Direct Effects of TNF-α on Local Fuel Metabolism and Cytokine Levels in the Placebo-Controlled, Bilaterally Infused Human Leg

Increased Insulin Sensitivity, Increased Net Protein Breakdown, and Increased IL-6 Release

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Tumor necrosis factor-α (TNF-α) has widespread metabolic actions. Systemic TNF-α administration, however, generates a complex hormonal and metabolic response. Our study was designed to test whether regional, placebo-controlled TNF-α infusion directly affects insulin resistance and protein breakdown. We studied eight healthy volunteers once with bilateral femoral vein and artery catheters during a 3-h basal period and a 3-h hyperinsulinemic-euglycemic clamp. One artery was perfused with saline and one with TNF-α. During the clamp, TNF-α perfusion increased glucose arteriovenous differences (0.91 ± 0.17 vs. 0.74 ± 0.15 mmol/L, P = 0.012) and leg glucose uptake rates. Net phenylalanine release was increased by TNF-α vs. placebo, with concomitant increases in appearance and disappearance rates. Free fatty acid kinetics was not affected by TNF-α, whereas interleukin-6 (IL-6) release increased. Insulin and protein signaling in muscle biopsies was not affected by TNF-α. TNF-α directly increased net muscle protein loss, which may contribute to cachexia and general protein loss during severe illness. The finding of increased insulin sensitivity, which could relate to IL-6, is of major clinical interest and may concurrently act to provide adequate tissue fuel supply and contribute to the occurrence of systemic hypoglycemia. This distinct metabolic feature places TNF-α among the rare insulin mimetics of human origin. Diabetes 62:4023–4029, 2013

Originally, tumor necrosis factor-α (TNF-α) was identified as an endogenous pyrogen or “cachectin” (1) because of its biological properties of inducing fever, cachexia, and muscle protein loss in various states of disease (2–4). TNF-α is a key component of an inflammatory response and one of the most potent proinflammatory cytokines released by innate immune cells that induces release of other cytokines, including interleukin-6 (IL-6) (5,6). TNF-α plays an important role in the pathophysiology of sepsis, and there seems to be a relation between the TNF-α level and the severity of disease (7–9). Finally, TNF-α has been associated with states of constant low-grade inflammation, eventually leading to insulin resistance and overt diabetes (10,11). In line with this, it has been shown that plasma levels of TNF-α are correlated with BMI; weight loss leads to a decrease in plasma levels of TNF-α (12,13).

Systemic infusion of TNF-α induces insulin resistance and increased lipolysis in humans (6,14,15), whereas the effects on protein metabolism are less clear (16). A number of studies have shown that anti–TNF-α treatment increases insulin sensitivity in patients with inflammatory chronic diseases (17–19), whereas other reports have failed to confirm this relationship (20–23). Furthermore, studies investigating TNF-α neutralization in type 2 diabetic patients and in patients with metabolic syndrome show no effect of anti–TNF-α treatment on insulin sensitivity (24,25). TNF-α activates the hypothalamicpituitary axis and stimulates the release of stress hormones, such as epinephrine, glucagon, cortisol, and growth hormone into the blood (26,27), all of these counter-regulatory stress hormones generate insulin resistance (27–29), and glucocorticoids generate muscle loss (30). Thus, TNF-α invariably generates release of both other cytokines and stress hormones, and it is uncertain to which extent the metabolic actions of TNF-α are intrinsic or caused by other cytokines or stress hormones in humans.

The current study was therefore designed to define the direct metabolic effects of TNF-α in human muscle. Since all previous human studies assessing the metabolic actions of TNF-α have used systemic administration, making discrimination between direct and indirect effects impossible, we infused TNF-α directly into the femoral artery.
and compared the effects to the saline-infused contralateral leg.

RESEARCH DESIGN AND METHODS

Subjects. Eight healthy male volunteers, 23 ± 1 years of age and with a BMI of 23.9 ± 0.6 kg/m², were included in the study, after oral and written informed consent was obtained. An unremarkable medical history was confirmed during a structured interview, and normal blood test screening and physical examination were present. The study was approved by the Central Denmark Region Ethics Committee (M-2010-0076) in accordance with the Declaration of Helsinki. The study protocol was registered at www.clinicaltrials.gov (NCT01452058).

The subjects reported to the laboratory at 0700 h after an overnight fast. Vigorous physical exercise was not allowed for 2 days before participating in the study. The experiments were performed under thermoneutral conditions (21–23°C). After each experiment, the subjects were hospitalized overnight followed by a physical examination (including ultrasonography of both femoral arteries) every day in a period of 1 week.

