations and the *ob* mutation also generated wild-type, p55^{-/-}, p75^{-/-}, and p55^{-/-}-p75^{-/-} mice, which are homozygous for the *ob* mutation (*ob/ob*). The *ob/ob* animals developed early onset and severe obesity regardless of the TNFR allele they carried (Fig. 2A). Similar to the lean group, no significant difference was observed in the body weights of *ob/ob* animals that were wild type or mutant at TNFR loci (61.6 \pm 0.5, 62.6 \pm 1.1, 58.7 \pm 1.3, and 61.3 \pm 1.9 g for *ob/ob*, *ob/ob*-p55^{-/-}, *ob/ob*-p75^{-/-}, and *ob/ob*-p55^{-/-}-p75^{-/-} mice, respectively). These results indicate that the absence of TNF- α signaling did not significantly affect body weight in either the lean

or obese state (Fig. 2A) in this experimental model within the duration of our analysis. No heterozygosity effects were seen for any of the genes segregating in this cross with regard to total body weight (data not shown).

Body composition analysis and lipid profile

To determine the effect of TNF- α signaling from each of its receptors on adiposity, we determined the epididymal fat pad weights and performed total body composition analysis on both lean and obese animals. In the lean group, the wild-type animals had significantly smaller epididymal fat pads (Fig. 2B) compared with the p55^{-/-}-p75^{-/-} mice (0.55 \pm 0.07 g and 0.78 \pm 0.08 g, respectively). However, in the body composition analysis, fat mass per lean body mass was not significantly different between wild-type and receptor-deficient mice (0.064 \pm 0.009, 0.070 \pm 0.007, 0.060 \pm 0.005, and 0.067 \pm 0.007 mmol glycerol/g protein for the wild-type, p55^{-/-}, p75^{-/-}, and p55^{-/-}-p75^{-/-} mice, respectively). There was no significant difference in fat mass per lean body mass among obese animals (0.209 \pm 0.010, 0.204 \pm 0.013, 0.194 \pm 0.009, and 0.203 \pm 0.015 mmol glycerol/g protein for *ob/ob*, *ob/ob*-p55^{-/-}, *ob/ob*-p75^{-/-}, and *ob/ob*-p55^{-/-}-p75^{-/-} mice, respectively). These results suggest that the lack of either TNFR did not significantly effect the overall adiposity in the *OB/OB* or *ob/ob* mice.

In the obese group, all the genotypes developed a mild hyperlipidemia with increased plasma total triglyceride, glycerol, and FFA levels by 8 and 12 weeks of age compared with the lean animals (data not shown). Within the lean and obese animals, the lack of either or both of the TNFRs did not effect the plasma total triglyceride, glycerol, or FFA levels significantly. Stimulation of lipolysis by the nonspecific β -adrenergic receptor agonist isoproterenol (10 mg/kg) or β_3 -specific agonist CL 316,243 (0.1 mg/kg) in wild-type control and TNFR-deficient animals resulted in similarly elevated plasma glycerol and FFA levels (data not shown). These data indicate that lack of TNFRs did not affect either

