Evidence That Tumor Necrosis Factor-Alpha–Induced Hyperinsulinemia Prevents Decreases of Circulating Leptin During Fasting in Rats

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Administration of tumor necrosis factor-alpha (TNF-α) acutely increases leptin gene expression and circulating leptin concentrations in rodents and humans. Since TNF-α also induces hyperinsulinemia, and because insulin is a potent stimulator of leptin production, we hypothesized that elevated plasma insulin mediates TNF-α–induced increases of circulating leptin. To test this hypothesis, rats were made insulin-deficient with streptozotocin (STZ) and treated with subcutaneous implants that released insulin at a constant rate and thereby "clamped" insulin levels. STZ-diabetic and nondiabetic rats were injected with TNF-α or vehicle; plasma leptin, insulin, and glucose concentrations were measured during an initial 12-hour postinjection period of fasting and after a subsequent 12-hour period of refeeding. Food intake during the 12 hours after fasting was assessed as a physiologic correlate of changes in leptin concentrations. In nondiabetic rats, TNF-α increased plasma insulin ($P = .016$) and prevented the fasting-induced decrease of circulating leptin ($P = .004$) over the initial 12 hours compared with vehicle. Food intake during the refeeding period was 30% lower ($P = .008$) when the nondiabetic animals were injected with TNF-α. In contrast, TNF-α did not affect leptin concentrations in STZ-diabetic animals with clamped plasma insulin levels or their food intake during the refeeding period. These results suggest that TNF-α–induced hyperinsulinemia likely mediates the stimulatory effect of TNF-α on circulating leptin in vivo. Elevated leptin levels may in turn contribute to the effect of TNF-α to decrease food intake.

ADMINISTRATION OF tumor necrosis factor-α (TNF-α) has been reported to acutely increase leptin gene expression and circulating leptin concentrations in rodents and humans.[1-7] Whether TNF-α increases circulating leptin levels by directly stimulating leptin release from adipocytes is unclear. While some studies have shown that TNF-α stimulates leptin secretion from cultured adipocytes,[4-6,8] a number of others have shown that the cytokine inhibits leptin release.[8-12] However, those studies that also examined the effects of TNF-α on leptin gene expression demonstrated that it inhibits leptin mRNA levels.[8-14] This contrasts with the increase in leptin mRNA that is consistently observed after TNF-α administration in animals.[1,2,5-7] Thus, there is a discrepancy in the reported effects of TNF-α on leptin gene expression and secretion between studies performed in vitro and those conducted with whole animals.

Several studies have demonstrated that administration or infusion of either endotoxin, TNF-α or interleukin-1 (IL-1), induces alterations in glucose homeostasis that include an initial period of hyperglycemia followed by increases of plasma insulin and then a period of hypoglycemia.[15-17] TNF-α treatment has also been shown to induce insulin resistance in muscle, which would be expected to produce a compensatory increase of insulin secretion and hyperinsulinemia, but it does not appear to acutely affect insulin-mediated glucose uptake by adipose tissue.[18] Because insulin and adipocyte glucose metabolism are potent regulators of leptin production,[19-26] we hypothesized that the acute increase of plasma leptin following TNF-α administration in vivo results from a concomitant rise of plasma insulin concentrations, which acts to increase leptin production by adipose tissue.

To investigate whether TNF-α acutely increases circulating leptin levels by increasing plasma insulin concentrations, insulin-deficient streptozotocin (STZ)-diabetic rats were treated with subcutaneous implants that released a constant amount of insulin, which "clamped" plasma insulin concentrations and thereby prevented fluctuations of circulating insulin during the experiment. Treatment with insulin also prevented the large decrease of fat mass that accompanies STZ-induced insulin-deficient diabetes.[23,27] Plasma leptin in this insulin-treated diabetic rat model is responsive to supplemental insulin injections.[23] STZ-diabetic and nondiabetic control animals were then injected with TNF-α or vehicle and blood samples collected for measurement of plasma leptin, insulin, and glucose during an initial 12-hour postinjection period of fasting, and after a subsequent 12-hour period of refeeding. Based on the hypothesis that TNF-α stimulates the acute increase of leptin via an increase of circulating insulin, we expected that TNF-α would not affect circulating leptin concentrations in the insulin-clamped STZ-diabetic animals. In contrast, we expected that fasting would decrease plasma leptin in untreated nondiabetic animals,[28-31] but that TNF-α treatment would prevent this decrease by inducing an elevation of plasma insulin.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats weighing 350 to 450 g (Charles River, Wilmington, MA) were used for the studies. The animals were individually housed in hanging wire cages in temperature-controlled

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rooms, and fed Purina chow (Ralston-Purina, St Louis, MO) and deionized water ad libitum. The light/dark cycle was 12 hours on and 12 hours off, with lights on at 6 AM. The study protocol was approved by the University of California, Davis Animal Care and Use Committee.

