

Obesity and Diabetes in TNF- α Receptor-deficient Mice

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Abstract

TNF- α may play a role in mediating insulin resistance associated with obesity. This concept is based on studies of obese rodents and humans, and cell culture models. TNF elicits cellular responses via two receptors called p55 and p75. Our purpose was to test the involvement of TNF in glucose homeostasis using mice lacking one or both TNF receptors. C57BL/6 mice lacking p55 (p55^{-/-}), p75, (p75^{-/-}), or both receptors (p55^{-/-}p75^{-/-}) were fed a high-fat diet to induce obesity. Marked fasting hyperinsulinemia was seen for p55^{-/-}p75^{-/-} males between 12 and 16 wk of feeding the high-fat diet. Insulin levels were four times greater than wild-type mice. In contrast, p55^{-/-} and p75^{-/-} mice exhibited insulin levels that were similar or reduced, respectively, as compared with wild-type mice. In addition, high-fat diet-fed p75^{-/-} mice had the lowest body weights and leptin levels, and improved insulin sensitivity. Obese (*db/db*) mice, which are not responsive to leptin, were used to study the role of p55 in severe obesity. Male p55^{-/-}*db/db* mice exhibited threefold higher insulin levels and twofold lower glucose levels at 20 wk of age than control *db/db* expressing p55. All *db/db* mice remained severely insulin resistant based on fasting plasma glucose and insulin levels, and glucose and insulin tolerance tests. Our data do not support the concept that TNF, acting via its receptors, is a major contributor to obesity-associated insulin resistance. In fact, data suggest that the two TNF receptors work in concert to protect against diabetes. (*J. Clin. Invest.* 1998. 102:402–411.) Key words: insulin resistance • hyperglycemia • leptin • diet • cytokine

Introduction

Non-insulin-dependent diabetes mellitus (NIDDM)¹ is prevalent in Western societies, affecting over 5% of the United States population (1). NIDDM is the result of peripheral insu-

lin resistance, hepatic glucose overproduction, and impaired pancreatic insulin secretion (1–3). In addition, obesity is a common feature of NIDDM and a risk factor for its development. Genetic loci have been implicated in NIDDM (4–7), but actual genes in humans have yet to be identified.

Because the etiology of NIDDM includes the development of insulin resistance, genes contributing to this feature remain candidates for NIDDM. There is evidence that TNF- α plays a key role in mediating insulin resistance as a result of obesity (8–13). In obese humans (11, 12, 14) and numerous rodent models of obesity–diabetes syndromes (8, 15), there is a marked elevation in muscle and adipose TNF production, as compared with tissues from lean individuals. TNF levels can be reduced with weight loss (11, 12, 14) or after treatment with the insulin-sensitizing agent pioglitazone (15). Further, substantial improvements in insulin sensitivity were demonstrated in fatty rats (*fa/fa*) after neutralization of TNF by intravenous administration of a soluble TNF receptor–immunoglobulin G chimeric protein (8, 9). In contrast, treatment of NIDDM human subjects with an antibody specific for TNF had no effect on insulin sensitivity (16). Insulin resistance can be directly induced in cell culture or whole animals by treatment with TNF (10, 17, 18). Furthermore, there is evidence that TNF participates in dysregulation of hepatic glucose output (18, 19) and inhibits glucose-induced insulin secretion (20–22). Thus, these data provide a strong rationale for studying the mechanisms involved in TNF and obesity–diabetes syndromes (23).

TNF elicits cellular responses via two receptors: p55 and p75. The receptors are expressed in relatively different quantities on nearly all mammalian cells. In inflammation, the receptors activate both unique and synergistic responses (24–28). p55 can participate in all TNF-associated activities involving differentiation, proliferation, and apoptosis (29). Studies of receptor-deficient mice have established that p55 mediates the lethal effects of endotoxin and maintains resistance to bacterial infection (25, 26). p75 mediates tissue necrosis and promotes lymphocyte proliferation (28). The roles of each receptor in lipid metabolism and glucose transport are currently being investigated. For instance, TNF inhibits LPL gene transcription in cultured adipocytes via p55 (30). Activation of p55 in vitro results in impaired insulin-mediated glucose uptake (31). However, production of p75, but not p55, is increased in adipose tissue from human obese subjects (32). Thus, both receptors may be needed for normal glucose homeostasis with singular roles occurring during imbalanced states of obesity and insulin resistance.

Several laboratories have begun using mouse models to study the role of TNF in the development of insulin resistance (33, 34). Mice lacking expression of TNF ligand, which were made obese by gold thioglucose (GTG) injection, exhibited small but significant reductions in fasting plasma glucose levels, fed plasma insulin levels, and improved insulin responses to an oral glucose tolerance test as compared with obese wild-type mice, suggesting that TNF contributes modestly to reducing insulin sensitivity (33). Mice lacking TNF have also been

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Received for publication 20 January 1998 and accepted in revised form 2 June 1998.

1. Abbreviations used in this paper: GTG, gold thioglucose injection; i.p., intraperitoneally; NIDDM, non-insulin-dependent diabetes mellitus.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.
0021-9738/98/07/0402/10 \$2.00

Volume 102, Number 2, July 1998, 402–411

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induced to obesity by high-fat diet feeding (34). Although none of the diet-fed mice reached the extent of insulin resistance as seen for the gold thioglucose-injected animals, small improvements in insulin sensitivity were demonstrated in the TNF-deficient mice. In addition, obese (*ob/ob*) mice deficient in both TNF p55 and p75 receptors exhibited a transient improvement in fasting plasma glucose levels, and insulin levels were reduced by twofold as compared with *ob/ob* mice expressing receptors (34). Small but significant improvements in insulin resistance were seen based on glucose and insulin tolerance tests, but responses to these tests were still impaired as compared with the levels observed in lean mice, demonstrating that other factors besides TNF are playing a role in impaired glucose homeostasis observed in the *ob/ob* mouse.

In this report, we use a diet-induced model of obesity and *db/db* mice to test the hypothesis that TNF receptors contribute to obesity and insulin resistance. C57BL/6 wild-type, p55^{-/-}, p75^{-/-}, and p55^{-/-}p75^{-/-} mice were fed a high-fat diet known to elicit obesity and diabetes in C57BL/6 mice (35, 36). Mice lacking p55 receptors and homozygous for the leptin receptor mutation (*db/db*) were generated and compared with lean controls. Overall, our data did not support the concept that TNF, acting via its receptors, is a major contributor to obesity-associated insulin resistance. In fact, high-fat diet-fed p55^{-/-}p75^{-/-} mice exhibited marked fasting hyperinsulinemia, and glucose tolerance and insulin sensitivity were not improved in this strain as compared with wild-type animals. The data suggest that the two TNF receptors work in concert to protect against diabetes.