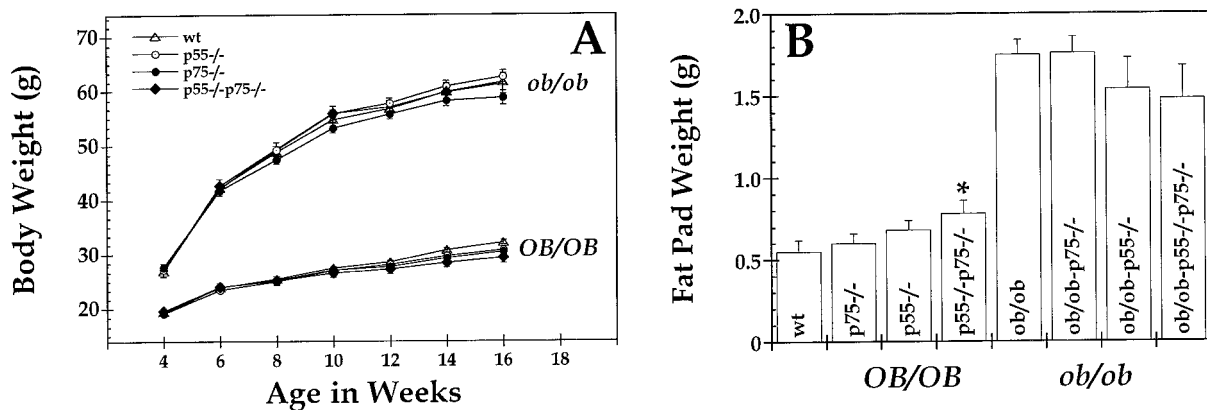


FIG. 2. Body weights of *OB/OB* (lean) and *ob/ob* (obese) wild-type, p55^{-/-}, p75^{-/-}, and p55^{-/-}-p75^{-/-} mice (A). In *OB/OB* group, n = 244, 97, 85, and 24 for wild-type, p55^{-/-}, p75^{-/-}, and p55^{-/-}-p75^{-/-} mice, respectively. In *ob/ob* group, n = 99, 23, 23, 17, for *ob/ob*, *ob/ob*-p55^{-/-}, *ob/ob*-p75^{-/-}, and *ob/ob*-p55^{-/-}-p75^{-/-} animals, respectively. Epididymal fat pad weights of lean (*OB/OB*) and obese (*ob/ob*) wild-type, p55^{-/-}, p75^{-/-}, and p55^{-/-}-p75^{-/-} mice (B). In *OB/OB* group, n = 16, 17, 16, and 10 for wild-type, p55^{-/-}, p75^{-/-}, and p55^{-/-}-p75^{-/-} mice, respectively. In *ob/ob* group, n = 13, 12, 10, and 7 for *ob/ob*, *ob/ob*-p55^{-/-}, *ob/ob*-p75^{-/-}, and *ob/ob*-p55^{-/-}-p75^{-/-} animals, respectively. Epididymal fat pads of 20-week-old male mice were dissected bilaterally and weighed. Comparisons between groups are done by ANOVA (Statview 4.01, Abacus Concepts, Berkeley, CA). Statistical significance is indicated by *, $P < 0.05$. Data are shown as mean \pm SEM.

the steady state lipid profile or β -adrenergic-stimulated lipolysis in *ob/ob* model of obesity.

Glucose homeostasis

To determine the effects of the absence of TNF signaling on glucose homeostasis in obese and lean animals, we measured plasma glucose and insulin concentrations in the TNFR-deficient animals after a 6-h daytime fast. All of the lean animals remained euglycemic during the experimental period, and there was no significant difference in plasma glucose among genotypes with the exception of the *p55*^{-/-}*p75*^{-/-} mice, which tended to have lower plasma glucose concentrations by 8 weeks of age when compared with all other genotypes (11) (12.242 ± 0.300 , 12.015 ± 0.341 , 12.424 ± 0.314 , and 9.739 ± 0.501 mmol/liter for wild-type, *p55*^{-/-}, *p75*^{-/-}, and *p55*^{-/-}*p75*^{-/-}, respectively, $P < 0.05$) (Fig. 3A). The plasma insulin levels of all lean mice also remained within normal boundaries throughout the study [53.058 ± 6.453 , 63.813 ± 10.396 , 67.039 ± 6.812 , and 34.775 ± 2.868

pmol/liter for wild-type, *p55*^{-/-}, *p75*^{-/-}, and *p55*^{-/-}*p75*^{-/-}, respectively, at 8 weeks of age (11)] (Fig. 3B). The lowest plasma insulin concentration among all genotypes however, was also observed in the lean *p55*^{-/-}*p75*^{-/-} mice ($P < 0.05$) (11).

In the obese group, the *ob/ob* animals (wild type at TNFR loci) developed a moderate and transient hyperglycemia by 8 weeks of age (22.527 ± 0.774 mmol/liter), which subsided by 12 weeks of age (Fig. 3A). The *ob/ob-p75*^{-/-} animals displayed an essentially identical pattern of plasma glucose levels (22.937 ± 1.820 mmol/liter at 8 weeks of age) as did the *ob/ob* mice during the entire study period. In contrast, the increases in plasma glucose levels at week 8 in the *ob/ob-p55*^{-/-} were minor (17.567 ± 0.865 mmol/liter) compared with the *ob/ob* and *ob/ob-p75*^{-/-} mice ($P < 0.05$). The plasma glucose levels observed in *ob/ob-p55*^{-/-}*p75*^{-/-} mice [18.568 ± 1.047 mmol/liter (11)] were similar to those observed in *ob/ob-p55*^{-/-} animals. The *ob/ob* animals also displayed a severe and progressive hyperinsulinemia during the course of study (Fig. 3B). The development of hyperinsulinemia in the *ob/ob-p75*^{-/-} animals was essentially identical to the *ob/ob* mice. However, the *ob/ob-p55*^{-/-} mice displayed significantly lower plasma insulin levels (at 12 weeks, 519.45 ± 111.13 pmol/liter) compared with the *ob/ob* (1129.30 ± 193.02 pmol/liter, $P < 0.05$) and *ob/ob-p75*^{-/-} (993.05 ± 190.01 pmol/liter, $P < 0.05$) mice throughout the study (Fig. 3B). Interestingly, the *ob/ob-p55*^{-/-}*p75*^{-/-} animals showed significantly less hyperinsulinemia [329.10 ± 46.61 pmol/liter (11)] than the *ob/ob-p55*^{-/-} mice, indicating that the impact of complete deficiency in TNF- α signaling exceeds that of p55 deficiency, at least as judged by plasma insulin levels.

