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Adipose Expression of Tumor Necrosis Factor–α: Direct Role in Obesity-Linked Insulin Resistance

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Tumor necrosis factor–α (TNF-α) has been shown to have certain catabolic effects on fat cells and whole animals. An induction of TNF-α messenger RNA expression was observed in adipose tissue from four different rodent models of obesity and diabetes. TNF-α protein was also elevated locally and systemically. Neutralization of TNF-α in obese *fa/fa* rats caused a significant increase in the peripheral uptake of glucose in response to insulin. These results indicate a role for TNF-α in obesity and particularly in the insulin resistance and diabetes that often accompany obesity.

Obesity and diabetes are among the most common human health problems in industrialized societies. Obesity, which is the result of an imbalance between caloric intake and energy expenditure, is highly correlated with insulin resistance and diabetes in experimental animals and humans. However, the molecular mechanisms that are involved in obesity-diabetes syndromes are still not clear. Because adipose tissue is the major site for energy storage and mobilization, many studies have been focused on finding abnormalities in adipocyte physiology or metabolism (*1*, *2*).

Several cytokines, such as TNF-α, have important metabolic effects (*3*, *4*), including direct effects on adipocyte metabolism. TNF-α acts in vitro on murine adipocytes to suppress expression of most adipose-specific genes, including the enzymes involved in lipogenesis (*5*, *6*). However, some of these effects are not observed in primary cultures of human or rat adipocytes (*4*, *7*). In vivo, TNF-α expression has been associated with catabolic states leading to a wasting syndrome, termed cachexia (*8*, *9*), but this effect of TNF-α has been challenged by several groups of investigators (*10*, *11*). TNF-α administration causes an increase in serum triglycerides and very low density lipoproteins in rats and humans (*10*, *12*). This hyperlipidemia is thought to be the result of decreased lipoprotein lipase activity and increased hepatic lipogenesis (*13*). TNF-α administration also has effects on appetite and gastrointestinal tract functions (*1*). Besides TNF-α, other cytokines, such as TNF-β, interleukin-1 (IL-1), IL-6, and interferon, also have profound effects on lipid metabolism (*4*). Furthermore, all of these cytokines affect glucose homeostasis in various tissues (*14*).

Our laboratory has been pursuing the role of cytokines in energy balance and fat metabolism, primarily because of their potential role as endogenous regulators of gene expression in fat tissue. We have shown that fat cells produce certain key components of the alternative pathway of complement, including adipsin–factor D (*15–17*). The local complement pathway appears to be dysregulated in several models of obesity and can be controlled in cultured adipocytes by certain cytokines, including TNF-α (*16*, *17*). This link between the immune system and energy metabolism has led us to investigate directly the regulation and role of cytokines in obesity-diabetes syndromes.

To examine the expression of the TNF-α gene in the tissues of lean (+/?) or obese (*db/db*) mice, we extracted total RNA from various tissues and organs and subjected them to RNA (Northern blot) analysis (Fig. 1). Endogenous expression was evident only in adipose tissue and spleen. The amount of TNF-α mRNA expression in spleen was not different in obese mice as compared with their lean litter mates. However, in adipose tissue the amount of TNF-α mRNA per unit of RNA was at least five- to tenfold elevated in obese animals, as compared with lean controls. TNF-β, IL-1α, IL-1β, and IL-6 were neither expressed in fat tissue nor regulated in any other organ in obesity (*18*). The earliest time of adipose expression of TNF-α examined was 6 to 7 weeks of age in *db/db* mice and 3 to 4 weeks of age in *fa/fa* rats (*18*), when animals are obese and insulin-resistant but not significantly hyperglycemic (*19*). TNF-α mRNA in fat tissue was elevated at these times.