The leg model. As previously described (31), the Seldinger technique was used to insert catheters into the femoral artery and vein of both legs, under local anesthesia (lidocaine, 10 mg/mL, AstraZeneca, Albertslund, Denmark). Femoral arteries and veins were visualized directly using ultrasonography (Vivid e, GE, Milwaukee, WI).

The proximal lumina of double-lumen arterial catheters were used for infusion of either TNF-α (batch 01430022, Beroum; Boehringer Ingelheim, Ingelheim am Rhein, Germany) or placebo (isotonic saline), respectively, in a single-blind, randomized manner. TNF-α was diluted in isotonic saline, infusion rate 6 ng/kg/h, administered continuously over 360 min. Blood samples were taken from the arterial catheter infusing placebo and from both venous catheters. One catheter was placed in a cubital vein for infusion of isotonic saline, metabolic tracers, insulin, amino acids, and glucose.

Femoral arterial blood flow was measured using Vivid e (GE Healthcare). In brief, angle-corrected pulsed-wave Doppler (blood flow velocity) measurements were performed at the tip of the catheter. Care was taken to avoid turbulent flow from the infused fluid. The diameters of the arteries were measured using the two-dimensional images, and the flow was estimated by calculating the mean flow from three measurements (each based on 10 pulse waves) during the last 20 min of the basal and clamp periods.

Hyperinsulinemic-euglycemic clamp. The study consisted of a 180-min basal period (referred to as basal), followed by a 180-min hyperinsulinemic-euglycemic clamp period (referred to as clamp). The infusion rate of insulin (Insulin Actrapid; Novo Nordisk, Copenhagen, Denmark) was 1.0 mU/kg/min i.v. Plasma glucose was clamped at 5 mmol/L by adjusting peripheral intravenous infusion of 20% glucose (glucose, 200 g/L; SAG; Copenhagen, Denmark) according to arterial plasma glucose measurements every 10 min immediately after sampling (Beckman Instruments, Palo Alto, CA). During the clamp, amino acids were infused (infusion rate, 1.056 mg/kg/h; Glavamin, 22.4 g N/L; Fresenius Kabi AB, Uppsala, Sweden) to avoid a decrease in amino acid levels and subsequent changes in insulin sensitivity.

Phenylalanine and palmitate kinetics. Total RNA was isolated from muscle biopsies using Trizol (Life Technologies, Boston, MA). RNA was quantified by measuring absorbance at 260 and 280 nm with a ratio ≥ 1.8. Integrity of the RNA was checked by visual inspection of the two ribosomal RNAs, 18S and 28S, on an agarose gel. cDNA was synthesized with the TaqMan Gold RT-PCR Kit (PerkinElmer, Boston, MA). Real-time PCR was performed with mRNA levels of β-2-microglobulin as a housekeeping gene. The following primers were used: β-2-microglobulin, GAG CCT ATC CAG CGT ACT CC and AAT GTC GGA TGG ATG AAA CCC; TLTC; ATG ATG TTC TTC AAC AGG AAC GAG TCC and GTG CAG GCC GCA GTA ACC GAT G; and GLUT4, CCC CAT TCC TTG GAT CAT CG and ATG TCC GCC TCC ACA TAC TGG. The analyses were performed in duplicate using the KAPA SYBR Fast qPCR Kit (Kapa Biosystems, Woburn, MA) in an iCycler from Bio-Rad Laboratories (Hercules, CA).

Data are presented as mean ± SEM. Statistical analysis was performed using two-factor repeated-measures ANOVA and paired Student t tests, as outlined in our original protocol. All ANOVA results refer to an overall main effect of TNF-α versus placebo; in addition, we have given P values for paired Student t tests comparing the two legs at specific time points. Normal distribution was assessed by inspection of QQ plots, and Wilcoxon signed rank tests were used to test data that were not normally distributed.

RESULTS

Mean blood pressure remained constant around 92 ± 2 mmHg, and axillary temperature was constant around 37.0 ± 0.2°C. Heart rate increased slightly from 65 ± 5 to
76 ± 4 bpm (P = 0.005). Arterial insulin levels increased from 40 ± 6 to 214 ± 52 pmol/L during the clamp (P < 0.0005).