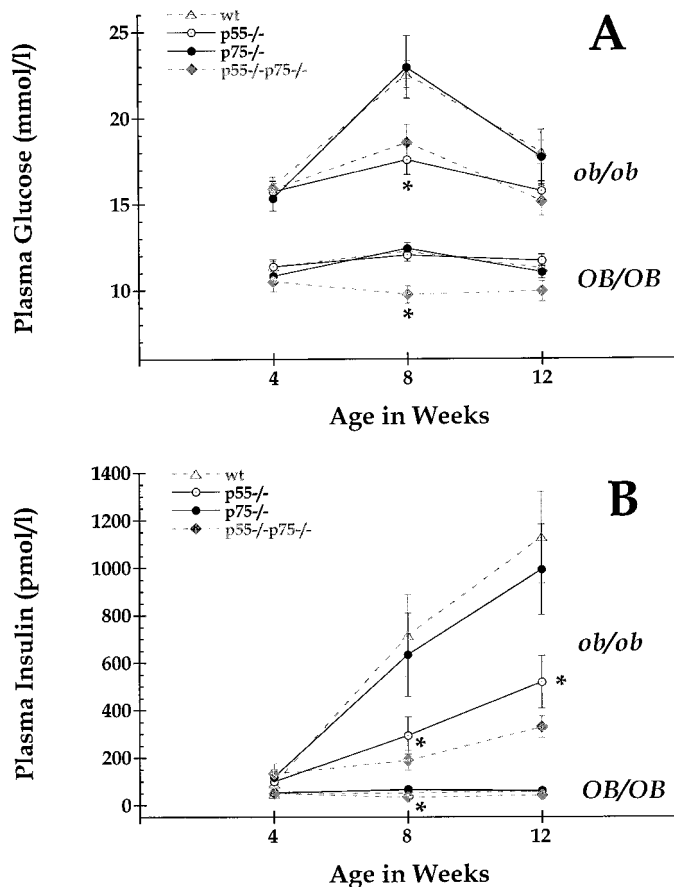


FIG. 3. Glucose homeostasis in *OB/OB* and *ob/ob*, wild-type, *p55*^{-/-}, *p75*^{-/-}, and *p55*^{-/-}*p75*^{-/-} mice during fasting. Glucose (A) and insulin (B) concentrations in plasma were measured, as described in *Materials and Methods*. Comparisons between groups are done by ANOVA. Statistical significance is indicated by *, $P < 0.05$. In *OB/OB* group, $n = 60$, 44 , 40 , and 23 , and in *ob/ob* group, $n = 51$, 23 , 23 , and 17 for wild-type, *p55*^{-/-}, *p75*^{-/-}, and *p55*^{-/-}*p75*^{-/-} mice, respectively. Data are shown as mean \pm SEM. Data for wild-type and *p55*^{-/-}*p75*^{-/-} animals are reprinted by permission from *Nature* (389:610–614). Copyright 1997 Macmillan Magazines Ltd.

Tolerance tests

At the end of the study, insulin sensitivity was determined by performing ip insulin (IITT) and glucose (IGTT) tolerance tests. The deficiency of each receptor alone had no effect on insulin sensitivity in the lean animals (*OB/OB*). However, both of these tests demonstrated increased insulin sensitivity in the *p55*^{-/-}*p75*^{-/-} mice compared with the wild-type animals [Fig. 4 (11)].

IITT and IGTT were also performed in obese (*ob/ob*) animals that were wild-type or mutant at the TNFR loci. The *ob/ob-p55*^{-/-} mice displayed a significantly stronger hypoglycemic response to an ip administered dose of insulin compared with the *ob/ob* and *ob/ob-p75*^{-/-} mice, demonstrating an improvement in insulin sensitivity (Fig. 4). The overall response to insulin in the *ob/ob-p55*^{-/-} mice tended to be smaller than that of the *ob/ob-p55*^{-/-}*p75*^{-/-} animals. Similar results were also obtained in the IGTT (Fig. 4). Following ip administration of glucose, the *ob/ob-p55*^{-/-} animals displayed blood glucose profiles consistent with increased insulin sensitivity relative to the *ob/ob* mice ($P < 0.05$). The response to IGTT in the *ob/ob-p55*^{-/-} mice was again greater than the *ob/ob-p75*^{-/-} but smaller than the *ob/ob-p55*^{-/-}*p75*^{-/-} animals. There was no significant difference between the *ob/ob* and *ob/ob-p75*^{-/-} in both of the tolerance tests (Fig. 4).

