Neutralization of Tumor Necrosis Factor Reverses Age-Induced Impairment of Insulin Responsiveness in Skeletal Muscle of Sprague-Dawley Rats

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Between 7 and 14 weeks of age, male Sprague-Dawley (S-D) rats exhibit a substantial increase in adiposity and a corresponding decrease in insulin-stimulated glucose transport in skeletal muscle. In S-D rats aged 3 months, daily administration of goat antimurine tumor necrosis factor (TNF) IgG (anti-TNF; 8 mg, subcutaneously, daily for 7 days) increased insulin-stimulated glucose transport in isolated strips of soleus muscle, compared to controls treated with nonimmune IgG (NI). The TNF content of muscle was markedly higher than that of fat or plasma. Treatment with anti-TNF decreased the mass of inguinal subcutaneous fat and the TNF content of skeletal muscle, but not the TNF content of fat or plasma. Treatment with anti-TNF also produced a nonsignificant trend for reduction in weight gain. Muscle mass and visceral fat mass were unchanged. These data suggest that skeletal muscle pools of TNF may play an important role in the development of insulin resistance.

DURING MATURATION, Sprague-Dawley (S-D) rats become obese and develop impaired responses to insulin in both liver and skeletal muscle.[1-4] We have reported that between 7 and 14 weeks of age, S-D males display a 50% or greater reduction in insulin-stimulated glucose transport in a variety of skeletal muscles.[4]

Several studies have identified tumor necrosis factor (TNF) as a mediator of hepatic insulin resistance. Cheung et al have demonstrated that delivery of a gene construct designed to prevent TNF action (TNF-receptor linked to mouse IgG) reduces hepatic glucose output in fa/fa Zucker rats without reducing adiposity.[5] We have previously shown that overnight infusion of TNF increases hepatic glucose output in S-D rats.[6] Hotamisligil has shown that TNF-receptor knockout in ob/ob mice improves glucose tolerance.[7] Several groups have reported that TNF interferes with insulin signal transduction.[5,8-12]

TNF is expressed in both visceral and subcutaneous fat.[13] Visceral fat has been identified as a potential source of TNF causing insulin resistance.[7] Increased visceral fat and elevated circulating TNF in humans are associated with insulin resistance.[14] Decreased visceral fat in rodents is associated with increased insulin responses in liver[1,2] and muscle.[15]

The main objective of the present study was to determine whether neutralization of TNF can restore insulin responsiveness in skeletal muscle of S-D rats. A second objective was to determine which tissue pools of TNF were reduced by treatment with anti-TNF.

MATERIALS AND METHODS

Animals

S-D male rats that were barrier-raised and viral pathogen-free were obtained at 3 months of age from Zivic-Miller Laboratories (Zellenople, PA). Rats were housed individually on a 12:12 light-dark cycle. Animal use was in accordance with the Guide to the Care and Use of Experimental Animals and was approved by the University of Florida Institutional Animal Care and Use Committee.

Experimental Design

Rats weighing 425 ± 25 (SD) g were injected daily, subcutaneously, for 7 days with 8 mg goat antimurine TNF IgG (anti-TNF) or goat nonimmune IgG (NI). We have previously shown that this dosing regimen produces measurable plasma antibody titers for at least 7 days.[16] Before treatment and at 2, 4, and 6 days after treatment, 200 μL heparinized blood was collected by tail tip amputation for analysis of plasma anti-TNF. Rats were fasted overnight before euthanasia on day 7, followed by harvesting of the following: soleus muscle for glucose transport studies, blood by cardiac puncture for serum analysis, and muscles and fat depots for measurement of wet weight and TNF content.

