# Skeletal Muscle Interleukin-6 and Tumor Necrosis Factor-$\alpha$ Release in Healthy Subjects and Patients With Type 2 Diabetes at Rest and During Exercise

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**To examine the influence of type 2 diabetes on cytokine release from the leg at rest and during exercise, 9 male type 2 diabetics (D) and 8 age-, gender-, $Vo_{2peak}$-, weight- and body mass index (BMI)-matched control subjects (C) were studied before and after 25 minutes of supine bicycle exercise at 60% $Vo_{2peak}$. Blood samples were obtained from a femoral artery and vein from 1 limb, and plasma was analyzed for glucose and the cytokines, interleukin (IL)-6 and tumor necrosis factor (TNF)-$\alpha$. Leg blood flow (LBF) was measured by thermodilution in the femoral vein, and net leg IL-6, TNF-$\alpha$, and glucose balance were calculated as the product of LBF and femoral arteriovenous (fa-v) glucose, IL-6, and TNF-$\alpha$ difference. Arterial plasma glucose and IL-6 were higher ($P < .05$) at rest in D compared with C, but there were no differences in arterial TNF-$\alpha$ concentrations at rest when comparing groups. Despite measurable arterial levels of both IL-6 and TNF-$\alpha$ in both groups at rest, there was not net leg release of either cytokine at rest. Exercise increased ($P < .05$) IL-6 release and glucose uptake in both D and C, and contracting leg glucose uptake was similar when comparing D with C. While not significant, there was a trend ($P = .1$) for augmented exercise-induced IL-6 release in D compared with C. In contrast, exercise did not result in TNF-$\alpha$ release in either D or C. These data demonstrate that basal circulating TNF-$\alpha$ is not elevated in patients with type 2 diabetes when matched for BMI with control subjects. The results also suggest that neither type 2 diabetic nor healthy skeletal muscle releases these cytokines at rest, indicating that organs other than skeletal muscle contribute to the elevated basal IL-6 in type 2 diabetics. In contrast with IL-6, exercise does not result in the release of TNF-$\alpha$ from the contracting limbs of either healthy subjects or patients with type 2 diabetes.**



THE INFLAMMATORY cytokines, tumor necrosis factor (TNF)-$\alpha$ and interleukin (IL)-6 have been associated with insulin resistance, obesity, and type 2 diabetes (for review, see Febbraio and Pedersen[1]). The role of TNF-$\alpha$ in the etiology of insulin resistance has been the subject of numerous investigations, and while it is generally well accepted that TNF-$\alpha$ is associated with insulin resistance in rodents (for review, see Moller[2]), the effect of TNF-$\alpha$ in the pathogenesis of human type 2 diabetes is the subject of controversy. TNF-$\alpha$ gene expression is augmented in the skeletal muscles of patients with type 2 diabetes,[3] while TNF-$\alpha$ secretion from adipose tissue is greater in these patients.[4] In addition, a marked reduction in TNF-$\alpha$ mRNA in morbidly obese patients after weight loss surgery positively correlates with an increase in GLUT4 mRNA in skeletal muscle.[5] However, while some studies have observed plasma TNF-$\alpha$ to be elevated in patients with type 2 diabetes,[6-8] others do not.[4,9] In addition, when TNF-$\alpha$ is pharmacologically blocked in patients with type 2 diabetes, insulin sensitivity is unaffected.[10] Hence, further studies examining the relationship between type 2 diabetes and circulating TNF-$\alpha$ in humans are warranted. Less is known of the association between IL-6 and insulin resistance and/or type 2 diabetes. Although type 2 diabetes is associated with an IL-6 gene polymorphism,[11] higher plasma concentrations of IL-6[12,13] and IL-6 release from adipose tissue,[14] there is no direct evidence that IL-6 causes insulin resistance, particularly in skeletal muscle. In fact, to our knowledge, no previous studies have examined skeletal muscle IL-6 expression or protein release in insulin-resistant muscle.