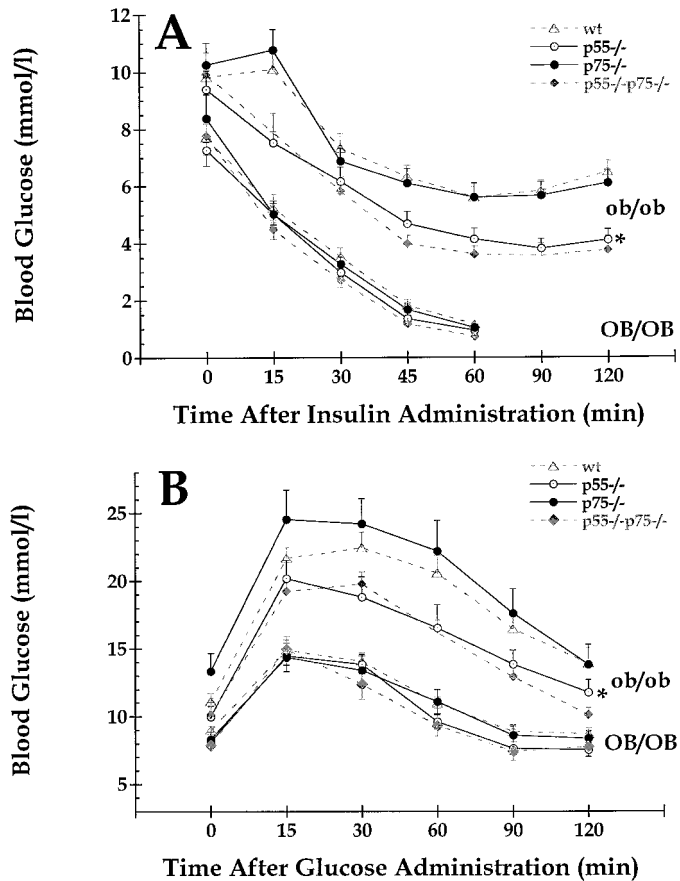


FIG. 4. Insulin (A) and glucose (B) tolerance tests were conducted after a six h fast. Lean (*OB/OB*) and obese (*ob/ob*) control mice (wild type at receptor loci) are compared with lean and obese *p75*^{-/-}, *p55*^{-/-} and *p55*^{-/-}*p75*^{-/-} littermates. Analysis of dynamics of responses to tolerance tests were done by ANOVA repeated measures analysis and statistical significance is indicated by *. In *OB/OB* group, n = 12, 9, 8 and 8, and in *ob/ob* group, n = 41, 23, 23 and 17 for wild type, *p55*^{-/-}, *p75*^{-/-} and *p55*^{-/-}*p75*^{-/-} mice, respectively. Data are shown as mean ± SEM. Data for wild type and *p55*^{-/-}*p75*^{-/-} animals are reprinted by permission from *Nature* (389:610–614) Copyright 1997 Macmillan Magazines Ltd.

Discussion

In addition to its antitumor and proinflammatory actions, TNF- α also modulates adipocyte biology and affects systemic glucose and lipid metabolism (49, 50). These metabolic functions include regulation of lipogenesis and lipolysis and blocking the action of insulin, especially in the context of obesity (5, 11, 49). The general mechanism of TNF- α -induced insulin resistance involves inhibition of insulin receptor signaling as demonstrated in variety of cell types (10, 20–22) and obese animals (11). Interestingly, in cultured 3T3-L1 adipocytes and 32D myeloid cells, antibody-mediated activation of both p55 and p75 receptors decreased insulin receptor signaling, although the role of p55 was significantly stronger than that of p75 (42). Recent experiments using primary human adipocyte cultures have also shown that although TNFR1 specific agonists mimic the inhibitory effects of TNF- α on insulin signaling, these effects can be blocked by an antibody antagonistic to TNFR2 (43) suggesting the involvement of both TNFRs in this activity. Although

the role of TNF- α in insulin action has been demonstrated in several *in vitro* experimental systems, no information exists on the *in vivo* role of each TNFR in this action. In this study, we examined the role of each TNFR in mediating the *in vivo* effects of TNF- α on glucose homeostasis in *OB/OB* and *ob/ob* mice with targeted null mutations in p55 or p75 TNFRs.