Methods

Animals and diets. C57BL/6CR (C57BL/6) male and female mice, 6–8 wk of age, were purchased from Charles River Laboratories (Wilmington, MA). Mice lacking either TNF receptor p55 (p55^{-/-}), p75 (p75^{-/-}), or both p55 and p75 (p55^{-/-}p75^{-/-}) have been described previously (37). The p55^{-/-} mice used in this study were developed directly in C57BL/6CR and are an inbred strain. The p75^{-/-} and double receptor-deficient animals represent, respectively, five and four backcrosses onto C57BL/6 as described (37).

C57BL/KsJ-m^{+/+}*db* (*db/db*) female mice, 6–8 wk of age, were purchased from the Jackson Laboratory (Bar Harbor, ME). Six of these females were crossed to six p55^{-/-} male mice, and progeny were randomly interbred for four to seven generations. During the initial breeding period, the *db* gene had not yet been cloned and, thus, selection for mice carrying the *db* allele was done by phenotyping the subsequent litter derived from candidate pups. Mouse lines from parents carrying at least one allele for p55^{-/-} which generated *db/db* progeny were interbred. Subsequently, direct typing for the *db* mutation was used to select progeny for further breeding using mutagenic PCR (38, 39). During this process we have created a new outbred population in which background alleles constitute an assortment of alleles from C57BL/KsJ and C57BL/6. This is a strength in that important effects due to p55 should be evident regardless of genetic background. One caveat is that genes tightly linked to *db* and p55 loci will likely reflect the alleles of the original parental strains (C57BL/KsJ and C57BL/6, respectively).

Only littermates were used for the *db/db* study. Because *db/db* mice are sterile, mice for this study were generated by intercrossing mice that were heterozygous for *db* and either heterozygous or homozygous for the p55-null allele. The genotype of lean mice were ^{+/+}*db* or ^{+/+} at the *db* locus, and varied in genotype at the p55 locus. Obese mice always showed homozygosity for *db*, but varied in genotype at the p55 locus. Data for mice heterozygous for p55 (p55^{-/-}) were com-

bined with data for mice homozygous for the wild-type p55 allele since these mice did not differ significantly in any of our parameters nor in separate investigations of immunological status (37).

Mice were maintained in a temperature-controlled (25°C) facility with a strict 12-h light/dark cycle and were given free access to food and water. Mice were fed pelleted rodent chow (Wayne Rodent BLOX 8604; Teklad, Madison, WI), which is low in fat (4% w/w) unless otherwise indicated. For the diet-induced obesity study, mice were fed either rodent chow diet or a high-fat diet (Diet #F1850; Bioserv Industries, Inc., Frenchtown, NJ), which contained 35.5% (w/w) fat (primarily lard) and 36.6% carbohydrate (primarily sucrose). This diet has been shown to induce obesity and diabetes in C57BL/6 mice (35, 36). Unless otherwise noted, food was removed from mice 4 h before the collection of blood from the retroorbital sinus into tubes containing anticoagulant (1 mM EDTA). Plasma was used immediately or stored at -70°C until analysis. Mice were killed by cervical dislocation. This project was approved by the Animal Care and Use Committee of the University of Washington (Protocol No. 2140-07).

Genomic PCR. Genotype analyses for alleles at p55, p75, and leptin receptor loci were performed using PCR. The PCR reaction to differentiate p55 wild-type and homologous mutation alleles contained ~ 100 ng DNA, 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM MgCl₂, 0.2 mM dNTP, 1 μM of primers, 2.5 U of Taq polymerase, and sterile dH₂O was added to a final volume of 50 μl. After an initial incubation for 5 min at 94°C, samples were incubated at 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min for 35 cycles, with a final incubation at 72°C for 5 min and 4°C for 10 min. Each reaction contained four primers: P60B (5'-GGATTGTCACGGTGCCTTGAAG-3'), P60E (5'-TGACAAGGACACGGTGTGTGGC-3'), PGK5-66 (5'-CCG-GTGGATGTGGAATGTGTG-3'), and P60-SPE (5'-TGCTGAT-GGGGATACATCCATC-3') that allowed differentiation of wild-type (120 bp) and p55-null (155 bp) alleles using 3% agarose gels (Cat. #A-6013; Sigma Chemical Co., St. Louis, MO).

PCR reactions for p75 alleles contained ~ 100 ng DNA, 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM MgCl₂, 0.2 mM dNTP, 2.5 U of Taq polymerase, and 1 μM of each primer (PGK5-66, p80-Kas, and p80i-1). The samples were incubated at 94°C for 1 min, 65°C for 1 min, and 72°C for 30 s for 35 cycles. Primer sequences are: p80-Kas (5'-AGAGCTCCAGGCACAAGGGC-3'), and p80i-1 (5'-AACGGGCCAGACCTCGGGT-3'). These primers allowed identification of wild-type (275 bp) and p75-null (160 bp) alleles using 3% agarose gels.

PCR reactions for the leptin receptor locus used two primers: db-F1 (5'-AGAACGGACACTCTTTGAAGTCTC-3') and db-R (5'-CATCAAAACCATAGTTTAGGTTTGTGT-3'). Reaction conditions were similar to those given above for p55. Samples were incubated at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 40 cycles. Approximately 5 μl of the PCR product was digested with 1 U of RsaI restriction enzyme at 37°C for 1–3 h. Digests were run on 3% agarose gels containing 1% low melting agarose. The *db* allele is digested yielding fragments of 108 and 27 bp, whereas the wild-type allele produces an uncut fragment of 135 bp.

Plasma insulin, leptin, and glucose analyses. Glucose levels were determined colorimetrically (Trinder glucose 500 Diagnostic Kit; Sigma Diagnostics, St. Louis, MO). Plasma insulin was measured using a radioimmunoassay kit (No. RI-13K; Linco Inc., St. Louis, MO) using rat insulin as the standard. Plasma leptin was measured using a mouse leptin radioimmunoassay kit (No. ML 82K; Linco Inc.).

Insulin sensitivity assay. Insulin sensitivity was evaluated essentially as described (36). Mice were fed the chow diet or the high-fat diet, fasted for 4 h, and injected intraperitoneally (i.p.) with pork insulin (Eli Lilly Inc., Indianapolis, IN) at 1 U per kg body weight. Insulin was diluted in sterile saline for a final injected volume of ~ 100 μl. Plasma was collected for glucose quantification before injection and at 15, 30, and 90 min after insulin injection. For the genetic study, lean and *db/db* mice, 18-wk-old, were fasted 4 h and then injected with either 1 U/kg or 1,000 U/kg body weight.