**Basal.** Arterial glucose concentrations were 5.04 ± 0.09 mmol/L, and blood flows were not different between the two legs during the basal period (Table 1). Basal arteriovenous (a-v) glucose differences were not significantly affected by TNF-α (0.067 ± 0.019 [TNF-α] vs. 0.083 ± 0.022 mmol/L [placebo]), although overall two-way (TW) ANOVA for repeated measurements revealed a TNF main effect (P = 0.025) (Fig. 1).

Phenylalanine rate of proteolysis and synthesis was increased by TNF-α (Table 1), represented by RaPhe (3.194 ± 297 [TNF-α] vs. 3.053 ± 132 μg/min [placebo], P = 0.003) and RdPhe (2.364 ± 200 [TNF-α] vs. 2.000 ± 123 μg/min [placebo], P = 0.025, respectively).

Overall TW ANOVA revealed an overall increased phenylalanine muscle release after TNF-α (P = 0.023), although basal phenylalanine muscle release was not significantly increased. Lactate release, FFAs, and palmitate kinetics were not affected by regional TNF-α infusion (Table 1).

**Cytokines.** As expected, venous concentrations of cytokine TNF-α were significantly higher in the TNF-α–treated leg, meaning that a-v differences were lower in the TNF-α leg (−41.05 ± 12.09 [TNF-α] vs. 9.85 ± 6.92 pg/mL [placebo], P = 0.011, after 60 min; −25.9 ± 15.5 [TNF-α] vs. 12.9 ± 16.3–26 pg/mL [placebo], P = 0.016, after 180 min) (Table 2).

TNF-α treatment induced a net release of IL-6 after 180 min (P = 0.016); cytokine IL-8 (P = 0.008) and IL-4 (P = 0.0289) release also increased. GM-csf, IFN-γ, IL-1β, IL-2, IL-5, and IL-10 were similar in both legs.

**Clamp.** Arterial glucose concentrations were clamped at 5.00 ± 0.04 mmol/L, and steady-state glucose infusion rates of 4.2 ± 0.6 mg/kg/min during the last 30 min of the clamp were recorded. During the hyperinsulinemic-euglycemic clamp, leg blood flows remained similar (Table 1). Glucose a-v differences were increased by TNF-α (0.921 ± 0.175 [TNF-α] vs. 0.744 ± 0.151 mmol/L [placebo], P = 0.012) (Fig. 1 and Table 1), as were leg glucose uptake rates (P = 0.036).

In contrast to the basal state, rates of proteolysis and synthesis were unaltered. Overall TW ANOVA revealed an overall increased phenylalanine muscle release after TNF-α (P = 0.023), although insulin-stimulated phenylalanine muscle release was not significantly increased. Lactate release, FFAs, and palmitate kinetics remained similar under both conditions (Table 1).

**Cytokines.** a-v Differences of TNF-α (−17.75 ± 8.18 [TNF-α] vs. −3.18 ± 3.85 pg/mL [placebo], P = 0.008), IL-6 (−24.61 ± 11.51 [TNF-α] vs. 1.95 ± 3.40 pg/mL [placebo], P = 0.016), and IL-8 (−12.90 ± 7.71 [TNF-α] vs. 1.54 ± 3.40 pg/mL [placebo], P = 0.027) were lower in the TNF-α–treated leg (Table 2). GM-csf, IFN-γ, IL-1β, IL-2, IL-4, IL-5, and IL-10 were similar in both legs.

**Biopsies.** To assess whether insulin signaling was improved in the TNF-α–treated leg, we measured phosphorylation of Akt and AS160 and expression of GLUT4. Phosphorylation of Ser473 on Akt responded to insulin stimulation, but not to TNF-α stimulation (Fig. 2A). Phosphorylation of AS160 was detected with antibody against PAS sites, and it responded to insulin with no effect of TNF stimulation (Fig. 2B). As an indication of carbohydrate oxidation regulation, phosphorylation of Ser293 (site 1) and Ser300 (site 2) on PDH-E1α was examined (Fig. 2C and D).
PDH phosphorylation was unaffected by TNF-α treatment and insulin stimulation, as was phosphorylation of ACC and AMPK (Fig. 2E and F). Phosphorylation of mTOR (protein kinase that suppresses autophagy) responded to insulin stimulation, but not to TNF-α stimulation (Fig. 2G). Neither protein expression (Fig. 2H and I) nor mRNA expression (data not shown) of GLUT1 and GLUT4 was affected by TNF-α.