Besides adipocytes, adipose tissue consists of vascular endothelial cells, smooth muscle cells, fibroblasts, local mast cells, and macrophages (*20*). To determine the source of TNF-α expression in adipose tissue, we separated mature adipocytes and nonadipose cells (stromal-vascular fraction), as described (*21*), and determined the amount of mRNA associated with these compartments. The majority of the TNF-α mRNA fractionated with the adipocytes, although some was also detected in the stromal-vascular fraction that contains nonadipocytes and less mature adipocytes

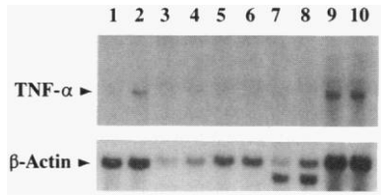

Fig. 1. Expression of TNF-α mRNA (indicated by the labeled arrow) in the tissues of lean and obese mice. Total RNA from tissues of 7- to 8-week-old, male, lean (+/?) and obese (*db/db*) animals (Jackson Laboratory) was extracted by a Cs chloride extraction protocol (*45*). Total RNA (20 μg) was denatured in formamide and formaldehyde at 55°C for 15 min and separated by electrophoresis in formaldehyde-containing agarose gels, as described (*44*). RNA was blotted onto Biotrans membranes that were ultraviolet cross-linked (Stratagene) and baked for 0.5 hours. Hybridization and washes were done as directed by the manufacturer. DNA probes were radioactively labeled to specific activities of at least 10^9 dpm/μg with [α-^{32}P]dCTP (6000 Ci/mmol) by the random priming method (*44*). Lanes 1 and 2, epididymal fat; lanes 3 and 4, liver; lanes 5 and 6, kidney; lanes 7 and 8, skeletal muscle; and lanes 9 and 10, spleen. Odd-numbered lanes, from lean animals; even-numbered lanes, from obese animals. β-Actin mRNA is shown as a control for the loading and integrity of the RNA. Lean mice are designated as +/? because +/+ and *db*/+ animals have not been differentiated.

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(Fig. 2). These results suggested that adipocytes express TNF-α mRNA in vivo and are the major source of the elevated amounts of mRNA expression in adipose tissue. In situ hybridization will be needed to conclusively localize TNF-α mRNA.

Because metabolic profiles differ among various animal models of obesity and diabetes, analysis of multiple models should help separate the effects of hyperinsulinemia, hyperglycemia, and obesity from each other. The diabetes (*db/db*) and obese (*ob/ob*) mice are characterized by massive obesity, hyperphagia, variable hyperglycemia, insulin resistance, hyperinsulinemia, and impaired thermogenesis (*19*). However, diabetes is much more severe in the *db/db* model (*19*). Zucker (*fa/fa*) rats are severely obese, hyperinsulinemic, and insulin resistant (*19*), and the *fa/fa* mutation may be the rat equivalent of the *db* mutation (*22*). Tubby (*tub/tub*) mice are characterized by obesity, moderate insulin resistance, and hyperinsulinemia without significant hyperglycemia (*23*). Like the *db/db* mouse, the *ob/ob*, *tub/tub*, and *fa/fa* models exhibit a similar obesity-related expression of TNF-α mRNA in fat (Fig. 3).

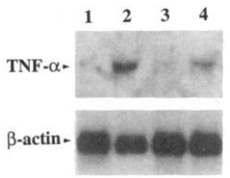

Fig. 2. TNF-α mRNA expression (indicated by the labeled arrow) in cell-fractionated adipose tissue. Epididymal fat pads were isolated from 12- to 13-week-old, male, lean (+/?) and obese (*db/db*) mice, washed in sterile PBS, minced, washed with KRB buffer (pH 7.4) containing 4% albumin and 5 mM glucose, and treated with collagenase (0.5 mg/ml) on a shaking platform at 37°C for 30 min (*21*). We filtered the incubation medium through Nitex screen filters (250-μm pore size) to remove undigested tissue. Adipocytes were then separated by their ability to float after low-speed centrifugation. To obtain total stromal-vascular fractions, we centrifuged the medium below the adipocyte layer at 200*g* for 10 min, and the pellets were washed three times with warm KRB buffer. Total RNA was extracted from the fractions as described in Fig. 1. Lanes 1 and 2, adipocyte fraction; lanes 3 and 4, stromal-vascular fraction. Odd-numbered lanes, lean animals; even-numbered lanes, obese animals. β-Actin mRNA is shown as a control for the loading and integrity of RNA.