Insulin dose response. Wild-type and p55^{-/-}p75^{-/-} male mice

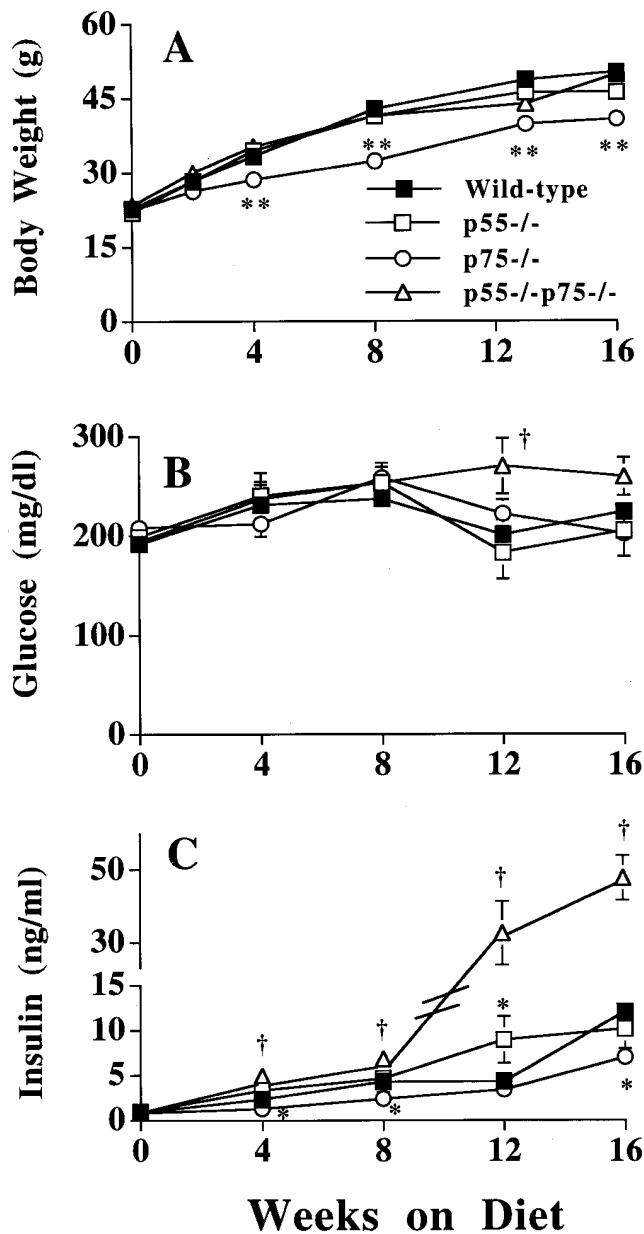


Figure 1. Body weights (A), and plasma glucose (B) and insulin concentrations (C) for male C57BL/6 (*wild-type*) ($n = 10-15$) and TNF receptor-deficient ($n = 5-15$) mice fed a high-fat diet for 16 wk as described in the text. Mice were fasted for 4 h before obtaining blood

were fed rodent chow or high-fat diets for 12 wk and fasted for 18 h. Mice were injected i.p. with 1, 2, or 5 U/kg insulin and plasma glucose levels, quantified immediately before and at 30 min after insulin injection.

Intraperitoneal glucose tolerance test. Mice were fed rodent chow or high-fat diet, fasted overnight (18 h), and injected i.p. with 10% glucose in sterile saline at a dose of 2 g glucose/kg body weight. Plasma was collected for glucose quantification before injection and at 15, 30, 120, and 240 min after injection. Insulin levels were evaluated in plasma taken before and 15 min after glucose injection. For the genetic study, lean and *db/db* mice, 12-wk-old, were fasted overnight before glucose injection. Glucose levels were monitored at 0, 15, 30, 60, and 120 min after injection.

Statistical analysis. Values are reported as mean \pm SEM. ANOVA analyses were used to determine interactions and Tukey post hoc tests were applied to determine differences between means. In some cases, the Student's *t* test was used to compare independent means. Pearson correlation analyses were performed to evaluate relationships between measured parameters. $P < 0.05$ was accepted as statistically significant.

Results

Diet study. C57BL/6 *wild-type* and TNF receptor-deficient mice (*p55*^{-/-}, *p75*^{-/-}, *p55*^{-/-}*p75*^{-/-}) of both genders were fed rodent chow or a high-fat diet for 16 wk. Body weights and plasma glucose, insulin, and total leptin concentrations were determined. Sensitivities of these mice to injections of glucose and insulin were also evaluated.

Among male mice fed rodent chow, no differences in body weights, glucose, or insulin levels were observed between genotypes (data not shown). Feeding the high-fat diet to male mice resulted in increases in body weight, and plasma glucose, insulin, and leptin levels that were strain specific. First, although *wild-type*, *p55*^{-/-}, and *p55*^{-/-}*p75*^{-/-} mice showed similar body weight gains throughout the 16-wk feeding study, *p75*^{-/-} mice consistently gained less weight than *wild-type* mice (Fig. 1 A). Second, at 12 and 16 wk of high-fat diet feeding, plasma glucose and insulin levels were elevated in double receptor-deficient mice, as compared with *wild-type*, *p55*^{-/-}, and *p75*^{-/-} mice (Fig. 1, B and C). As shown in Table I, elevations in insu-

for quantification of glucose and insulin. * $P < 0.05$ and ** $P < 0.001$ indicate significant differences between *p75*^{-/-} or *p55*^{-/-} and *wild-type* mice, and † $P < 0.05$ indicates significant differences between *p55*^{-/-}*p75*^{-/-} and *wild-type* mice by two-way ANOVA.

Table I. Effect of Fasting Time on Glucose, Insulin, and Leptin Levels in Male Mice Fed the High-fat Diet for 16 wk

Genotype	Glucose (mg/dl)		Insulin (ng/ml)		Leptin (ng/ml)	
	4 h	18 h	4 h	18 h	4 h	18 h
Wild-type	224 \pm 9	190 \pm 9*	12.0 \pm 1.6 ^a	3.6 \pm 0.6 ^{a,b*}	138 \pm 21 ^a	126 \pm 11 ^a
<i>p55</i> ^{-/-}	205 \pm 26	158 \pm 28	10.1 \pm 2.2 ^a	2.7 \pm 0.5 ^{a*}	89 \pm 11 ^{a,b}	84 \pm 16 ^b
<i>p75</i> ^{-/-}	202 \pm 23	144 \pm 19	7.0 \pm 1.1 ^a	1.9 \pm 0.5 ^{a*}	68 \pm 6 ^b	27 \pm 2 ^{c**}
<i>p55</i> ^{-/-} <i>p75</i> ^{-/-}	259 \pm 16	145 \pm 23*	47.7 \pm 11 ^b	5.4 \pm 1.0 ^{b*}	84 \pm 25 ^{a,b}	80 \pm 7 ^{b*}

Values are presented as the mean \pm SEM for $n = 5$. Letters are used to denote the presence of significant differences between genotypes within each parameter (glucose, insulin, or leptin) and time group. Values that share at least one letter are not significantly different. Values with different letters indicate $P < 0.05$ between genotypes. * $P < 0.05$ versus 4-h fast; ** $P < 0.001$ versus 4-h fast.