Phosphorylation of GSK3-α and GSK3-β and phosphorylation of Thr50S on Akt responded similarly to insulin stimulation, but not to TNF-α stimulation (Fig. 2H and I) nor to TNF-α stimulation in both legs, but there was no effect of TNF-α (data not shown).

**DISCUSSION**

This study was designed to define the direct metabolic effects of TNF-α in human muscle and more specifically to test whether local placebo-controlled leg infusion of TNF-α directly affects insulin sensitivity and protein and lipid metabolism. The main outcome of the study was that TNF-α directly increases basal phenylalanine rates of appearance (protein breakdown) and disappearance (protein synthesis), the overall effects being an increased net phenylalanine release reflecting increased breakdown. Furthermore, TNF-α increased insulin sensitivity in terms of increased glucose uptake during a systemic hyperinsulinemic glucose clamp. Intramyocellular insulin and mTOR signaling, lipid metabolism, and release of lactate were unaffected, whereas local release of certain cytokines, including IL-6, distinctly increased.

The observation that TNF-α increases insulin sensitivity is intriguing and may hold a major therapeutic potential if the underlying mechanisms can be targeted. Previous studies have reported that systemic TNF-α administration induces insulin resistance and increases lipolysis in humans (6,15), but no studies have examined the local effects of TNF-α on muscle glucose uptake in humans. It is well described that bacterial lipopolysaccharide induces a TNF-α response (37), and it has been reported that lipopolysaccharide administration after a latency of 1–2 h may induce increased peripheral insulin sensitivity (27,38),

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### Table 2: Cytokine a-v differences

| Cytokine (pg/mL) | Placebo | TNF-α | P-value | Placebo | TNF-α | P-value | Placebo | TNF-α | P-value |
|------------------|---------|-------|---------|---------|-------|---------|---------|-------|---------|
| IFN-γ (pg/mL)    | 13.47   | 1.94  | 0.003   | 13.67   | 2.41  | 0.007   | 13.17   | 2.25  | 0.013   |
| IL-6 (pg/mL)     | 1.28    | 0.46  | 0.001   | 1.09    | 0.32  | 0.006   | 1.08    | 0.34  | 0.007   |
| IL-8 (pg/mL)     | 1.42    | 0.28  | 0.005   | 1.56    | 0.72  | 0.006   | 1.42    | 0.54  | 0.005   |
| GM-CSF (pg/mL)   | 0.54    | 0.02  | 0.003   | 0.36    | 0.19  | 0.004   | 0.36    | 0.19  | 0.003   |

P-values calculated by paired Student t test and Wilcoxon signed rank test (where appropriate). Data are presented as mean ± SD.
a potential mechanism that may contribute to hypoglycemia in the clinical setting of sepsis (39). Studies in rats have also shown that induction of sepsis increased glucose uptake by 67%, secondary to increased GLUT1 expression (40), and studies in dogs have reported that TNF-α may directly stimulate glucose uptake (41). Taken together, these observations suggest that TNF-α may inflect a bimodal metabolic response, which may decrease as well as increase peripheral muscle insulin sensitivity. In our study, increased insulin sensitivity was not mediated by increased GLUT1 expression.

The mechanisms whereby TNF-α leads to an increase in muscle glucose uptake and insulin sensitivity are not clear. In this context, the recorded increase in IL-6 release from the TNF-α–infused leg is of particular interest; previous studies have shown an increased circulating level of IL-6 after systemic TNF-α administration (6), and our study complements these observations by showing a substantial increase in IL-6 release from the working muscle (42). A number of studies suggest that IL-6 may act to increase muscle insulin sensitivity. Treatment of muscle cells in vitro with IL-6 increased both basal glucose uptake and GLUT4 translocation from intracellular compartments to the plasma membrane, and infusion of IL-6 into healthy humans during a clamp increased glucose infusion rate (43). When IL-6 was infused into femoral artery in healthy subjects, there was no effect on leg glucose metabolism; this design, however, did not involve a saline-infused control leg, and it remains possible that local insulin-like effects of IL-6 were counterbalanced by a concomitant systemic stress hormone response with elevations of, for example, epinephrine, growth hormone, and cortisol (44). The concept that IL-6 mediates the insulin-like actions of TNF-α is not supported by the fact that phosphorylation of AMPK remained unchanged in the TNF-α–treated leg; systemic IL-6 infusion has been demonstrated to increase skeletal muscle AMPK (43).