Induction of Diabetes and Insulin Implant Placement

Insulin-deficient diabetes was induced with subcutaneous injections of freshly prepared STZ (Sigma, St Louis, MO) at a dose of 40 mg/kg in ice-cold 0.5 mol/L citrate buffer (pH 4.5). A second dose of STZ (40 mg/kg) was administered 24 hours later. This regimen produces insulin-deficient diabetes (plasma glucose >25 mmol/L) in more than 95% of treated animals without inducing renal failure or losses from hypoglycemia.[32] Nondiabetic animals received injections of citrate buffer only. At the time of the second STZ injection, all of the STZ-diabetic animals[12] received 1.5 implants impregnated with bovine insulin (Linplant, Toronto, Canada) placed subcutaneously through a 14-gauge needle under ketamine-xylazine (30:10 mg/kg, respectively) anesthesia. These implants slowly release insulin (~2 U/d) for up to 40 days. The nondiabetic rats were sham-implanted.

TNF-α Response Study

Experiments were conducted 2 weeks following the induction of diabetes. Tail blood samples were collected from both diabetic (n = 12) and nondiabetic (n = 8) rats for baseline measurements at 8 AM of the day prior to the experiment. On the day of the experiment, diabetic and nondiabetic rats were randomly selected so that half of the animals in each group would receive either TNF-α or phosphate-buffered saline (PBS). Then, 100 μg/kg recombinant human TNF-α (BioSource International, Camarillo, CA) or the equivalent volume of PBS was injected intraperitoneally. Blood samples were collected at 2 PM and 8 PM of the same day and at 8 AM of the following day (ie, 6, 12, and 24-hour postinjection). Because TNF-α suppresses food intake[33] and since leptin concentrations are regulated by food intake,[28-30] after injection, the animals were fasted until 8 PM to control for treatment effects on food intake. The animals were then allowed to eat ad libitum. Baseline food intake was measured over the 24-hour period from 8 AM of the day preceding to 8 AM of the day of the experiment. Food intake was also assessed over the 12 -hour period after fasting between 8 PM of the day of injection and 8 AM of the following day. After 1week, all of the animals were "crossed-over" so rats that received a PBS injection the first time received a TNF-α injection the second time and vice versa.

Assays

Plasma leptin concentrations were measured in duplicate with a specific radioimmunoassay for rat leptin (Linco Research, St Louis, MO) as previously described.[23] Plasma insulin concentrations were measured in duplicate with a radioimmunoassay for rat insulin using rat insulin standards (ICN Diagnostic Division, ICN, Costa Mesa, CA) according to the method of Yalow and Berson with minor modifications[34]; the intra- and interassay variation are 7.0% and 9.0%, respectively. Plasma glucose and lactate were measured with a glucose analyzer (Yellow Springs Instruments, Yellow Springs, OH).

Data Analysis for TNF-α Response Study

To compare treatment effects on plasma leptin, insulin, glucose, and lactate concentrations between TNF-α and PBS injection for diabetic rats and between TNF-α and PBS injection for nondiabetic rats, the logarithm of the area under the curve (AUC) for these parameters (0 to 12 hours postinjection) was calculated for each animal to generate summary measurements that were then analyzed by a paired *t* test (2-tailed).[35] If an overall significant effect of TNF-α was found, individual time points were analyzed with a paired *t* test for comparisons within a treatment group (ie, baseline and postinjection responses). The effects of TNF-α at 24 hours postinjection were analyzed with a paired *t* test (2-tailed). *P* values less than .05 were considered statistically significant. Data are expressed as means ± SEM.