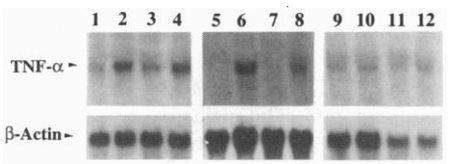

Fig. 3. Adipose expression of TNF-α mRNA (indicated by the labeled arrow) in different rodent models of genetic and chemically induced obesity or diabetes. Total RNA (20 μg) from epididymal fat pads of different animal models (*46*) was subjected to Northern blot analysis, as described in Fig. 1. Lane 1, +/? lean mice; lane 2, *ob/ob* obese mice; lane 3, +/? lean mice; lane 4, *db/db* obese mice; lane 5, +/? lean rat; lane 6, *fa/fa* obese rat; lane 7, *tub*/+ lean mice; lane 8, *tub/tub* obese mice; lane 9, Swiss-Webster lean mice; lane 10, MSG-treated Swiss-Webster obese mice; lane 11, Wistar nondiabetic rat; and lane 12, STZ-treated Wistar diabetic rat. All animals were males; +/?, *ob/ob*, +/?, *db/db*, *tub*/+, and *tub/tub* mice were 12- to 13-weeks-old; +/? and *fa/fa* rats were 7- to 8-weeks-old. β-Actin mRNA is shown as a control for the loading and integrity of the RNA.

We also examined the monosodium glutamate (MSG) model for chemically induced obesity (*24*), in which obesity is less severe than in the genetic models and develops without hyperphagia, hyperinsulinemia, and insulin resistance. There is no induction of TNF-α mRNA in MSG-treated animals (Fig. 3). Finally, we tested the streptozotocin (STZ) model for chemically induced diabetes to examine the effects of hyperglycemia in the absence of obesity. STZ-treated animals are deficient in insulin and severely hyperglycemic (*19*). STZ-treated rats did not exhibit induction of TNF-α expression in fat tissue. These results suggest that TNF-α induction is best correlated with severe obesity and insulin resistance. Detection of elevated TNF-α gene expression in four independent animal models suggests that this may be a general phenomenon in these disorders.

An important question is whether the differences in mRNA amounts are reflected in the amounts of local and systemic (circulating) TNF-α protein. Local protein production was examined in explanted adipose tissue, and a significant amount of TNF-α secretion was observed. When expressed as the mass of TNF-α secreted per unit of tissue DNA, the obese adipose tissue secreted approximately twice as much TNF-α as the lean tissue did (*25*). The amounts of TNF-α in circulation were determined by the enzyme-linked immunosorbent assay (ELISA) in plasma of 24 control and *db/db* animals. Only 6:24 (25%) lean animals had detectable amounts of TNF-α protein, with concentrations ranging from 25 to 97.7 pg/ml (mean ± SE, 61.53 ± 11.9). In obese animals, TNF-α protein was detectable in 14:24 (58.3%), with concentrations ranging from 34 to 165.6 pg/ml (85.6 ± 10.0). These differences in the fraction of lean or obese animals having detectable TNF-α amounts in the plasma were statistically significant, with a P value of <0.05, indicating that TNF-α protein in circulation is also elevated in obese animals. However, the circulating protein concentrations detected in plasma were low (*26*).

Most studies examining the effects of TNF-α on adipocytes have reported a general suppression of fat cell gene expression and in some cases a dedifferentiation response (*6, 27–29*). However, the interpretation of most of these studies is complicated by the fact that high doses of mixed cytokines or human TNF-α were often used, and it is now known that recombinant human TNF-α only binds to one of the two murine TNF receptors (*30*). Because of the results presented above, we have examined the chronic effects (10 to 15 days) of low-dose (50 pM; 2 ng/ml) murine TNF-α treatment on cultured murine fat cells. This treatment did not cause any phenotypic changes in 3T3-F442A adipocytes (*18*). We then examined the pattern of specific mRNA, especially that for adipsin and Glut4, the insulin-sensitive glucose transporter that is expressed in muscle and fat. Both of these genes are expressed in a differentiation-dependent manner in adipocytes and are specifically down-regulated in obesity–insulin resistance syndromes (*16, 17, 31*). Long-term treatment of adipocytes with TNF-α led to down-regulation of Glut4 mRNA (Fig. 4A). This down-regulation is not general for most fat-specific genes because no changes were observed in the mRNA amounts for the fatty acid–binding protein aP2 and glycerophosphate