The observed increase in insulin sensitivity was not reflected in altered intramyocellular insulin signaling to GLUT4 translocation and PDH activation. It has been

FIG. 2. Intramyocellular signaling during infusion of TNF-α in one femoral artery and saline in the other femoral artery in healthy volunteers. Muscle biopsies were obtained simultaneously from both lateral vastus muscles at t = 120 min (basal period) and t = 210 min (clamp). Phosphorylation of Akt Ser473 (A), AS160 (B), PDH site 1 (C) and 2 (D), AMPK (E), ACC (F), and mTOR (G); expression of GLUT1 (H) and GLUT4 (I). White bars, basal period; black bars, clamp. *P value <0.05, basal vs. clamp.
shown that systemic infusion of TNF-α inhibits Akt protein phosphorylation (6) in humans, but it is likely that those observations may also relate to systemic stress hormone release, because TNF-α, in fact, has been shown to induce Akt phosphorylation in cultured myoblasts (45). Our study examined the direct effect of TNF-α in the human leg without any systemic interference and clearly shows that the direct effect of TNF-α is to improve muscle insulin sensitivity in the presence of increased local IL-6 release and unaltered intramyocellular insulin signaling.

In accordance with studies using systemic TNF-α administration, we also recorded increased muscle protein breakdown after local TNF-α exposure; despite more modest increments in muscle protein synthesis, TW ANOVA revealed an overall catabolic increase in net breakdown. The pathophysiological mechanisms underlying proteolysis and protein synthesis are unknown. Our results suggest that the direct effects of TNF-α to induce muscle proteolyis and protein synthesis are not dependent on stimulation of intramyocellular mTOR signaling. TNF-α has been shown to induce proteolysis by urokinase-type plasminogen activator and β-catenin activation (46), and inducible nitric oxide synthase has been shown to be an important mediator of TNF-α–induced cachectic muscle loss (47). Muscle protein loss in septic shock patients was shown to be associated with elevated IL-6 levels, and addition of anti–IL-6 to septic shock plasma reduced the loss of myosin by ~25% (48). In addition, IL-6 has been shown to decrease amino acid concentrations and muscle protein turnover, with a subsequent small increase in net muscle loss (49). It is of interest that TNF-α increased leg release of IL-8. Exercise has shown to induce IL-8 expression in human skeletal muscle (50). To our knowledge, no specific metabolic role has been assigned to IL-8, but it appears attractive to speculate that the increased IL-8 levels observed in some studies of diabetes and poor metabolic control, to some extent, merely reflect low-grade inflammation and increased TNF-α activity.

In the current study, we used a novel method to study the direct effects of TNF-α in the bilaterally perfused placebo-controlled leg. The method has been validated and is well suited for investigations of metabolites and hormones with a short half-life and a good penetration into muscle and adipose tissue (31). It is a methodological strength that it excludes day-to-day intrapersonal variability and minimizes analytical variation. On the other hand, the a-v model has some limitations. One of the most prominent is the large variability in blood flow, which can introduce a substantial variability in calculated a-v substrate fluxes. In our experience, pure a-v concentration differences are generally less prone to type 2 errors. In our studies, we opted for a constant intra-arterial infusion of 6 ng/kg/h for 6 h of TNF-α. This was based on previous systemic studies (15,16), considering that femoral artery blood flow only represents a minor proportion of cardiac output. It remains possible that other modes of administration, other dosages, and other time points of sampling would have yielded other results. Thus, it should be underlined that data obtained during acute elevation of TNF-α with a continuous infusion in the leg of very healthy subjects do not necessarily apply to conditions in obese and prediabetic patients with chronic low-grade systemic inflammation.

In summary, constant intra-arterial TNF-α infusion directly increases insulin-stimulated glucose uptake and net phenylalanine release in the perfused human leg without detectable effects on FFA metabolism or lactate release. These effects could, to some extent, be due to increased local IL-6 release and activity. The finding of paradoxically increased insulin sensitivity is of major clinical interest, albeit the precise metabolic mechanisms must be defined in the process of targeting specific insulin mimetic agents.

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E.B. wrote the protocol, performed experiments, analyzed the data, performed statistical analyses, and wrote the manuscript. R.R.N. performed experiments and reviewed the manuscript. M.H.V. and A.B.M. performed laboratory experiments and reviewed the manuscript. N.J., M.B., T.-K.H., L.H., S.B.P., H.P., and R.S.B. contributed to performing the experiments and reviewed the manuscript. J.O.L.J. reviewed and edited the manuscript. N.M. wrote the protocol, analyzed the data, performed statistical analyses, and wrote the manuscript. N.M. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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