We have recently demonstrated that IL-6 is expressed in resting human skeletal muscle,[15,16] and that muscle is capable of releasing this protein.[16,17] Although it has been demonstrated that adipose tissue release of IL-6 contributes to circulating IL-6 levels at rest, whether the skeletal muscle contributes to this release and whether any release is augmented in patients with type 2 diabetes is unknown. Likewise, although TNF-$\alpha$ expression in skeletal muscle is augmented in patients with type 2 diabetes,[3] whether this increased expression results in an increase in protein release, thereby contributing to the elevated circulating TNF-$\alpha$ levels often observed in patients with type 2 diabetes, is unclear.

Recent studies have demonstrated that muscle contraction rapidly increases IL-6 gene expression,[15-18] the nuclear transcriptional activity of IL-6,[18] and IL-6 protein release from skeletal muscle.[16,17] Although TNF-$\alpha$ is expressed in skeletal muscle, no studies have examined whether contraction releases this cytokine from skeletal muscle in a manner similar to that of IL-6. Hence, the aim of the present study was to examine the release of IL-6 and TNF-$\alpha$ from skeletal muscle at rest and during exercise in healthy subjects and patients with type 2 diabetes.

## MATERIALS AND METHODS

### *Subjects*

Nine type 2, diabetic males aged 48 ± 4 (mean ± SD) and 8 controls (46 ± 5 years) participated in the study, which was part of a larger study [19] approved by the Alfred Hospital Ethics Committee, and

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conducted in accordance with the Declaration of Helsinki. All subjects were nonsmokers, free of coronary disease with a body mass index (BMI) $< 35\ kg \cdot m^{-2}$. Control subjects did not take any medication and had fasting and post-2-hour 75-g oral glucose load plasma glucose levels $< 6.1\ mmol/L^{-1}$, while type 2 diabetics had fasting plasma glucose $> 7\ mmol/L^{-1}$ and/or post-75-g oral glucose load plasma glucose levels of $> 11.1\ mmol/L^{-1}$. Of the type 2 diabetics, 7 were controlled by diet, and 2 were medicated with metformin (half-life 3.5 hours), and 1 of these was also taking gliclazide (half-life 12 hours). These 2 subjects who were medicated had metabolic responses similar to the 7 nonmedicated subjects.[19] All were normally active, but were not specifically exercise trained.

### *Subject Preparation*

The 2 medicated diabetics did not take their medication the night prior to or the morning of the screening or experimental days, and thus had a 24 hour drug-free period before all measurements. Peak pulmonary oxygen uptake ($V_{O_{2peak}}$) was determined during continuous incremental upright cycling to volitional exhaustion on an electronically-braked ergometer (Ergo-line 900 ergometer; Bitz, West Germany). Expired air was analyzed for volume, $O_2$, and $CO_2$ using calibrated analyzers (Cosmed Quark; $b^2$, Rome, Italy). During a subsequent visit, subjects were familiarized with supine cycling during a 30-minute bout on an electronically-braked ergometer (Siemens-Elema; 380B ergometry system, Väsby, Sweden) at a workload eliciting 60% of the upright $V_{O_{2peak}}$ as previously described.[19,20]

### *Experimental Design and Procedures*

On the experimental day, subjects performed a 25-minute bout of supine cycling at 60% ± 2% $V_{O_{2peak}}$. Leg blood flow (LBF) was measured and arterial and venous femoral blood was obtained at rest and immediately before the cessation of exercise. Subjects were requested to refrain from exercise, alcohol, and caffeine for 24 hours before the experimental trial. After an overnight fast, subjects attended the Alfred Hospital at 8 AM. Teflon catheters were placed in the right femoral artery (3.0F, Cook Australia, Brisbane, Australia) and right femoral vein (4.0F, Cook Australia) under local anesthetic (1% lidocaine; Astra, Sydney, Australia) using strict aseptic conditions. The artery was cannulated ~ 2 cm and the vein ~ 4 cm below the inguinal ligament, and the catheters advanced 10 cm and 4 cm centrally, respectively. A thermistor probe (Edslab 94-030-2.5F; Baxter Healthcare, Irvine, CA) was inserted through a side arm in the venous catheter and advanced ~ 8 cm beyond the catheter tip.[21] After resting for ~ 30 minutes, LBF was measured, and blood samples were simultaneously obtained from the 2 catheters. Subjects then cycled at the predetermined workload for 25 minutes. Immediately prior to the cessation of exercise, these sampling procedures were repeated.