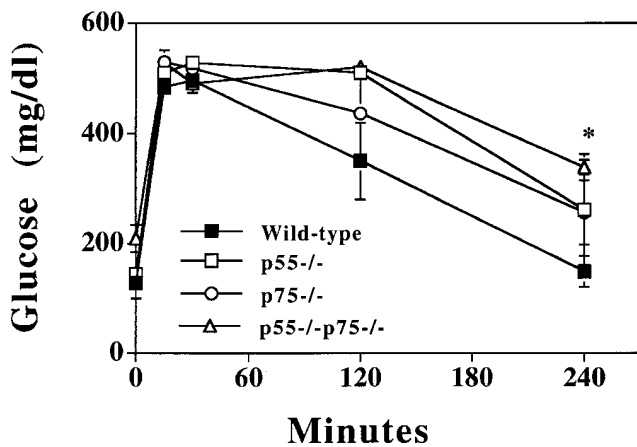


Figure 2. Intraperitoneal glucose tolerance test for male C57BL/6 (wild-type) and TNF receptor-deficient mice ($n = 5$) fed a high-fat diet for 6 wk. Mice were fasted overnight (18 h) before intraperitoneal glucose injection at a dose of 2 g/kg body weight. * $P < 0.05$ indicates a significant difference between $p55^{-/-}p75^{-/-}$ and wild-type mice at 240 min by two-way ANOVA.

lin and glucose were seen for double receptor-deficient mice under conditions of moderate fasting (4 h) and semi-starvation (18 h) (40). In contrast to double receptor-deficient mice, the $p75^{-/-}$ mice displayed reduced insulin levels as compared with wild-type mice (Fig. 2 C; Table I). Third, the levels of circulating leptin were modulated by TNF receptor genotype. Male mice fed chow exhibited a twofold range in plasma leptin levels after fasting for 4 h (13–27 ng/ml) and 18 h (9–21 ng/ml), and the lowest levels were seen for $p75^{-/-}$ at both fasting times (e.g., $P < 0.03$ between $p75^{-/-}$ and wild-type mice at 4 h, $n = 5$). Upon feeding the high-fat diet, plasma leptin levels were increased 6–10-fold (Table I). Among strains lacking one or both

TNF receptors, leptin levels were lower than in wild-type mice by 35–50% (Table I). High-fat fed $p75^{-/-}$ were the only mice to display a decrease in leptin levels with increasing fasting time.

Chow-fed male mice showed no differences in glucose disposal profiles after glucose injection among all the TNF receptor genotypes (data not shown). For male mice fed the high-fat diet for 6 wk, wild-type mice tended to show the most efficient clearance of injected glucose (Fig. 2). Lack of both TNF receptors resulted in the poorest clearance at 240 min, which was significant as compared with wild-type males (338 mg/dl versus 149 mg/dl, $P < 0.003$).

Insulin sensitivity was examined in several separate experiments. First, insulin sensitivity assays were performed on chow and high-fat fed mice of all four genotypes. Mice were fed diets for 16 wk and fasted for 4 h before i.p. insulin injection and glucose monitoring over 90 min. Among male mice fed rodent chow, $p55^{-/-}$ and $p55^{-/-}p75^{-/-}$ mice were unresponsive to an insulin dose of 1 U/kg, but wild-type and $p75^{-/-}$ mice showed a reduction in plasma glucose that reached 50% of initial levels by 30 min (data not shown). All genotypes were unresponsive to the 1 U/kg insulin dose after high-fat diet feeding (data not shown). Second, wild-type and $p55^{-/-}p75^{-/-}$ mice were further evaluated under conditions of reduced circulating insulin obtained after an 18-h fast (e.g., Table I). Chow-fed animals of both strains reduced plasma glucose ~ 44 and 65% after insulin injections of 1 and 2 U/kg, respectively (Fig. 3). In contrast, responses were blunted in high-fat fed mice. In particular, the double receptor-deficient mice showed no response to 1 U/kg and responses were lower than wild-type animals at insulin doses of 2 and 5 U/kg, suggesting that loss of both TNF receptors resulted in significantly more insulin resistance as compared with wild-type mice.

For female mice fed rodent chow, no differences in body weights or diabetogenic parameters were seen among the genotypes (data not shown). Upon feeding the high-fat diet for

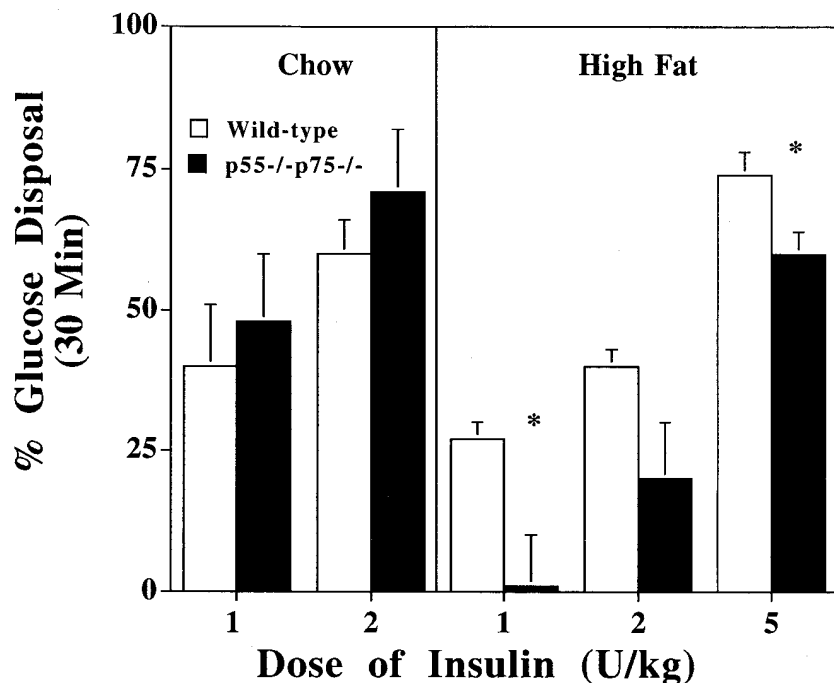


Figure 3. Insulin dose response assay. Male C57BL/6 (wild-type) and $p55^{-/-}p75^{-/-}$ mice were fed rodent chow or a high-fat diet for 12 wk and fasted overnight before intraperitoneal insulin injection. Insulin was injected at a dose of 1 and 2 U/kg for mice fed rodent chow and 1, 2, and 5 U/kg for mice fed the high-fat diet. Glucose levels were quantified before and 30 min after insulin injection. Results are presented as percent change in glucose at 30 min as compared with initial concentrations. * $P < 0.05$ indicates significant differences between $p55^{-/-}p75^{-/-}$ ($n = 5$) and wild-type mice ($n = 5$).