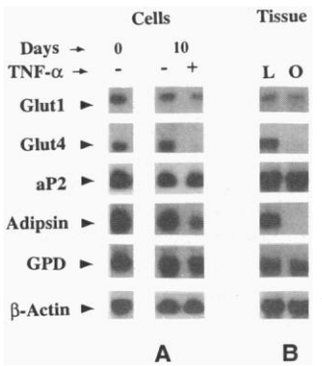

Fig. 4. Effect of chronic treatment with murine TNF-α on adipocyte gene expression in 3T3-F442A cells. (**A**) Murine 3T3-F442A adipocytes were cultured and differentiated in 10% fetal calf serum and insulin (5 μg/ml), as described (*17, 47*). Adipocyte conversion was essentially complete by 7 days after confluence. Day 0 represents the time when cells were fully differentiated. Adipocytes were then treated with 50 pM recombinant murine TNF-α (Genzyme) for 10 days in the same medium or left untreated. Total RNA was extracted from adipocytes, as described (*17, 47*), and subjected to Northern blot analysis and probed with cDNAs for Glut1, Glut4, aP2, adipsin–complement factor D, GPD, and β-actin. (**B**) Total RNA (20 μg) from epididymal fat pads of 12- to 13-week-old, male, +/? lean (L) and *db/db* obese (O) animals was subjected to Northern blot analysis and probed with the same markers as in (A).

dehydrogenase (GPD). Glucose transporter type 1 (Glut1) and β-actin mRNA were also unaffected. However, a reduction in adipsin mRNA was evident (Fig. 4A). The gene expression pattern of these cells is similar to that of adipose tissue in obese animals (Fig. 4B), where Glut4 and adipsin mRNA expression are also severely deficient but most other fat-specific genes are expressed fairly normally (*16*, *17*). These results suggest that TNF-α could be a key mediator of abnormal gene expression in obesity-diabetes syndromes and may affect glucose homeostasis.

To understand the role of the expression of TNF-α, we attempted to neutralize TNF-α in vivo and ask whether this treatment would affect glucose homeostasis of genetically obese and insulin-resistant animals. For neutralization, a recombinant, soluble TNF-α receptor (TNFR)–immunoglobulin G (IgG) chimeric protein was used (*32*). This molecule was administered intravenously into *fa/fa* rats daily, for 3 days (200 μg per rat), and a steady blood concentration of 47.69 ± 4.79 ng/ml was established (*32*). We then examined in vivo insulin sensitivity by using two-step hyperinsulinemic-euglycemic clamps (*33*). Plasma insulin concentrations after two doses of constant insulin infusion (5 and 25 mU per kilogram of body mass per minute) were similar in control and TNFR-IgG–treated animals (*33*). Plasma glucose concentrations (Fig. 5A) and glucose infusion rates to maintain euglycemia under hyperinsulinemia (Fig. 5B) were stable in both groups of animals during the clamps. However, at all time points and at the two different insulin doses, TNFR-treated animals required two to three times more glucose to maintain normal blood glucose concentrations, indicating a greater response to insulin.

Insulin regulation of glucose homeostasis has two major components: stimulation of peripheral glucose uptake and suppression of hepatic glucose output (*34*, *35*). Using tracer studies in the glucose clamps, we determined which portion of the insulin response was affected by the soluble receptor. As illustrated in Fig. 6, A and B, insulin-stimulated peripheral glucose utilization rate (Rd) was two- to threefold higher in the TNFR-treated animals, whereas hepatic glucose output (HGO) was unaffected (Fig. 6, C and D). Thus, the neutralization of TNF-α affects the sensitivity to insulin in obese-diabetic animals. This effect is predominantly seen as an increased peripheral glucose uptake.