### *LBF*

Right femoral venous blood flow was measured by constant-rate infusion of cold saline according to the thermodilution principle.[22,23] Cold (5°C to 8°C) saline was drawn from a reservoir and then immediately infused through the tip of the femoral venous catheter, which was perforated with 4 side holes to facilitate infusate dispersion using an Angiomat 3000 Injector (Leibel-Flarsheim; Sybron, Cincinnati, OH). At rest, LBF was measured at an infusion rate of $0.7\ mL \cdot s^{-1}$ for 20 seconds. During exercise, LBF was measured using a constant infusion rate of between 1.5 to 2.4 $mL \cdot s^{-1}$ for 15 seconds, titrated to produce ~ 0.9°C to 1.2° C decrease in blood temperature.[22] The coefficient of variation in blood flow measurement during exercise was 6.7%.[19]

### *Blood Sampling and Analysis*

Simultaneous blood samples were drawn from the femoral artery and vein at rest and immediately before the cessation of exercise. At each sampling time, 4 mL blood was placed in a fluoride/oxalate tubes, placed on ice, then centrifuged at 1,500*g* at the cessation of each trial with the plasma frozen at −80°C for later glucose analysis using enzymatic, spectrophotometric techniques with a Cobas-BIO centrifugal analyzer (Roche Diagnostic Systems, Basel, Switzerland). Blood samples for IL-6 and TNF-$\alpha$ measurement were drawn into precooled glass tubes containing EDTA. The tubes were spun immediately at 1,500*g* for 15 minutes at 4°C, and the plasma was stored at −80°C until analyses were performed. Enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) were used to measure IL-6 and TNF-$\alpha$ in plasma. According to R&D Systems, the ELISA kits are insensitive to the addition of the recombinant forms of the soluble receptors (sIL-6R) and (sTNF-$\alpha$ R) and the measurements, therefore, correspond to both soluble and receptor-bound specific cytokines. The intra-assay coefficient of variation for the kit is <6%.[24] The product of arterial-venous (a-v) difference and LBF was used to calculate leg glucose uptake and leg net IL-6 and TNF-$\alpha$ release (v-a).

### *Statistics*

Descriptive data are presented as means ± SD while all comparative data are expressed as means ± SEM. Group characteristics were compared using unpaired *t* tests. Resting and postexercise measures were compared using a 2-way (group x time) analysis of variance (ANOVA) with repeated measures. A SigmaStat (version 2.03, SPSS, Chicago, IL) program was used to compute these statistics.

## RESULTS

There were no differences in age (46 ± 2 *v* 48 ± 1 years), weight (85.5 ± 4.5 *v* 88.2 ± 4.8 kg) or body mass index (25.4 ± 1.0 *v* 28.1 ± 1.4 $kg \cdot m^{-2}$) for C and D, respectively. C and D exercised at the same absolute (91 ± 8 *v* 97 ± 7 W) and relative workloads (61 ± 2 *v* 60% ± 2% $V_{O_{2peak}}$) for C and D, respectively. LBF was not different between C and D before exercise (0.35 ± 0.05 *v* 0.44 ± 0.06; $l \cdot min^{-1}$ for D and C), but was lower ($P < .05$) in diabetics after 25 minutes of exercise (2.82 ± 0.20 *v* 3.37 ± 0.36; $l \cdot min^{-1}$ for D and C).