Previously, we and others have demonstrated that obese mice with a null mutation in TNF- α have significantly improved insulin sensitivity (11, 12). Furthermore, even in the most severe genetic model of obesity (*ob/ob*), the lack of TNF- α function through targeted mutations in both TNFRs confers a significant but incomplete improvement in insulin sensitivity (11). In this study, we show that the p55 TNFR is primarily responsible for mediating the effects of TNF- α on insulin signaling *in vivo*. Obese mice that lack only the p55 TNFR (*ob/ob-p55*^{-/-}) show an improvement in insulin responsiveness, whereas the p75-deficient animals (*ob/ob-p75*^{-/-}) are indistinguishable from their wild-type (*ob/ob*) littermates. The *ob/ob-p55*^{-/-} mice have lower steady state glucose and insulin levels, indicating improved glucose homeostasis. Furthermore, both the IITT and IGTT demonstrate a significantly increased hypoglycemic response and glucose disposal rate in the *ob/ob-p55*^{-/-} mice, consistent with an improvement in insulin sensitivity. Overall, the increase in insulin sensitivity in *ob/ob-p55*^{-/-} mice approaches that of the *ob/ob-p55*^{-/-}*p75*^{-/-} animals indicating that signaling via p55 TNFR is primarily responsible for TNF- α -mediated insulin resistance in these animals. Because the genetic material of these animals still carry a small portion from the 129 strain, the possibility remains that an unknown gene close to the p55 or p75 loci may be responsible for the phenotype. This possibility seems highly unlikely for several reasons. First, in a smaller breed, the responses to IITT in *ob/ob-p55*^{-/-} mice on a pure C57Bl/6 or C57Bl/6/129 mix (mice used in this report) were indistinguishable (data not shown). Second, two independent strains of obese TNF- α -deficient (TNF- α ^{-/-}) mice also demonstrated a similar improvement in obesity-related insulin resistance as the receptor deficiency (11, 12).

Although the *ob/ob-p75*^{-/-} animals were indistinguishable from the *ob/ob* mice in all of the measures tested, the *ob/ob-p55*^{-/-} animals demonstrated slightly lower insulin sensitivity compared with the *ob/ob-p55*^{-/-}*p75*^{-/-} mice. In light of these data comparing the *ob/ob-p55*^{-/-} to *ob/ob-p55*^{-/-}*p75*^{-/-} animals, it is possible that the p75 receptor does in fact play a smaller role in insulin sensitivity. This might be more critical in human disease, because several studies have implicated TNFR2 in insulin resistance in humans. Adipose tissue TNFR2 (p80 receptor) expression is elevated in obese humans, and the level of expression is related to insulin sensitivity (51). Recently, Eckel *et al.* (43), demonstrated that TNFR2 is essential for TNF- α -induced inhibition of insulin signaling in human adipocytes. It is not clear whether this effect of TNFR2 is mediated by direct signaling through TNFR2 or whether TNFR2 is regulating the rate of association between TNF- α and TNFR1 (ligand passing) as demonstrated by Tartaglia *et al.* (52). Additional experimental models will be necessary to establish the role of p75 TNFR in TNF- α -mediated insulin resistance *in vivo*.

Although deficiency of each receptor alone had no effect on insulin sensitivity in the lean animals (*OB/OB*), increased

insulin sensitivity was observed in mice lacking both receptors (p55^{-/-}p75^{-/-}). The p55^{-/-}p75^{-/-} mice showed both a stronger hypoglycemic response to insulin and a higher glucose disposal rate as well as lower steady state glucose and insulin measurements when compared with the wild-type animals. A similar improvement in insulin sensitivity in the lean state has also been observed in TNF- α -deficient mice (11, 12), indicating that TNF- α might play a role in the physiological control of glucose homeostasis in the lean state.

There is still a significant degree of insulin resistance in the *ob/ob*-p55^{-/-}p75^{-/-} and *ob/ob*-p55^{-/-} animals compared with lean controls, indicating that TNF- α action through the two known TNFRs plays a partial role in mediating the insulin resistance associated with the *ob/ob* phenotype. Previous work in obese mice lacking TNF- α function demonstrated varying degrees of protection from obesity-induced insulin resistance based on the model studied. The lack of TNF- α resulted in a partial protection in mice with hypothalamic obesity, whereas a more significant level of protection was seen in a diet-induced model of obesity (11, 12), suggesting that the choice of experimental model or the severity of the phenotype may effect the outcome. In addition, in this genetic model of obesity, there is no difference in FFA levels between the *ob/ob* and *ob/ob*-p55^{-/-}p75^{-/-} animals described in this study, whereas lower levels of FFA have been observed in obese mice lacking TNF- α in a diet-induced model of obesity. This result is similar to that seen in hypothalamic obesity, where the lack of TNF- α did not result in a significant difference in the plasma FFA levels, although lower plasma triglyceride levels have been observed in lean TNF- α -deficient mice. Therefore, in severe cases of obesity, such as in the *ob/ob* mouse or in goldthio-glucose treatment, it is possible that other factors may overcome the protective effect of TNF- α deficiency. At the moment, it is not clear whether TNF- α deficiency results in different phenotypes based on the etiology of obesity or genetic background or whether it might differ from TNFR deficiency. Further experiments should clarify these questions.