RESULTS

Baseline Parameters

Prior to treatment with TNF-α or PBS injection, the mean baseline values for plasma leptin, insulin, glucose, and lactate concentrations, food intake, and weight within the 2 experimental groups (STZ-diabetic or nondiabetic) were not significantly different from each other (Table 1). Similar to a recent study we conducted with this insulin-treated, STZ-induced diabetic rat model,[27] insulin levels in the diabetic rats were elevated compared with nondiabetic animals ($P < .05$). The insulin implants normalized food intake and plasma lactate in the diabetic animals, while leptin concentrations were slightly, but not significantly, lower. Insulin treatment did not completely normalize plasma glucose concentrations ($P < .05$). Prolonged insulin infusion can induce insulin resistance in muscle but not adipose tissue.[36] This likely explains why insulin treatment was able to normalize plasma leptin but not glucose. The inability to normalize glucose levels is also indicative of the importance of hepatic insulin delivery to glucose homeostasis; subcutaneously administered insulin does not preferentially increase insulin concentrations within the hepatic portal vein as when insulin is secreted from the pancreas. Nonetheless, the purpose of the implants was to clamp insulin concentrations and to prevent large decreases of fat mass in the STZ-diabetic rats.

Table 1. Mean Baseline Plasma Leptin, Insulin, Glucose, and Lactate Concentrations, Food Intake, and Weight, Prior to TNF-α or PBS Injection

	PBS	TNF-α
Leptin (ng/mL)		
Nondiabetic	7.0 ± 1.1	7.0 ± 0.8
Diabetic	5.9 ± 1.0	4.6 ± 1.0
Insulin (pmol/L)		
Nondiabetic	145.8 ± 32.4	159.6 ± 34.2
Diabetic	553.2 ± 61.2*	658.8 ± 147.0*
Glucose (mmol/L)		
Nondiabetic	8.1 ± 0.2	8.0 ± 0.2
Diabetic	12.8 ± 1.8*	17.1 ± 2.2*
Lactate (mmol/L)		
Nondiabetic	2.6 ± 0.2	2.7 ± 0.2
Diabetic	2.7 ± 0.1	3.2 ± 0.3
Food intake (g/24 h)		
Nondiabetic	31.5 ± 1.0	31.2 ± 1.3
Diabetic	32.3 ± 1.2	31.9 ± 1.7
Weight (g)		
Nondiabetic	454.8 ± 11.5	455.1 ± 9.9
Diabetic	448.1 ± 9.4	432.9 ± 7.3

NOTE. Differences in mean baseline values prior to PBS or TNF-α injection, within the nondiabetic or diabetic group, and between groups were tested by a paired and 2-sample *t* test, respectively. Values are mean ± SEM (n = 8 diabetic rats; n = 12 nondiabetic rats).
*$P < .05$ *v* nondiabetic animals.