Antibody

Polyclonal goat antimurine TNF IgGs were prepared as we have previously reported[16] using the Ribi adjuvant system containing 0.5 mg each of monophosphoryl lipid A, trehalose dimycolate, and cell wall skeleton in 0.2% Tween 80 (Ribi ImmunoChemical, Hamilton, MT). The serum IgG fraction was obtained by polyethylene glycol precipitation and column chromatography using DEAE Bio-Gel A (Bio-Rad Laboratories, Richmond, CA). The neutralizing capacity of the anti-TNF IgG fraction in the L929 cytotoxicity assay was 6.5 and 9.0 $\times\times$ 10^5 50% neutralizing units/mg IgG against recombinant TNF-α and serum TNF from lipopolysaccharide (LPS)-treated rats, respectively. Nonimmune goat IgG was prepared in the same way and had no detectable TNF neutralizing activity. Neither IgG preparation demonstrated effective binding to LPS, interleukin-1, or interferon-γ in an enzyme-linked immunosorbent assay (ELISA) protocol.

TNF-α Content of Serum, Muscle, and Fat

Extracts of tibialis anterior (TA) and red gastrocnemius muscles were prepared by the method of Murase et al.[17] Muscle tissue was sonicated in 20 vol of buffer containing 1 mol/L NaCl, 2% bovine serum albumin, 0.1 mol/L Tris-HC1, 2 mmol/L Na_2EDTA, 80 U of aprotinin/L, and 0.02% NaN_3, pH 7.6. Following centrifugation at 14,000 revolutions/min for 30 minutes, the supernatants were frozen for later TNF analysis. TNF in serum and in extracts of muscle and fat

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was measured by an ELISA (BioSource International, Camarillo, CA) that is specific for rat TNF and has a sensitivity of 0.7 pg/mL.

Glucose, Insulin, and Free Fatty Acids

Serum glucose was measured by the Trinder method (Sigma Chemical, St Louis, MO). Serum insulin was measured by radioimmunoassay (Linco Research, St Louis, MO). Free fatty acid content was measured using a colorometric assay kit that relies on fatty acids as substrate for enzymatic acylation of coenzyme A (Wako Chemicals, Neuss, Germany).

Muscle glucose transport was measured according to the protocol of Dohm et al in rats fasted for 24 hours.[18] Briefly, 25-mg strips of soleus muscle were mounted at resting length in acrylic plastic clips and incubated for 60 minutes in Krebs Ringer Henseleit buffer containing 1% BSA. Muscle strips were then transferred to fresh buffer and incubated for an additional 60 minutes with [^{3}H]-2-deoxyglucose ([^{3}H]-2-DOG; 10^6 dpm/mL, 5 mmol/L) and [^{14}C]-sorbitol, 10^5 dpm/mL, 20 mmol/L (ICN Pharmaceuticals, Costa Mesa CA) ± a maximal concentration of insulin (16,000 μU/mL). Following incubation, the tissue was rinsed, weighed, solublized, and radioactivity was counted. Glucose transport was estimated as the specific uptake of [^{3}H]-2-DOG and expressed as nmol/g tissue/min. The nonspecific component of tissue-associated radioactivity was estimated using the nontransportable analog [^{14}C]-sorbitol and was subtracted. Our data (not shown) and that Dohm et al[18] have confirmed that [^{3}H]-2-DOG transport is linear over 60 minutes.

Statistical Analysis

Differences among means were tested using Students' *t* test or analysis of variance (ANOVA), with *P* values less than .05 defined as the threshold of significance. Post-hoc analysis was performed using Fischer's exact least significant difference (LSD) test. Values are reported as means ± SEM.

RESULTS

We treated maleS-D rats aged 3 months with goat anti-TNF 8 mg, ssubcutaneously, daily for 7 days. Control rats were treated with goat NI. Active anti-TNF IgG was assessed by the ability of plasma to inhibit TNF-induced cytotoxicity in L929 cells. We consistently detected active antibody in the plasma throughout the 7-day period of treatment. Food intake was unaffected by treatment (29.8 ± 2.7 g/d for anti-TNF *v* 30.5 ± 2.9 for NI).