Neutralization attempts using antibodies against TNF- α in several obese rodent models including the *ob/ob* mice have generated conflicting results. For example, the use of a neutralizing antibody has failed to generate significant changes in insulin sensitivity in the *ob/ob* mice (G.S. Hotamisligil, unpublished observations), whereas the genetic absence of TNF- α function significantly improved insulin sensitivity of these animals (this study) (11), as well as animals suffering from dietary or hypothalamic obesity (11, 12). In addition, the use of a synthetic compound that blocks TNF- α production has been effective in increasing insulin sensitivity in obese diabetic yellow KK (KKA^y) mice (53). Taken together, these observations might reflect either the lack of effectiveness of neutralizing antibodies or the duration of the treatments to block TNF- α action, which primarily occurs in an autocrine/paracrine fashion. This might have important implications in human disease, because the use of neutralizing antibodies have also failed to generate effects on the insulin sensitivity of obese individuals with established diabetes (54). These studies also illustrate the need for better characterization of the individuals who are likely to benefit from anti-TNF- α

treatments. If a long-term and highly effective blockade of TNF- α is necessary to prevent its effects on insulin action *in vivo*, alternative modalities might be necessary to definitively address the therapeutic potential of TNF- α in human disease.

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References

- Kuczmarski RJ, Flegal KM, Campbell SM, Johnson CL 1994 Increasing prevalence of overweight among US adults. *JAMA* 272:205–211
- Flegal KM, Carrol MD, Kuczmarski RJ, Johnson CL 1998 Overweight and obesity in the United States: prevalence and trends, 1960–1994. *Int J Obes* 22:39–47
- Olefsky JM, Molina JM 1990 Insulin resistance in man. In: Rifkin H, Porte DJ (eds) *Diabetes mellitus*. 4th ed. Elsevier Science Publishing, New York, pp 121–153
- Rewers M, Hamman RF 1995 Risk factors for non-insulin-dependent diabetes. In: Harris MI, Cowie CC, Stern MP, Boyko EJ, Reiber GE, Bennet PH (eds) *Diabetes in America*, 2nd ed. National Institutes of Health, Bethesda, MD, pp 179–220
- Hotamisligil GS, Spiegelman BM 1994 TNF α : a key component of obesity-diabetes link. *Diabetes* 43: 1271–1278
- Lang CH, Dobrescu C, Bagby GJ 1992 Tumor necrosis factor impairs insulin action on peripheral glucose disposal and hepatic glucose output. *Endocrinology* 130:43–52
- Hofmann C, Lorenz K, Braithwaite SS, Colca JR, Palazuk BJ, Hotamisligil GS, Spiegelman BM 1994 Altered gene expression for tumor necrosis factor- α and its receptors during drug and dietary modulation of insulin resistance. *Endocrinology* 134:264–270
- Hamann A, Benecke H, Le Marchand-Brustel Y, Susulic VS, Lowell BB, Flier JS 1995 Characterization of insulin resistance and NIDDM in transgenic mice with reduced brown fat. *Diabetes* 44:1266–1273
- Hotamisligil GS, Shargill NS, Spiegelman BM 1993 Adipose expression of tumor necrosis factor- α : direct role in obesity-linked insulin resistance. *Science* 259:87–91
- Hotamisligil GS, Murray DL, Choy LN, Spiegelman BM 1994 TNF- α inhibits signaling from insulin receptor. *Proc Natl Acad Sci USA* 91:4854–4858
- Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS 1997 Protection from obesity-induced insulin resistance in mice lacking TNF- α function. *Nature* 389:610–614
- Ventre J, Doebber T, Wu M, Macnaul K, Stevens K, Pasparakis M, Kollias G, Moller DE 1997 Targeted disruption of the tumor necrosis factor- α gene-metabolic consequences in obese and nonobese mice. *Diabetes* 46:1526–1531
- Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM 1995 Increased adipose expression of tumor necrosis factor- α in human obesity and insulin resistance. *J Clin Invest* 95:2409–2415
- Kern PA, Saghizadeh M, Ong JM, Bosch RJ, Deem R, Simolo RB 1995 The expression of tumor necrosis factor in adipose tissue: regulation by obesity, weight loss, and relationship to lipoprotein lipase. *J Clin Invest* 95:2111–2119
- Saghizadeh M, Ong JM, Garvey WT, Henry RR, Kern PA 1996 The expression of TNF α by human muscle: relationship to insulin resistance. *J Clin Invest* 97:1111–1116
- Katsuki A, Sumida Y, Murashima S, Murata K, Takarada Y, Ito K, Fujii M, Tsuchihashi K, Goto H, Nakatani K, Yano Y 1998 Serum levels of tumor necrosis factor- α are increased in obese patients with noninsulin-dependent diabetes mellitus. *J Clin Endocrinol Metab* 83:859–862
- Pfeiffer A, Janott J, Mohlig M, Ristow M, Rochlitz H, Busch K, Schatz H, Schifferdecker E 1997 Circulating tumor necrosis factor alpha is elevated in male but not in female patients with type II diabetes mellitus. *Horm Metab Res* 29:111–114
- Fernandez-Real JM, Gutierrez C, Ricart W, Casamitjana R, Fernandez-Castanar M, Vendrell J, Richart C, Soler J 1997 The TNF- α gene Nco I polymorphism influences the relationship among insulin resistance, percent body fat, and increased serum leptin levels. *Diabetes* 46:1468–1472
- Norman RA, Bogardus C, Ravussin E 1995 Linkage between obesity and a marker near the tumor necrosis- α locus in Pima Indians. *J Clin Invest* 96:158–162
- Feinstein R, Kanety H, Papa MZ, Lunenfeld B, Karasik A 1993 Tumor necrosis factor- α suppresses insulin-induced tyrosine phosphorylation of insulin receptor and its substrates. *J Biol Chem* 268:26055–26058
- Kroder G, Bossenmaier B, Kellerer M, Capp E, Stoyanov B, Mühlhölfer A, Berti L, Horikoshi H, Ullrich A, Haring H 1996 Tumor necrosis factor- α - and