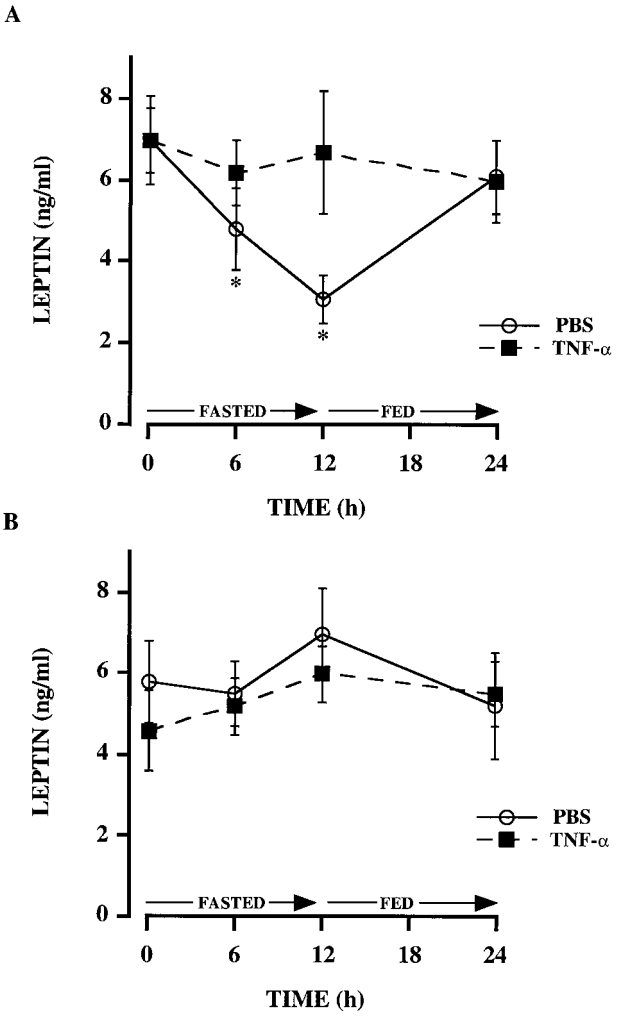

Fig 1. Effect of TNF-α injection on plasma leptin concentration in (A) nondiabetic and (B) diabetic rats. Animals were fasted for 12 hours following injection with 100 μg/kg TNF-α or PBS. Blood samples were collected at 6, 12, and 24 hours postinjection. Mean ± SEM; n =8 nondiabetic and n = 12 diabetic rats. *P < .005 *v* baseline.

Plasma Leptin Concentrations

As expected, when vehicle (PBS) was administered to nondiabetic rats, plasma leptin concentrations decreased markedly from baseline levels during the 12-hour postinjection period of fasting (Δ = -4.1 ± 0.7 ng/mL; P = .0012) (Fig 1A). In contrast, when injected with TNF-α, leptin levels did not decrease during the fast, and 12-hour postinjection concentrations were not different from baseline levels (Δ = -0.7 ± 0.9 ng/mL). Over the entire 12-hour period, plasma leptin concentrations were higher following TNF-α injection compared with vehicle (P = .004); at 12 hours, mean leptin concentrations were 2 times higher when the animals received TNF-α compared with PBS. After 12 hours of refeeding (ie, at 24 hours postinjection), plasma leptin concentrations were restored to baseline levels following PBS treatment and were not different from those at that time point following TNF-α treatment.

In contrast to nondiabetic animals, plasma leptin concentrations did not decrease during fasting in diabetic rats in which plasma insulin was clamped and were not affected by TNF-α administration over the 12-hour fasting period (P = .29; not significant) or at the 24-hour time point (Fig 1B).

Plasma Insulin Concentrations

TNF-α increased insulin concentrations in nondiabetic rats over the 12-hour postinjection period compared with PBS (P = .016) (Fig 2A); at 6 hours, mean insulin levels were 1.7 times greater than when the animals were injected with vehicle. Insulin concentrations were maximally increased from baseline levels at 6 hours (Δ = +216.4% ± 87.5%; P = .015) following TNF-α administration, whereas insulin levels did not change after injection of PBS.

As expected, when insulin levels were clamped with slow-release insulin implants in diabetic rats, TNF-α administration did not affect plasma insulin concentrations over the 12-hour postinjection period (P = .71) (Fig 2B). There was a slight tendency for insulin levels to increase over the 24-hour period but the change was not significant.