Table 1. Effect of Anti-TNF on Body Composition

	NI	Anti-TNF
Plantaris muscle (g)	0.44 ± 0.025	0.43 ± 0.014
Gastrocnemius muscle (g)	2.12 ± 0.058	2.06 ± 0.062
Tibialis anterior muscle (g)	0.80 ± 0.031	0.791 ± 0.019
EDL muscle (g)	0.216 ± 0.009	0.217 ± 0.004
Combined muscle (g)	3.58 ± 0.107	3.50 ± 0.085
Perirenal fat (g)	1.06 ± 0.156	0.79 ± 0.14
Epididymal fat (g)	6.48 ± 0.557	5.72 ± 0.44
Retroperitoneal fat (g)	5.72 ± 0.82	5.04 ± 0.86
Combined visceral fat (g)	13.26 ± 1.39	11.55 ± 1.36
Inguinal fat (g)	9.82 ± 0.72	6.98 ± 0.92*
Body weight gain (g)	32.78 ± 3.49	21.97 ± 5.27

NOTE. Body weight gain is for the first six days of treatment.
Abbreviations: EDL, extensor digitorum longus, NI, nonimmune
*Values are means ± SE, N = 8.
$P < .05$ *v* NI.

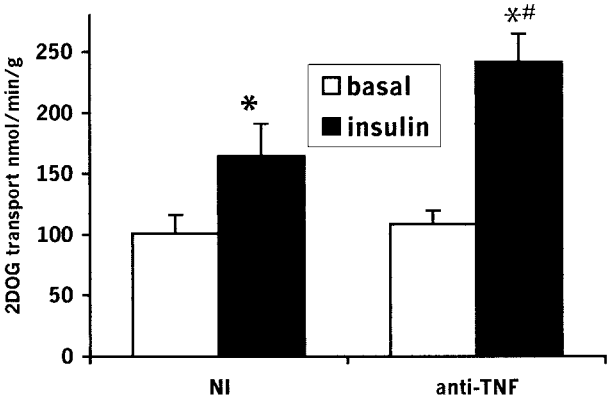

Fig 1. Treatment with anti-TNF increases in vitro glucose transport in soleus muscle. Following treatment with anti-TNF or NI IgG for 6 days, rats were fasted 24 hours and killed. Strips of soleus muscle were isolated and incubated with [^{3}H]-2-DOG for 1 hour ± a maximal concentration of insulin as described in the Methods. Treatment with anti-TNF did not affect basal transport of [^{3}H]-2-DOG, but significantly elevated insulin-stimulated transport. *$P < .05$ *v* basal; #$P < .05$ *v* NI. Values are means ± SE; N = 8 for NI group, 7 for anti-TNF group.

Body Composition

Treatment with anti-TNF caused a significant 29% reduction in the inguinal deposit of subcutaneous fat (Table 1). There was also a nonsignificant trend toward reduced weight gain during the first 6 days of treatment ($P = .103$). The masses of several muscles and of several depots of visceral fat were unchanged.

Insulin Responsiveness

Rats were fasted overnight and insulin-stimulated transport of [^{3}H]-2DOG was measured in isolated strips of soleus muscle (Fig 1). Basal [^{3}H]-2DOG transport was 100.93 ± 15.15 nmol/min/g in NI controls and was unchanged by treatment with anti-TNF (108.45 ± 11.34 nmol/min/g). Insulin-stimulated [^{3}H]-2DOG transport was significantly elevated by treatment with anti-TNF (164.50 ± 26.64 nmol/min/g for NI *v* 241.53 ± 23.08 for anti-TNF).

TNF, Glucose, Insulin, and Free Fatty Acids

Treatment with anti-TNF did not alter concentrations of glucose, insulin, or free fatty acids in fasting serum (Table 2). Anti-TNF significantly reduced the TNF content of tibialis anterior muscle (by 42%) and red gastrocnemius muscle (by 35 %), but did not alter TNF content in serum, in inguinal subcu-

Table 2. Effect of Treatment With Anti-TNF on Concentrations of Glucose, Insulin, Free Fatty Acids, and TNF in Fasting Serum

	NI	Anti-TNF
Glucose (mg/dL)	183.4 ± 23.9	167.4 ± 8.5
Insulin (ng/mL)	3.01 ± 1.15	2.46 ± 0.69
Free fatty acids (mEq/L)	0.534 ± 0.091	0.497 ± 0.065
TNF (pg/mL)	31.9 ± 8.2	25.8 ± 10.5

NOTE. Values are means ± SE; N = 8.
*$P < .05$ *v* NI.