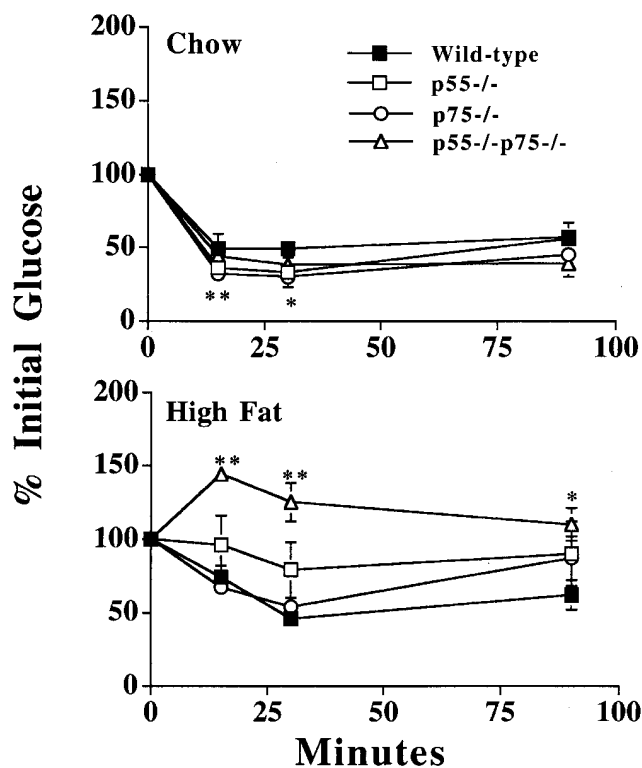


Figure 4. Insulin sensitivity assay. Female C57BL/6 (*wild-type*) and TNF receptor-deficient mice were fed rodent chow (*top*) or a high-fat diet (*bottom*) for 16 wk and fasted for 4 h before intraperitoneal insulin injection at a dose of 1 U/kg body weight ($n = 5$). Plasma glucose was quantified before injection and at 15, 30, and 90 min after injection. In the top panel, $*P < 0.05$ and $**P < 0.001$ indicate significant differences between $p75^{-/-}$ and wild-type mice. In the bottom, $*P < 0.05$ and $**P < 0.001$ indicate significant differences between $p55^{-/-}p75^{-/-}$ and wild-type mice.

16 wk, female mice showed significant increases in body weight (from 17–18 g to 43–45 g, $P < 0.0001$, $n = 5-10$), and plasma concentrations of glucose (from 120 mg/dl to 180 mg/dl, $P < 0.05-0.0001$, $n = 5$), insulin (from 1 ng/ml to 1.8–4 ng/ml, $P < 0.02-0.0001$, $n = 5$), and leptin (from 12 ng/ml to a range of 128–180 ng/ml, $P < 0.06-0.0001$, $n = 5$). However, no significant differences were seen among the four genotypes. Glucose tolerance profiles in females, as evaluated using intraperi-

toneal glucose tolerance tests after 6 wk of diet, were nearly identical among the four genotypes (data not shown). Profiles of glucose disposal as a result of injected insulin were similar among female mice fed chow (Fig. 4 A) and among the high-fat diet with the exception of the $p55^{-/-}p75^{-/-}$ strain that showed no response (Fig. 4 B).

Genetic study. Previous work in vitro demonstrated that activation of p55 resulted in impaired insulin-mediated glucose uptake, suggesting that this receptor was responsible for TNF induction of insulin resistance (31). Although we saw no improvement of diabetogenic parameters in $p55^{-/-}$ mice, as compared with wild-type mice in the diet-induced model, differences may become evident under more severe conditions of obesity such as seen in *db/db* mice. Body weights and diabetogenic parameters were evaluated in male and female lean and obese littermates from our colony of *db/db* mice. Male and female obese mice showed marked weight gains between the ages of 5 and 20 wk (from ~20 to 54 g for $n = 7-8$ mice of each gender), which were more than two times greater than those of lean littermates (from ~13 to 25 g for $n = 7-13$ mice of each gender). However, no significant differences in body weights due to loss of the p55 receptor were seen.

Plasma glucose and insulin levels increased markedly with age for male and female *db/db* mice and were elevated significantly, as compared with lean littermates at most of the time points (Table II). Among female obese mice, and obese males of 5 and 10 wk of age, no significant differences were observed between p55 genotypes. However, for obese male mice of 20 wk of age, those lacking p55 showed a 38% decrease in plasma glucose and nearly a fourfold increase in plasma insulin concentrations (Table II).

Glucose disposal profiles after glucose injection evaluated in 18 h fasted obese animals at 12 wk of age showed no significant differences between p55 genotypes, with the exception of males at 240 min (Fig. 5). However, when calculated as percent change of glucose after glucose injection, profiles between male genotypes were nearly identical (data not shown).

Sensitivity to injected insulin was severely blunted in 4 h fasted obese mice, as compared with lean littermates as illustrated in Fig. 6. At 1 U/kg of insulin, obese mice showed absolutely no decrease in plasma glucose levels regardless of their gender or p55 genotype, demonstrating profound insulin resistance (data not shown). Treating obese mice with 1,000 U/kg of insulin induced glucose clearance to 50% of initial levels by 30 min, but there was no improvement in insulin sensitivity with loss of p55 (Fig. 6). Overall, loss of p55 did not provide

Table II. Plasma Glucose and Insulin Levels for Lean and Genetically Obese (*db/db*) Mice at 5, 10, and 20 wk of Age

Gender	Genotype	Glucose (mg/dl)			Insulin (ng/ml)		
		5 wk	10 wk	20 wk	5 wk	10 wk	20 wk
Males	Lean	141±10 (10)	153±8 (11)	132±22 (9)	0.93±0.07 (10)	1.04±0.2 (12)	0.93±0.5 (12)
	<i>db/db</i>	220±36* (7)	296±33** (5)	454±72** (6)	5.62±1.4** (6)	5.01±1.3** (6)	8.11±2.5** (6)
	$p55^{-/-}db/db$	165±52 (5)	261±38** (6)	277±48*** (5)	6.49±2.5* (5)	6.79±1.8** (5)	29.3±13*** (4)
Females	Lean	142±21 (6)	134±24 (5)	135±9 (6)	0.83±0.1 (6)	0.62±0.1 (6)	0.66±0.1 (7)
	<i>db/db</i>	137±31 (7)	234±36* (7)	306±54* (7)	5.48±3.1 (7)	7.00±2.1* (7)	19.3±6* (10)
	$p55^{-/-}db/db$	136±7 (7)	185±20 (9)	343±49* (6)	5.62±1.2* (6)	9.71±3.3* (10)	12.4±4* (10)

Mice were fasted for 4 h before plasma measurements. Values are presented as the mean±SEM (n). $*P < 0.05$ versus lean mice. $**P < 0.001$ versus lean mice. $^{\ddagger}P < 0.05$ between obese genotypes.