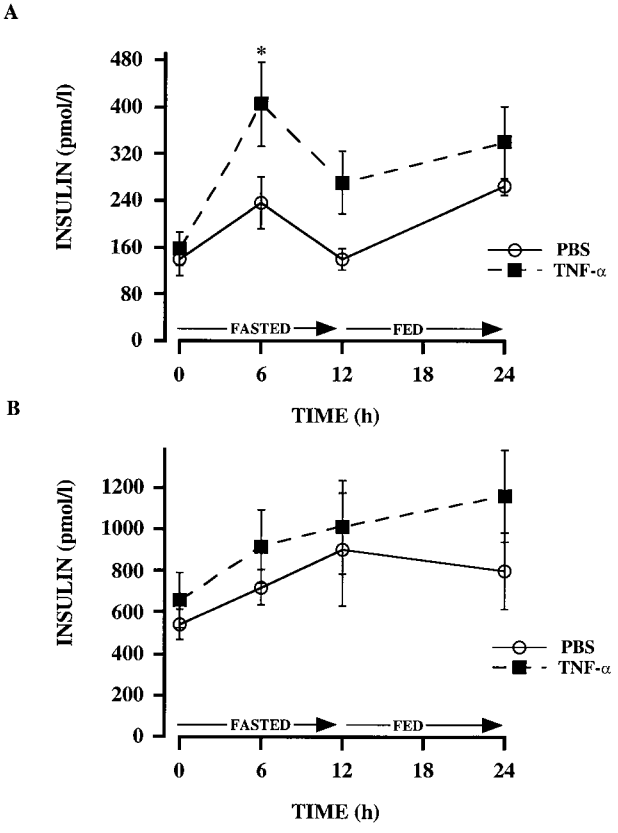

Fig 2. Effect of TNF-α injection on plasma insulin concentrations in (A) nondiabetic and (B) diabetic rats. Animals were fasted for 12 hours following injection with 100 μg/kg TNF-α or PBS. Blood samples were collected at 6, 12, and 24 hours postinjection. Mean ± SEM; n = 8 nondiabetic and n = 12 diabetic rats. *P < .05 *v* baseline.

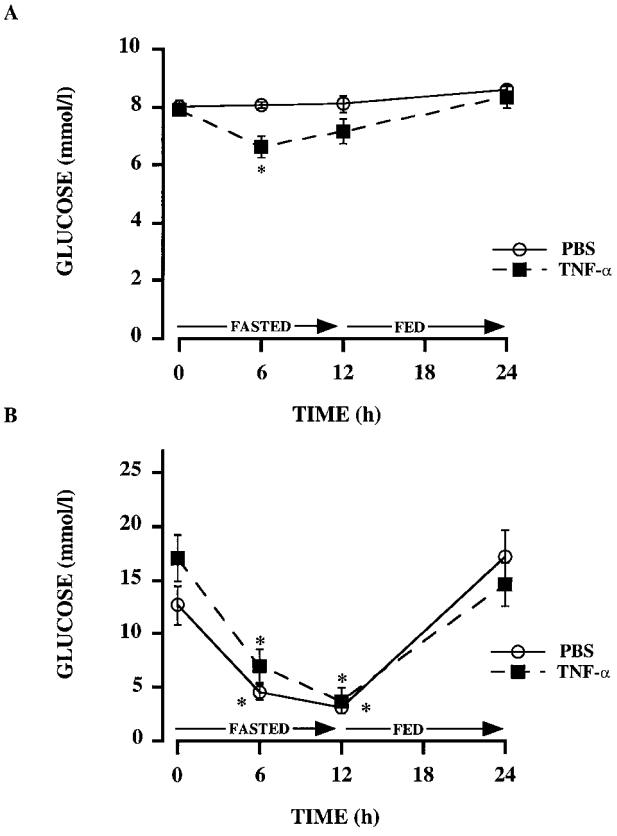

Fig 3. Effect of TNF-α injection on plasma glucose concentrations in (A) nondiabetic and (B) diabetic rats. Animals were fasted for 12 hours following injection with 100 μg/kg TNF-α or PBS. Blood samples were collected at 6, 12, and 24 hours postinjection. Mean ± SEM; n = 8 nondiabetic and n = 12 diabetic rats. *P < .005 *v* baseline.

Plasma Glucose Concentrations

Plasma glucose concentrations decreased modestly over the 12-hour postinjection period after TNF-α administration in nondiabetic rats ($P = .01$) (Fig 3A); at 6 and 12 hours, mean plasma glucose concentrations were 82% and 88%, respectively, of the glucose levels observed following injection with vehicle. Glucose concentrations were maximally decreased from baseline after TNF-α administration at 6 hours ($\Delta = -16.4\% \pm 4.3\%$; $P = .006$), whereas glucose levels did not change after PBS administration.

Due to the continuous insulin release by the implants, plasma glucose levels decreased markedly from baseline over the 12-hour period of fasting in the diabetic animals; however, plasma glucose concentrations in diabetic rats were not different between the TNF-α and PBS treatments over the 12-hour postinjection period ($P = .16$) (Fig 3B). At 12 hours, glucose concentrations were similarly decreased from baseline following TNF-α or PBS injection by 76.9% ± 6.4% and 71.3% ± 5.1%, respectively ($P < .001$ for both). Thus, although plasma glucose was slightly, but not significantly, higher prior to TNF-α injection, the pattern of glucose changes was similar after TNF-α or vehicle injection. For both nondiabetic and diabetic rats, plasma glucose concentrations at the 24-hour time point, after the rats were allowed to refeed for 12 hours (Fig 3A and B), were similar to baseline levels and not different between the TNF-α and PBS treatments.