- hyperglycemia-induced insulin resistance: evidence for different mechanisms and different effects on insulin signaling. *J Clin Invest* 97:1471–1477
22. Hotamisligil GS, Peraldi P, Budavari A, Ellis R, White MF, Spiegelman BM 1996 IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF- α - and obesity-induced insulin resistance. *Science* 271:665–668
 23. Hotamisligil GS, Budavari A, Murray DL, Spiegelman BM 1994 Reduced tyrosine kinase activity of the insulin receptor in obesity-diabetes: central role of tumor necrosis factor- α . *J Clin Invest* 94:1543–1549
 24. Kirchgessner TG, Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS 1997 TNF alpha contributes to obesity related hyperleptinemia by regulating leptin release from adipocytes. *J Clin Invest* 100:2777–2782
 25. Sarraf P, Frederich RC, Turner EM, Ma G, Jaskowiak NT, Rivet III DJ, Flier JS, Lowell BB, Fraker DL, Alexander HR 1997 Multiple cytokines and acute inflammation raise mouse leptin levels: potential role in inflammatory anorexia. *J Exp Med* 185:171–175
 26. Grunfeld C, Zhao C, Fuller J, Pollock A, Moser A, Friedman J, Feingold KR 1996 Endotoxin and cytokines induce expression of leptin, the ob gene product, in hamsters. A role for leptin in the anorexia of infection. *J Clin Invest* 97:2152–2157
 27. Souza SC, Yamamoto MT, Franciosa MD, Lien P, Greenberg AS 1998 BRL 49653 blocks the lipolytic actions of tumor necrosis factor- α . *Diabetes* 47:691–695
 28. Stephens JM, Pekala PH 1991 Transcriptional repression of the GLUT4 and C/EBP genes in 3T3-L1 adipocytes by tumor necrosis factor- α . *J Biol Chem* 266:21839–21845
 29. Tartaglia LA, Goeddel DV 1992 Two TNF receptors. *Immunol Today* 13:151–153
 30. Smith CA, Farrah T, Goodwin RG 1994 The TNF receptor superfamily of cellular and viral proteins: activation, costimulation and death. *Cell* 76:959–962
 31. Rothe M, Wong SC, Henzel WJ, Goeddel DV 1994 A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. *Cell* 78:681–692
 32. Rothe M, Sarma V, Dixit VM, Goeddel DV 1995 TRAF2-mediated activation of NF- κ B by TNF receptor 2 and CD40. *Science* 269:1424–1427
 33. Hsu H, Xiong J, Goeddel DV 1996 The TNF receptor 1-associated protein TRADD signals cell death and NF- κ B activation. *Cell* 81:495–504
 34. Hsu H, Huang J, Shu HB, Baichwal V, Goeddel DV 1996 TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. *Cell* 4:387–396
 35. Hsu H, Shu HB, Pan MG, Goeddel DV 1996 TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell* 84:299–308
 36. Pfeffer K, Matsuyama T, Kundig TM, Wakeham A, Kishihara K, Shahinian A, Wiegmann K, Ohashi PS, Kronke M, Mak TW 1993 Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to *L. monocytogenes* infection. *Cell* 73:457–467
 37. Erickson SL, de Sauvage FJ, Carver-Moore K, Pitts-Meek S, Gillett N, Sheehan KCF, Schreiber RD, Goeddel DV, Moore MW 1994 Decreased sensitivity to tumor necrosis factor but normal T cell development in TNF receptor-2-deficient mice. *Nature* 372:560–563