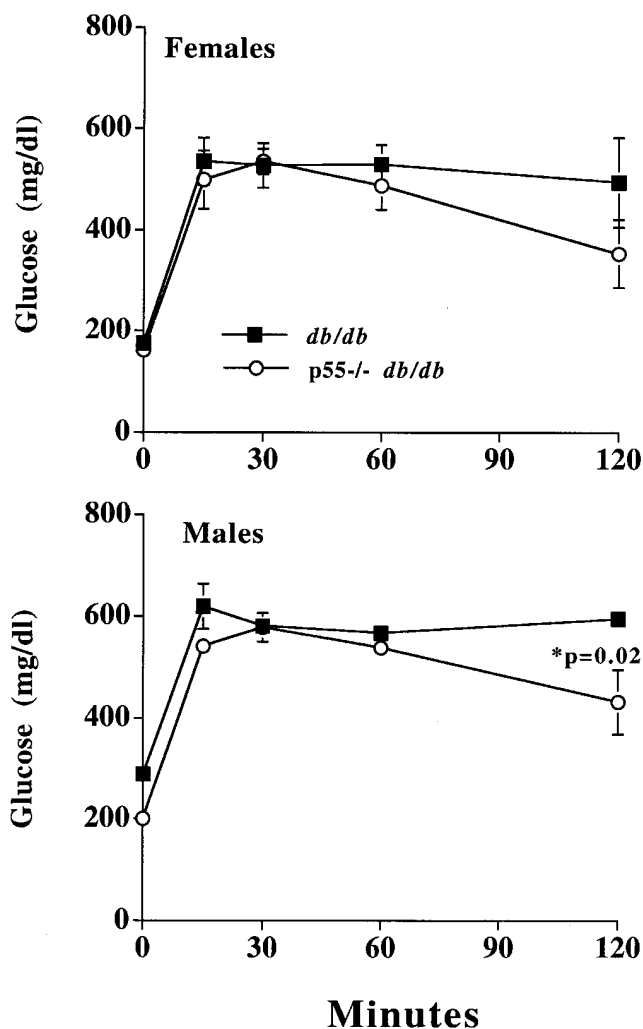


Figure 5. Intraperitoneal glucose tolerance test for *db/db* and *p55^{-/-} db/db* mice. Female (top) or male (bottom) mice at 12 wk of age were fasted overnight (18 h) before glucose injection at a dose of 2 g/kg body weight ($n = 5-7$). A significant difference ($P = 0.02$) was seen between *p55^{-/-} db/db* and *db/db* male mice at 120 min after injection.

significant improvement in diabetic phenotypes with the exception of circulating glucose and insulin levels in older male *db/db* mice.

Discussion

This study is the first to evaluate the relevance of individual TNF receptors, p55 and p75, in diabetes-obesity syndromes in the mouse. Mice lacking one or both of the TNF receptors were examined in conjunction with obesity and diabetes as induced by the *db* mutation in the leptin receptor or by feeding a high-fat/high-carbohydrate diet. The overall findings do not support a major role for improvement of diabetes by deletion of TNF receptors. In fact, the presence of at least one class of TNF receptor was required to protect diet-induced obese mice from overt hyperinsulinemia.

The most striking observation from this work were data ob-

tained from mice deficient in both p55 and p75 receptors. Within 4 wk of feeding the high-fat diet, male *p55^{-/-} p75^{-/-}* mice exhibited plasma insulin levels that were significantly elevated as compared with other genotypes. These animals were not hypoglycemic, indicating that the excess insulin was required to prevent hyperglycemia. In addition, glucose disposal after acute insulin injection was impaired in *p55^{-/-} p75^{-/-}* male and female mice fed the high-fat diet, and by male mice fed rodent chow. Thus, loss of TNF activity, via loss of both receptors, was not associated with improvements in insulin resistance. These data suggest that p55 and p75 are required for maintaining glucose homeostasis.

Previous work in vitro demonstrated that activation of p55 resulted in impaired insulin-mediated glucose uptake, suggesting that this receptor was responsible for TNF induction of insulin resistance (31). We tested this idea in two models of obesity. In a diet-induced obesity model, the loss of p55 yielded no significant effects on glucose and insulin levels. Further, measures of glucose and insulin sensitivity showed little variation in responses between wild-type and *p55^{-/-}* mice. We also examined p55 function in mice carrying the *db* mutation at the leptin receptor, which is a more severe model of obesity and insulin resistance than the diet model. Longitudinal studies showed no effect of the p55 receptor on diabetogenic parameters among female *db/db* mice. For male mice at 20 wk of age, the loss of p55 was associated with reduced fasting glucose levels. However, this change was not consistent with overall improvement of insulin resistance as evidenced by the elevated insulin levels. In addition, glucose tolerance and insulin sensitivity in both male and female *db/db* mice were improved only

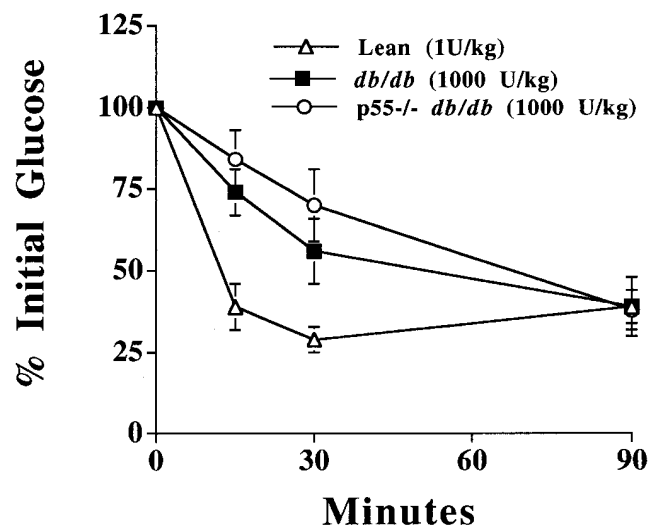


Figure 6. Insulin sensitivity assay for mice derived from a genetic cross with *db/db*. Mice at age 18-20 wk were fasted for 4 h before intraperitoneal injection of insulin. Plasma glucose levels were quantified before insulin injection and at 15, 30, and 90 min after injection. Data is presented as a percent of the glucose level obtained before insulin injection. Lean mice ($n = 10$) were injected with an insulin dose of 1 U/kg body weight. No response was observed for obese mice ($n = 5$) at this dose (not shown). Obese mice were injected with an insulin dose of 1,000 U/kg to obtain a significant decrease in glucose levels. No significant differences in percent glucose disposal were observed between *db/db* mice ($n = 5$) and *p55^{-/-} db/db* mice ($n = 4$).

modestly or not at all by loss of p55, although these parameters were evaluated for younger mice (12 wk). Taken together, results from the two models suggest that loss of p55 did not improve insulin resistance.