Table 3. Effect of Treatment With Anti-TNF on TNF Content of Muscle and Fat

	NI	Anti-TNF
Retroperitoneal fat (pg/g)	895.8 ± 164.3	731.1 ± 241.5
Epididymal fat (pg/g)	323.6 ± 110.5	407.5 ± 101.2
Inguinal fat (pg/g)	744.3 ± 267.8	868.4 ± 357.6
Tibialis anterior muscle(pg/g)	2,434.7 ± 328.7	1,418.3 ± 235.7*
Gastrocnemius muscle(pg/g)	4,469.8 ± 472.5	2,899.9 ± 293.4*

NOTE. Values are means ± SE; N = 8.
*$P < .05$ *v* NI.

taneous fat or in the retroperitoneal and epididymal depots of visceral fat (Table 3).

DISCUSSION

TNF is a proinflammatory cytokine that was first identified because of its anticancer activity and is now known to play a complex role in the response to injury and infection.[19] TNF has been implicated in several pathological states, including endotoxin lethality,[20] ischemia-reperfusion injury,[21] Crohn's disease,[22] tumor-induced cachexia,[16] and hepatic insulin resistance.[5,6] TNF may cause hepatic insulin resistance by inhibition of insulin signaling. Hotamisligil et al have shown that in myeloid 32D cells, TNF induces a serine phosphorylation of insulin receptor substrate (IRS-1), resulting in an inhibition of subsequent insulin-induced tyrosine phosphorylation of IRS-1.[8] Neutralization of TNF is known to improve hepatic insulin responsiveness in rodents. Cheung et al have demonstrated that delivery of a gene construct designed to prevent TNF action (TNF-receptor linked to mouse IgG) increases insulin suppression of hepatic glucose output in fa/fa Zucker rats without reducing adiposity.[5] To our knowledge, the possible role of TNF in skeletal muscle insulin resistance has not been previously explored.

We have reported that between 7 and 14 weeks of age, male S-D rats exhibit a 50% reduction in maximal insulin-stimulated glucose transport in skeletal muscle, a 15% increase in fasting serum glucose, and a 2.6-fold rise in fasting insulin.[4] In the present study, a 1-week treatment with anti-TNF reversed the impairment in muscle insulin responsiveness, but did not lower fasting glucose or insulin. Goodman et al have shown that insulin sensitivity is reduced in skeletal muscle of maturing S-D male rats.[3] More studies will be required to determine whether anti-TNF can restore insulin sensitivity as well as the maximum response to insulin.

Although TNF may play a role in the development of insulin resistance, it is not known which tissue pools of TNF are critical to the process. TNF is overexpressed in visceral fat and it has been hypothesized that visceral fat is a source of systemic TNF causing insulin resistance. In humans, elevated circulating TNF is associated with insulin resistance.[14] However, the present study demonstrates that TNF levels are much higher in muscle than in fat or serum and that anti-TNF specifically reduces the TNF content of muscle, but not fat or serum. The failure of anti-TNF to reduce TNF content in fat may be due to the low perfusion of that tissue, resulting in limited access of anti-TNF to adipocytes. Our values for serum TNF are similar to those obtained by others for S-D rats.[23,24] Murase et al found that in Wistar rats, TNF content of muscle is also higher than that of fat or serum.[17]

In addition to TNF, free fatty acids released from visceral fat have been proposed as mediators of insulin resistance. Surgical removal of visceral fat from insulin-resistant rats improves insulin suppression of hepatic glucose output.[1,2] Infusion of free fatty acids is known to decrease the rate of glucose disposal in euglycemic/hyperinsulinemic clamp studies.[25] However, we found the improved muscle insulin responses resulting from anti-TNF treatment were associated with, at most, a small decrease in visceral fat and no change in plasma free fatty acids. There may be 2 independent means of reversing skeletal muscle insulin resistance.

We conclude that TNF is a mediator of reduced muscle insulin responses in maturing S-D rats. We also conclude that impaired muscle insulin responses result from TNF present in muscle tissue, although it is not clear whether the TNF is of local or systemic origin.

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