Insulin resistance, defined as a smaller than expected biological response to a given dose of insulin, is a ubiquitous correlate of obesity. Many of the pathological consequences of obesity are thought to involve insulin resistance. These include hypertension, hyperlipidemia, and noninsulin-dependent diabetes mellitus (NIDDM). Most NIDDM patients are obese, and a central and early component in the development of NIDDM is insulin resistance (*34*). It has been demonstrated that a postreceptor abnormality develops during the course of insulin resistance, in addition to the insulin receptor down-regulation during the initial phases of this disease (*35*). Several studies on glucose transport systems as potential sites for such a postreceptor defect have demonstrated that both the quantity and function of Glut4 is deficient in insulin-resistant states of rodents and humans (*31*). A lack of a normal pool of insulin-sensitive glucose transporters could theoretically render an individual insulin resistant (*35*). However, some studies have failed to show down-regulation of Glut4 in human NIDDM, especially in muscle, the major site of glucose disposal (*36*).

The mechanistic link between obesity and insulin resistance is not understood. Much attention has been focused on the role of free fatty acids as potential mediators of insulin resistance (*37*). Free fatty acid concentrations are typically elevated in obesity, and fatty acids have been shown to affect insulin sensitivity in vitro and in vivo (*37*). The data presented here suggest that TNF-α is a mediator of insulin resistance, through its fat- and obesity-linked secretion. A two- to threefold increase in insulin-stimulated glucose utilization is observed after administration of a soluble TNF-α

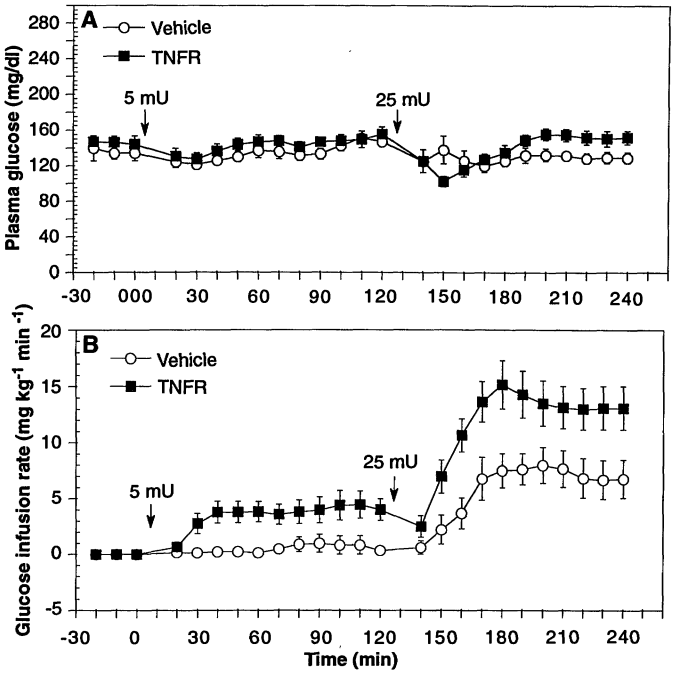

Fig. 5. Glucose control during hyperinsulinemic-euglycemic clamps. (**A**) Plasma glucose concentrations. (**B**) Glucose infusion rates. Male, 7- to 9-weeks-old, *fa/fa* rats (Charles River Laboratories, Massachusetts) were intravenously treated with 200 μg of TNFR-IgG per rat (n = 8) or vehicle [20% glycerol in PBS (n = 5)] for three consecutive days, and ~16 hours after the last treatment, glucose clamps were performed on conscious animals (*33*). The values represent the mean ± SE of plasma glucose and glucose infusion rates of all animals in each group at the given time points.