The role of p75 in weight gain and diabetes was tested by comparing mice with and without functional p75 receptors in the diet-induced obesity model. Surprisingly, p75^{-/-} male mice fed the high-fat diet gained less weight as compared with other TNF receptor genotypes, including wild-type animals. In addition, fasting insulin levels were reduced in this strain, yet glucose levels were comparable to other strains, which is consistent with an improvement in chronic insulin sensitivity. Alternatively, loss of p75 may alter other pathways involved in modulating plasma glucose levels. At this point, we cannot differentiate between these possibilities. One explanation for reduced weight gain could be increased sensitivity to leptin leading to reduced food intake, and this is currently being examined.

The attenuation of body weight in p75^{-/-} was gender specific. Differences in body composition as a function of gender are commonly observed. For instance, a greater proportion of body fat was retained in females overexpressing skeletal muscle lipoprotein lipase (41) or lacking UCP expression in brown adipose tissue (42). Overall body weight gain was greater in females transgenic for growth hormone (43) and fat stores were proportionately larger in females overexpressing β 3-adrenergic receptor (44). In contrast, *ob/ob* females lacking expression of NPY showed reduced body weight, as compared with males (45). Overall, sulfotransferases and other genetic factors particular to specific background strains may modify the extent of body weight and compositional changes due to genetic engineering.

Leptin levels are generally correlative with body weight or adiposity (46–48). Although leptin levels were lowest for p75^{-/-} mice, which showed the lowest body weights among males, leptin levels did not reflect body weight for the three remaining genotypes. Wild-type, p55^{-/-}, and the double receptor-deficient mice exhibited similar body weights and body lengths (nose to anus, data not shown), but wild-type mice showed greater leptin levels. On a molecular level, the lack of association of leptin with body weight suggests either inefficient expression of leptin or greater leptin sensitivity when TNF receptor(s) are absent. Based on work by Kirchgessner et al. (49), TNF influences leptin release from intracellular storage pools. Our data suggest that both receptors are required for efficient leptin release, as male mice lacking one or both receptors exhibited reduced leptin levels.

Because mice lacking one or more TNF receptors expressed circulating leptin, and leptin levels increased markedly with diet-induced obesity, factors other than TNF can mediate leptin synthesis and release. Such factors may include circulating insulin (50), which was chronically elevated in diet-induced obese mice.

It is of interest to compare our results to studies using TNF ligand-deficient (TNF^{-/-}) mice (33, 34) and *ob/ob* mice made deficient in both TNF receptors (34). A summary of mice created to lack TNF or its receptors and resulting major phenotypic changes from control strains with respect to diabetes is illustrated in Fig. 7. Uysal et al. (33) and Ventre et al. (34) examined wild-type and TNF^{-/-} that were lean or induced to obesity using a high-fat/high-carbohydrate diet or gold thioglucose, respectively. Ventre et al., but not Uysal et al., observed

that lean TNF^{-/-} mice showed modest but significantly reduced body weights as compared with wild-type mice. Loss of TNF did not prevent the development of obesity after diet or GTG treatments, which is consistent with the high-fat diet-induced weight gain seen in our studies of TNF double receptor-deficient mice.

Uysal et al. observed that chow-fed TNF^{-/-} mice had modestly reduced plasma glucose levels when compared with wild-type animals, but this difference between genotypes was lost upon high-fat diet feeding. These diet-treated TNF^{-/-} mice did exhibit modest reductions in plasma insulin (from 2 to 0.5 ng/ml), and modest improvements in glucose and insulin tolerances as compared with wild-type. However, glucose and insulin tolerance data was not presented for rodent chow controls and, thus, it is difficult to assess how much protection the loss of TNF afforded these animals.

The GTG-treated mice (33) were more obese and insulin resistant as compared with the diet-treated mice (34). Although GTG-treated TNF^{-/-} mice showed reductions in fasting insulin and glucose levels, and an improvement in glucose tolerance, insulin sensitivity was not fully restored, suggesting that TNF was not the predominant mediator of these metabolic disturbances (33). Our studies of mice lacking either p55 or p75 are in general agreement with those of the previous studies (33, 34). In older male *db/db* mice lacking p55, evidence of modest improvement in plasma glucose levels and glucose tolerance was observed. However, the decrease in plasma glucose was likely due to the markedly increased plasma insulin levels. In diet-induced obese p75^{-/-} male mice, modest improvements in diabetogenic parameters of body weight, plasma insulin, and leptin levels were seen. However, none of these mice showed a resolution of phenotypes classically associated with insulin resistance.

Several results for TNF^{-/-} obese mice (33, 34) are in contrast to our findings in p55^{-/-}p75^{-/-} obese mice. Major differences were that in our study, p55^{-/-}p75^{-/-} mice fed the high-fat diet exhibited increased plasma insulin levels, and no improvement in either glucose or insulin tolerance. Differences in genetic backgrounds of mice used in the previous studies (crosses between C57BL/6 and 129) and our study (C57BL/6) could possibly contribute to differences in results. Alternatively, TNF^{-/-} animals experienced TNF receptor pathway signaling driven by lymphotoxin, known to be a ligand for TNF receptors (51). Although previous work (8, 11) failed to demonstrate the presence of lymphotoxin in lean or obese rodent adipose tissue, careful studies of lymphotoxin expression and its possible role in body weight regulation are needed.