Food Intake

As a result of insulin treatment the diabetic rats were not hyperphagic and baseline food intake was similar for diabetic and nondiabetic animals. During the 12-hour refeeding period after the fasting period, food intake was decreased by approximately 30% in the nondiabetic animals after TNF-α administration compared with when the animals received PBS (18.5 ± 1.8 *v* 26.1 ± 0.6 g; $P = .008$) (Fig 4). In contrast, 12-hour food intake during this period was not different between the TNF-α and PBS treatments in insulin-treated STZ-diabetic rats (25.3 ± 2.0 *v* 27.6 ± 1.1 g; $P = .4$).

DISCUSSION

When the proinflammatory cytokines TNF-α or IL-1 are administered to animals, they induce acute increases of circulating leptin concentrations.[1-7,37] Lipopolysaccharide (LPS) also increases leptin levels, although TNF-α and IL-1 likely mediate that effect.[4,38] In the present study, we demonstrated that TNF-α administration prevented leptin levels from decreasing during fasting in nondiabetic rats; leptin levels after 12 hours of fasting were nearly 2-fold greater when TNF-α was administered compared with when vehicle was injected. TNF-α also induced a nearly 2-fold increase of plasma insulin concentrations at 6 hours that preceded this difference in leptin levels. In contrast, TNF-α injection did not affect circulating leptin concentrations in STZ-diabetic rats with subcutaneous insulin implants that clamped plasma insulin concentrations. Thus, increases of plasma insulin appear necessary in order for TNF-α to maintain leptin levels during fasting.

There are discrepancies in the reported effects of TNF-α on leptin gene expression and secretion between studies conducted in vitro and those performed in vivo. The inhibitory effect of

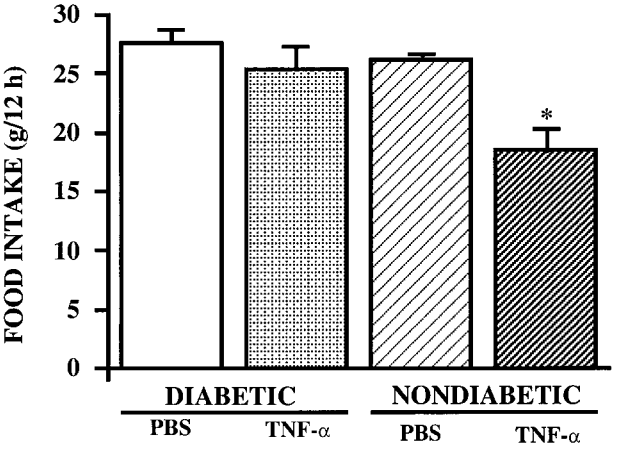

Fig 4. Effect of TNF-α injection on food intake in nondiabetic and diabetic rats. Animals were fasted for 12 hours following injection with 100 μg/kg TNF-α or PBS. Food intake was assessed for the 12 to 24 hours postinjection period. Mean ± SEM; n = 8 nondiabetic and n = 12 diabetic rats. *P < .005 *v* PBS.

incubation with TNF-α ($\geq$24 hours) on leptin production was demonstrated with cultured rat, mice and human adipocytes.[8-13] One of those reports did demonstrate that a 4- to 8-hour exposure to TNF-α increased leptin release from human adipocytes.[8] A few other studies also demonstrated that TNF-α stimulates leptin release from adipocytes. One reported that TNF-α maximally stimulated leptin secretion from 3T3-L1 adipocytes at 6 hours by possibly regulating the release of leptin from preformed pools,[6] while another showed that an 8-hour exposure to TNF-α stimulated leptin release from primary mouse adipocytes.[4,5] However, several reports showed that prolonged treatment (>24 hours) of cultured adipocytes with TNF-α inhibited leptin mRNA levels.[8-14] Leptin gene expression was also decreased in 3T3-L1 or mouse brown adipocytes after only a 4- to 6-hour treatment with TNF-α.[6,10] We have observed that an even shorter exposure (3 hours) to TNF-α did not affect leptin mRNA levels in 3T3-L1 adipocytes, but it potently inhibited insulin-induced leptin mRNA accumulation (unpublished observation, July 2001). Thus, results generated in vitro contrast with those from in vivo experiments, which showed that leptin mRNA was increased in the adipose tissue between 3 and 12 hours following TNF-α administration.[1,2,5-7]