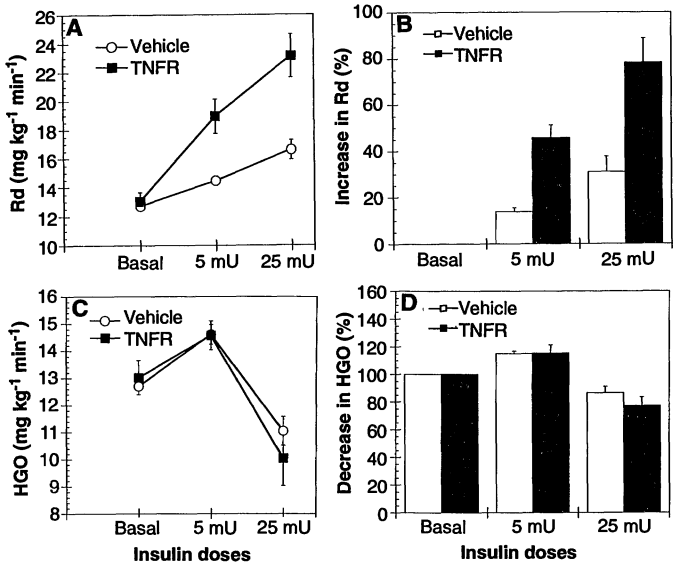

Fig. 6. Effect of TNFR-IgG infusion on glucose homeostasis in *fa/fa* rats. Rd (**A** and **B**) and HGO (**C** and **D**) were calculated as described (*33*, *41*). Rd increased 45.65 and 78.26% over basal amounts after the 5 and 25 mU/kg per minute infusion, respectively, in TNFR-treated animals. The same doses of insulin infusions resulted in 13.84 and 31.02% increases over basal amounts in controls. The values represent the mean ± SE of Rd and HGO of all animals in each group.

receptor to the Zucker (*fa/fa*) rat. This does not represent a complete reversal of insulin resistance, because these animals are at least six to eight times as resistant to insulin-stimulated glucose uptake, as compared with their lean litter mates (*38*). In addition, no effect of the soluble receptor was observed on HGO, another site of insulin resistance. However, it is possible that the length of treatment (3 days), dosing regimen (200 μg per rat), or the reagent itself (soluble human receptor) may not have been optimal to achieve complete neutralization of the action on the rat receptor of endogenous rat TNF-α. Additional experiments with other reagents against TNF-α and in other animal models will be necessary to determine the extent of this cytokine's role in obesity-linked insulin resistance.

Studies have suggested an association of TNF-α with states of peripheral insulin resistance, especially in infection. First, it is established that a biological mediator or mediators generated during infection interfere with insulin's actions and lead to profound metabolic alterations (*9, 29, 39*). Second, incorporation of glucose into lipids is decreased after short-term treatment of 3T3-L1 cells with supernatants of activated macrophages (*40*). Third, treatment of L6 myotubes and 3T3-L1 adipocytes with recombinant TNF-α causes down-regulation of Glut4 expression (*29*). However, the specificity of TNF-α's effect on Glut4 mRNA in fat cells was not clear because the expression of many or most other fat cell genes was also affected (*29*). Finally, a paper has directly demonstrated that chronic, low-level administration of TNF-α to rodents induces systemic insulin resistance (*41*). It remains to be determined whether the effects of TNF-α in this study (*41*) and in our neutralization studies here are due primarily to the modulation of Glut4 gene expression or to broader effects on the insulin signaling pathways.

How can a putative role for TNF-α in obesity-linked insulin resistance be reconciled with its possible role in cachexia (*8*)? This appears to be a question of the hormonal milieu of the organism and the relative concentrations of this cytokine (*42*). The concentrations produced in the obese rodents or those that yield insulin resistance when given exogenously (*41*) are far lower than those that can induce a variety of other symptoms, including cachexia (*8*). These dose-dependent differences in biological effects are interesting, considering data that demonstrate the existence of at least two different receptor systems that have different affinities for TNF (*30, 43*). The receptor systems responsible for a variety of effects of TNF-α are under investigation in many laboratories.

TNF-α could be affecting insulin-stimulated glucose uptake in obese rodents by lowering glucose use by fat, muscle, or both. Whereas this could occur by means of circulating TNF-α protein, it is also plausible that local secretion in adipose depots or in the fat tissue that pervades the muscles of obese animals could inhibit insulin-dependent glucose uptake. In addition to the elevated TNF-α production per cell or per unit of RNA that we have demonstrated, obese animals and humans (by definition) have an excessive amount of adipose tissue that frequently includes an elevated number of fat cells. This would tend to exacerbate the overproduction of TNF-α, whether the effects are achieved locally or systemically. It is important to determine whether obesity in humans is associated with the elevation of TNF-α expression in fat. The data presented here suggest a role for TNF-α in insulin resistance; studies should address the possible involvement of this cytokine in other aspects of obesity, such as fat cell hyperplasia or hypertrophism, hyperlipidemia, and the development of frank diabetes.