Our results are also seemingly at odds with a report in which p55^{-/-}p75^{-/-} mice were crossed with mice with a defective leptin gene, *ob/ob*, creating mice which lacked leptin and both TNF receptors (34). Improved fasting glucose and insulin levels, and improved responses to glucose and insulin were reported for *ob/ob* p55^{-/-}p75^{-/-} mice as compared with the *ob/ob* controls despite similar body weights between strains. However, differences in plasma glucose between TNF receptor genotypes were small and transient in nature. Also, it is not clear which gender was used in these studies and results could be skewed by an uneven distribution of genders between TNF receptor groups. But, perhaps the major difference between the p55^{-/-}p75^{-/-}*ob/ob* and p55^{-/-}p75^{-/-} strains used by Uysal et al. and our work, respectively, is the difference in leptin ac-

Modulation of Obesity and Diabetes by TNF and Leptin Genotypes

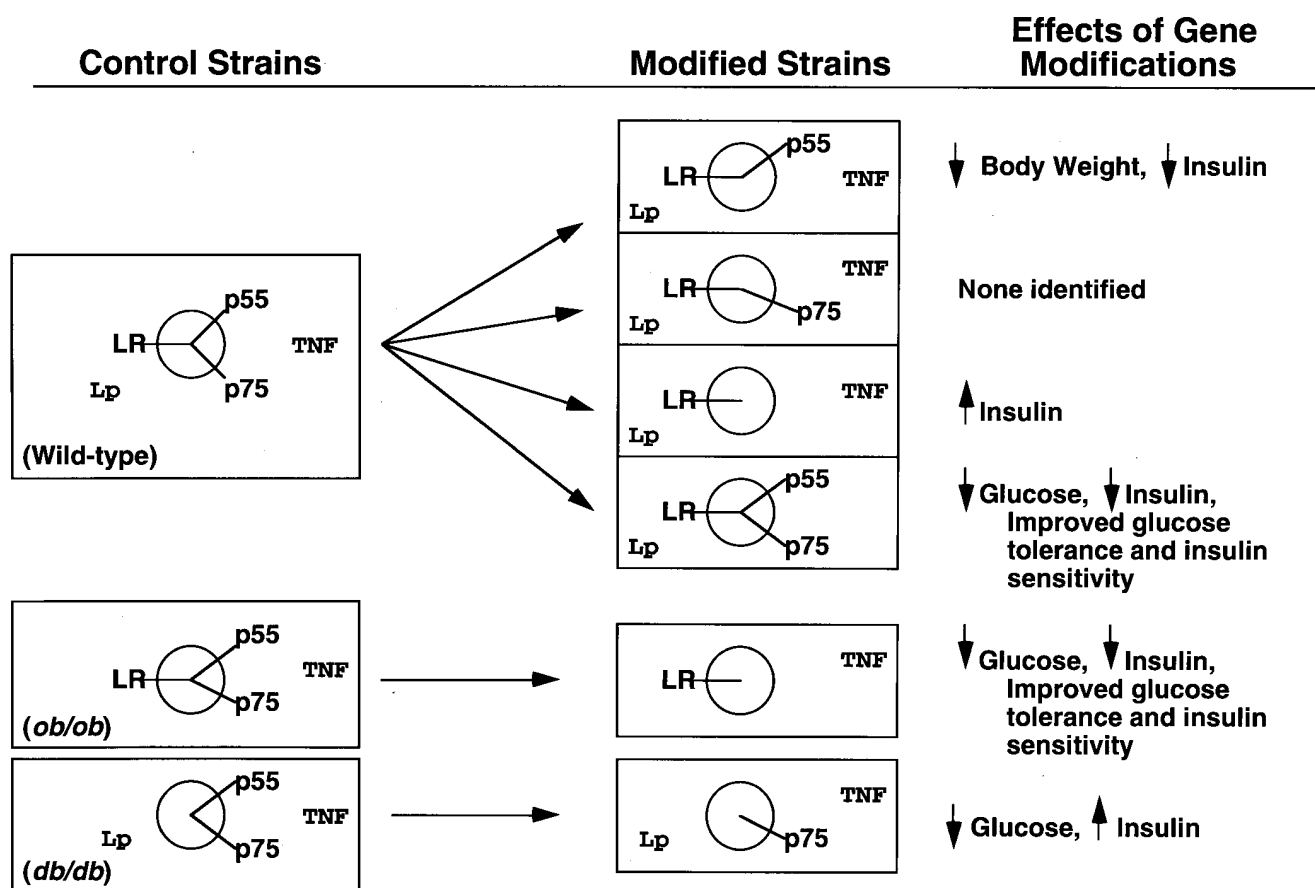


Figure 7. Summary of genetically diverse strains used to determine the contribution of TNF and leptin to the modulation of body weight and diabetes. Control strains shown on the left side of this figure are wild-type (C57BL/6), *ob/ob*, and *db/db* mice. Wild-type mice express circulating TNF and leptin (Lp), as well as receptors for TNF (p55 and p75) and leptin (LR). *ob/ob* mice express TNF and both TNF receptors, but not leptin. *db/db* mice express TNF and both TNF receptors, but not functional leptin receptors. The lack of functional leptin signaling causes hyperphagia leading to obesity and eventual diabetes in the *ob/ob* and *db/db* strains. Wild-type mice have been modified by homologous recombination to lack expression of p75, p55, both receptors, or TNF ligand (33, 34, 37). *ob/ob* (34) and *db/db* (this report) mice were modified to lack both TNF receptors or p55, respectively. Major phenotypic changes in diabetogenic parameters (body weight, plasma glucose and insulin, glucose tolerance, and insulin sensitivity) with respect to control strains are summarized on the right for male mice.

tivity, making it difficult to directly compare results from the two studies. Leptin is a multifunctional molecule, reported to impair insulin action in adipocytes (51) and an hepatic cell line (52), decrease insulin secretion from pancreatic islets (53, 54), and control appetite via actions in the brain (55, 56). We speculate that there is a combined role for leptin and TNF in modulating insulin sensitivity such that the loss of both TNF receptors while maintaining leptin activity (*p55^{-/-}p75^{-/-}* mice) is deleterious to glucose metabolism. On the other hand, loss of leptin or leptin receptor activity while maintaining TNF activity (*ob/ob* or *db/db* mice) also results in aberrant glucose metabolism. Loss of both TNF receptors and leptin (*p55^{-/-}p75^{-/-}ob/ob*) resulted in a more dramatic protection from obesity-induced insulin resistance.

In summary, this is the first report to evaluate the role of individual TNF receptors in glucose homeostasis. Our data do

not support a major role for TNF receptors in the induction of insulin resistance associated with obesity. Loss of p55 did not improve diabetes phenotypes in a diet-induced model of obesity, nor in female *db/db* mice. Although loss of p55 did reduce plasma glucose levels in older male *db/db* mice, this was accompanied by severe hyperinsulinemia. Diet-induced obese mice lacking both receptors exhibited hyperinsulinemia in a moderately fasted state, and insulin levels remained elevated upon severe fasting. This suggests that TNF receptors may be required for normal glucose homeostasis.

Acknowledgments

We are indebted to Jacques Peschon (Immunex Research and Development Corporation, Seattle, WA) for the TNF receptor-deficient

mice, genotyping protocols, and helpful suggestions concerning this manuscript.

This work was supported by the National Institutes of Health (Bethesda, MD) grants HL52848 (R.C. LeBoeuf) and DK47473 (S.C. Chua, Jr.), and NRSA grant HL07247 (S.A. Schreyer).

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