 38. Tartaglia LA, Ayres TM, Wong GHW, Goeddel DV 1993 A novel domain within the 55 kD TNF receptor signals cell death. *Cell* 74:845–853
 39. Evans TJ, Moyes D, Carpenter A, Martin R, Loetscher H, Lesslauer W, JC 1994 Protective effect of 55- but not 75-kD soluble tumor necrosis factor receptor-immunoglobulin G fusion proteins in an animal model of gram-negative sepsis. *J Exp Med* 180:2171–2179
 40. Baker SJ, Reddy P 1996 Transducers of life and death: TNF receptor superfamily and associated proteins. *Oncogene* 12:1–9
 41. Zheng L, Fisher G, Miller RE, Peschon J, Lynch D, Lenardo MJ 1995 Induction of apoptosis in mature T cells by tumor necrosis factor. *Nature* 377:348–351
 42. Peraldi P, Hotamisligil GS, Buurman WA, White MF, Spiegelman BM 1996 Tumor necrosis factor (TNF)- α inhibits insulin signaling through stimulation of the p55 TNF receptor and activation of sphingomyelinase. *J Biol Chem* 271:13018–13022
 43. Liu LS, Spelleken M, Röhrig K, Hauner H, Eckel J 1998 Tumor necrosis factor- α acutely inhibits insulin signaling in human adipocytes. *Diabetes* 47:515–522
 44. Bruce AJ, Boling WB, Kindy MS, Peschon J, Kraemer PJ, Carpenter MK, Holtzberg FW, Mattson MP 1996 Altered neuronal and microglial responses to excitotoxic and ischemic brain injury in mice lacking TNF receptors. *Nature Med* 2:788–794
 45. Peschon JJ, Torrance DS, Stocking KL, Glaccum MB, Otten C, Willis CR, Charrier K, Morrissey PJ, Ware CB, Mohler KM 1998 TNF receptor-deficient mice reveal divergent roles for p55 and p75 in several models of inflammation. *J Immunol* 160:943–952
 46. Green EL 1981 Mating systems. In: *Genetics and probability in animal breeding experiments*. Oxford University Press, New York, pp 114–185
 47. Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ 1979 Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18:5294–5299
 48. Salmon DMW, Flatt JP 1985 Effect of dietary fat content on the incidence of obesity among ad libitum fed mice. *Int J Obes* 9:443–449
 49. Grunfeld C, Feingold KR 1991 The metabolic effects of tumor necrosis factor and other cytokines. *Biotherapy* 3:143–158
 50. Beutler B, Cerami A 1989 The biology of cachectin/TNF- α primary mediator of the host response. *Ann Rev Immunol* 7:625–655
 51. Hotamisligil GS, Arner P, Atkinson RL, Spiegelman BM 1997 Differential regulation of the p80 tumor necrosis factor receptor in human obesity and insulin resistance. *Diabetes* 46:451–455
 52. Tartaglia LA, Pennica D, Goeddel DV 1993 Ligand passing: The 75-kDa tumor necrosis factor (TNF) receptor recruits TNF for signaling by the 55-kDa TNF receptor. *J Biol Chem* 268:18542–18548
 53. Morimoto Y, Nishikawa K, Ohashi M 1997 KB-R7785, a novel matrix metalloproteinase inhibitor, exerts its antidiabetic effect by inhibiting tumor necrosis factor- α production. *Life Sci* 61:795–803
 54. Ofei F, Hurel S, Newkirk J, Sopwith M, Taylor R 1996 Effects of an engineered human anti-TNF- α antibody (CDP571) on insulin sensitivity and glycemic control in patients with NIDDM. *Diabetes* 45:881–885