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25. Periuterinal fat pads were dissected under sterile conditions; massed; rinsed once in sterile phosphate-buffered saline (PBS); once with Krebs-Ringer bicarbonate (KRB) buffer (pH 7.4) with 4% bovine serum albumin (BSA); minced; and incubated in a medium (1.5 ml of media per gram of tissue) containing Dulbecco's modified Eagle's: Ham's F-12 in a 1:1 mixture (Sigma), supplemented with 0.5% (w/v) endotoxin-free BSA (ICN Biomedicals); 1 nM 3,3′,5-triiodo-L-thyronine (Sigma); 5 mM glutamine; penicillin (6.35 mg/liter); and streptomycin (5 mg/liter) in 6-well tissue culture plates (Nunclon) at 37°C. Fat pads from two lean mice and one obese mouse (12- to 13-week-old females) were used in each experiment. The culture plates were gently swirled every 15 min, and 250 ml of conditioned medium was collected at 0.5, 1, and 8 hours and frozen in liquid nitrogen. We performed the TNF-α assays by using an ELISA (Endogen, MA). Standard curves were simultaneously generated with murine TNF-α (Endogen) diluted in the medium described above. At the end of the experiment, total DNA was extracted from the fat tissues used in each experiment, as described (*44*), and TNF-α values were calculated as picograms of TNF-α per milligram of DNA. At 0.5, 1, and 8 hours, TNF-α secretions from lean ($n = 3$) fat tissue explants were 188.1 ± 44.6, 269.0 ± 50.5, and 265.4 ± 61.2 pg per milligram of DNA (means ± SE), respectively. Secretions from obese ($n = 4$) fat tissue explants at the same time points were 317.5 ± 25.2, 381.6 ± 12.1, and 506.9 ± 40.8 pg per milligram of DNA, respectively. Total secreted proteins and the pattern of secretion were similar in lean and obese groups at these time points as determined by SDS–polyacrylamide gel electrophoresis (PAGE). The nonlinearity of TNF-α that accumulated with increased time probably represents the progressive loss of cellular activity after removal of the tissue from the animals. Statistical analysis of the results was done with repeated measures, and the amount of TNF-α production from lean and obese fat tissue was found to be statistically significant ($P < 0.05$).
26. Approximately 1 ml of blood was collected by cardiac puncture from 12- to 13-week-old male mice into EDTA-containing microfuge tubes. Plasma was separated by centrifugation and immediately frozen in liquid nitrogen. TNF-α concentrations were determined with an ELISA (Endogen). Standard curves were simultaneously generated with murine TNF-α (Endogen). The TNF-α values for lean (6:24) mice were 25.0, 32.1, 53.7, 78.1, 82.6, and 97.7 pg/ml and for obese (14:24) mice were 34.0, 38.0, 43.9, 64.7, 69.1, 69.2, 70.4, 98.8, 100.4, 100.4, 102.8, 102.8, 138.3, and 165.6 pg/ml. Differences in the fraction of detectable TNF-α protein in lean and obese groups were compared with chi-square analysis and found to be statistically significant ($P < 0.05$).