Administration of TNF-α, IL-1, or LPS to rodents stimulates glycogenolysis and/or gluconeogenesis resulting in hyperglycemia followed by increases of circulating insulin concentrations.[16,39-42] This hyperinsulinemia then leads to enhanced glucose utilization by particular tissues such as adipose tissue, and hypoglycemia results when total glucose utilization, which also includes non–insulin-mediated glucose uptake by macrophage-rich tissues,[42-44] excedes glucose production.[45-47] Because insulin-mediated glucose utilization is a potent stimulus for leptin production by adipose tissue,[24-26] it likely mediates the effect of TNF-α, IL-1, and LPS to increase circulating leptin in vivo. Insulin's role as a mediator is further supported by several studies which showed that it acutely increases circulating leptin and leptin mRNA levels in rodents,[28,30,48,49] and directly stimulates leptin release and gene expression in cultured adipocytes.[5,21,50] In the present study, TNF-α clearly increased circulating insulin concentrations in the nondiabetic rats. Although not directly confirmed, this hyperinsulinemia probably increased insulin-mediated glucose utilization by adipose tissue, which contributed to the observed decrease in plasma glucose, and led to the stimulation of leptin production that maintained plasma leptin during fasting. This mechanism is supported by the inability of TNF-α -injection to alter leptin in diabetic rats in which plasma insulin levels were clamped.

An alternative interpretation for our results is that prolonged exposure to supraphysiological insulin concentrations due to the implants in the diabetic rats, may have somehow blunted the stimulation of leptin production by TNF-α. For example, adipocytes isolated from rats chronically treated with insulin were demonstrated to have decreased insulin responsiveness.[51] If hyperinsulinemia indeed mediates TNF-α's stimulatory effect on leptin production, chronic insulin treatment could impair the adipose tissue response to elevated plasma insulin in rats. However, one group showed that prolonged insulin infusion does not impair white adipose tissue insulin-mediated glucose utilization in STZ-diabetic rats,[52] and we have demonstrated that plasma leptin in our insulin-treated diabetic rat model is responsive to supplemental insulin injections.[26] We have also observed that glucocorticoid (dexamethasone) administration can still acutely increase plasma leptin in these animals (unpublished observation, April 2000). Thus, the adipose tissue of chronic insulin-treated STZ-diabetic rats is responsive to other leptin secretagogues. We believe the most likely explanation for the lack of effect of TNF-α to increase leptin secretion in the diabetic rats was that insulin levels were clamped and could not increase after TNF-α administration.

Based on the ability of TNF-α and other proinflammatory agents to decrease food intake and increase circulating leptin in rodents, it was proposed that leptin mediates the anorexia and weight-loss associated with infectious diseases and inflammation.[2] However, TNF-α modulates other factors that can regulate food intake, such as cholecystokinin (CCK), or it may affect hypothalamic signaling molecules that act downstream of leptin, such as α-melanocyte–stimulating hormone (α-MSH) and neuropeptide Y (NPY).[33] Thus, leptin's role as a mediator of TNF-α's effect on food intake is unclear. However, in the present study, TNF-α decreased the refeeding response in nondiabetic rats, and at the time refeeding was initiated, plasma leptin was 2 times higher compared with when they were administered vehicle. Together with the observation that TNF-α did not affect food intake or leptin in diabetic rats, these data suggest that leptin was a mediator of the TNF-α–induced decrease of food intake. This interpretation is supported by numerous studies which demonstrated that leptin administration decreases food intake in rodents.[31,53-55] In one related study, intracerebroventricular leptin injections produced dose-dependent decreases of food intake at 4 and 24 hours that were comparable for STZ-diabetic and nondiabetic rats,[54] indicating that STZ-diabetic rats respond normally to the effects of leptin to inhibit feeding.

In summary, TNF-α prevented the decline of circulating leptin concentrations during fasting in nondiabetic rats but had no effect on leptin levels in STZ-diabetic rats with subcutaneous implants that clamped insulin levels. These results suggest that TNF-α–induced hyperinsulinemia mediates the effect of TNF-α to increase plasma leptin levels in vivo.

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