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33. On arrival, Zucker obese rats (*fa/fa*) were housed for at least 1 week before experimental procedures. Surgeries for the placement of jugular vein and carotid artery catheters were done under sterile conditions with ketamine and xylazine (intramuscular) anesthesia. After surgery, all rats were allowed to regain consciousness and placed in individual cages. TNFR-IgG (200 μg per rat in 200 μl of volume) or vehicle (20% glycerol in PBS; 200 μl per rat) was administered through the jugular vein after complete recovery and for the following 2 days. Sixteen hours after the last treatment, hyperinsulinemic-euglycemic clamps were performed. Rats were placed in restrainers, and a bolus of 4 μCi of [3-^{3}H] glucose (Du Pont Biotechnology Systems) was administered, followed by a continuous infusion of the tracer at a dose of 0.2 μCi/min (20 μl/min). Two hours after the start of the tracer infusion, three blood samples (0.3 ml each) were collected at 10-min intervals (from −20 to 0 min) for basal measurements. An insulin infusion was then started (5 mU/kg per minute), and we took 100-μl blood samples every 10 min to monitor plasma glucose. A 30% glucose solution was infused with a second pump, based on the plasma glucose concentrations, in order to reach and maintain euglycemia. Once a steady state was established at 5 mU of insulin per kilogram of body mass per minute (stable glucose infusion rate and plasma glucose), three additional blood samples (0.3 ml each) were obtained for measurements of glucose, [3-^{3}H]glucose, and insulin (from 100 to 120 min). A higher dose of insulin (25 mU/kg per minute) was then administered, glucose infusion rates were adjusted for the second euglycemic clamp, and blood samples were taken from 220 to 240 min. Glucose-specific activity was determined in deproteinized plasma, and the calculations of Rd and HGO were made as described (*41*). Plasma insulin concentrations at the basal period and after the 5 and 25 mU/kg per minute infusions were 102.6 ± 9.4, 188.4 ± 41.4, and 667.4 ± 76.0 ng/ml (mean ± SE) in controls and 95.46 ± 12.4, 200.5 ± 23.6, and 659.1 ± 39.7 ng/ml in TNFR-treated animals.
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46. The *ob/ob*, *db/db*, and *tub/tub* obese mice and their lean controls were obtained from Jackson Laboratories (Bar Harbor, ME); Zucker rat tissues were from F. Gregoire and M. R. C. Greenwood (University of California at Davis). MSG-treated (3 mg of MSG per gram of body mass was subcutaneously injected into neonatal mice, and tissues were collected 7 weeks later) mice were a gift from S. Ross (University of Illinois Medical School); STZ-treated (0.1 mg of STZ per gram of body mass was intraperitoneally injected into 5- to 6-week-old rats, and tissues were collected 8 days later) rats were from R. C. Kahn (Joslin Diabetes Center, Boston, MA). The cDNA clone for murine TNF-α was a gift of B. Beutler (University of Texas, Southwestern Medical Center).
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Requirement for CD8$^+$ Cells in T Cell Receptor Peptide–Induced Clonal Unresponsiveness

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T cell receptor (TCR) vaccination in rats prevents the development of experimental allergic encephalomyelitis (EAE), an animal model of multiple sclerosis. The mechanism of this potential immunotherapy was examined by vaccinating mice with an immunogenic peptide fragment of the variable region of the TCR V_β8.2 gene. Another immunogen that usually induces an immune response mediated by V_β8.2$^+$ T cells was subsequently inhibited because specific clonal unresponsiveness (anergy) had been induced. Depletion of CD8$^+$ cells before TCR peptide vaccination blocked such inhibition. Thus, the clonal anergy was dependent on CD8$^+$ T cells, and such immunoregulatory T cells may participate in the normal course of EAE.

Experimental allergic encephalomyelitis (EAE) is an autoimmune disease induced in animals by immunization with myelin basic protein (MBP) and is considered to be a model for the human demyelinating disease multiple sclerosis (*1*). It has served as an important animal model in the development of strategies for immunotherapy. The studies reported here are based on the model developed by Vandenbark and co-workers and Howell and co-workers (*2*, *3*) in which TCR peptide vaccination prevented the subsequent development of EAE in rats after immunization with MBP. Because the mechanism of such TCR peptide–based vaccination has not been elucidated, we transferred this TCR peptide vaccination into a mouse model to study mechanisms of induced unresponsiveness. We found that T cell clonal anergy developed in T cells bearing the receptor against which such peptide-based vaccination was carried out. Furthermore, CD8$^+$ regulatory cells were involved in the induction of such anergy.

To test the immunogenicity of TCR peptide vaccination in mice, we immunized groups of DBA/2 and (PL/J × SJL)F_1 mice with several different peptides that corresponded to several different regions of the TCR β and α chains, including a peptide composed of amino acids 39 through 61 of the complementarity determining region 2 (CDR2) of V_β8.2 (V_β8.2 CDR2). Both DBA/2 and (PL/J × SJL)F_1 mice mounted a brisk proliferative response to the V_β8.2 CDR2 peptide but not to other tested peptides (*4*). We then investigated whether prior vaccination with the V_β8.2 CDR2 peptide could influence the immune response of DBA/2 mice to a synthetic peptide, the T cell response against which is predominantly mediated by V_β8.2$^+$ CD4$^+$ T cells (*5*). DBA/2 mice were vaccinated with the V_β8.2 CDR2 peptide 10 days before immunization with a synthetic peptide that consisted of amino acids 110

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