At rest arterial glucose concentration was higher ($P < .01$) in D compared with C, but leg glucose uptake was similar despite trends ($P = .07$) for greater leg a-v glucose difference in D compared with C. Arterial glucose concentration decreased from rest at the end of exercise in diabetics ($P < .01$), but not controls and remained higher ($P < .05$) in the D compared with the C during both trials ($P < .05$). Due to the lower LBF in D, mean glucose delivery at the end of exercise was not, however, different between C and D. Glucose uptake increased ($P < .05$) with exercise to a similar degree in both groups due to increases in both a-v glucose difference and LBF. Neither a-v glucose difference nor glucose uptake during exercise was different (0.94 ± 0.25 *v* 0.76 ± 0.12 $mmol \cdot min^{-1}$ for C and D, respectively) (Fig 1).

Arterial plasma IL-6 was higher ($P < .05$) in D compared with C at rest (Fig 2). However, no differences in arterial plasma IL-6 were observed when comparing D with C after exercise. No differences were observed in a-v IL-6 difference at rest or following exercise when comparing D with C. In contrast to arterial plasma IL-6 at rest, net IL-6 release was not different when comparing D with C at rest. However, IL-6

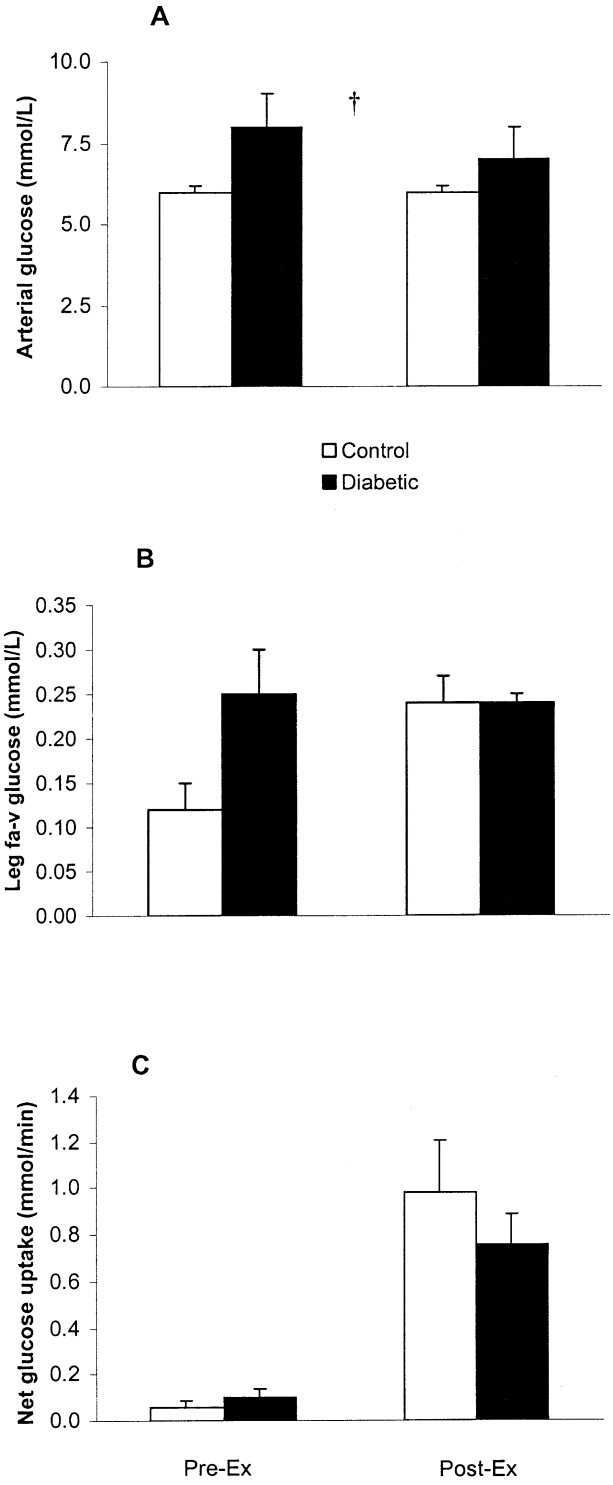


Fig 1. (A) Arterial glucose, (B) leg femoral arteriovenous glucose difference, and (C) net leg glucose uptake before (Pre-Ex) and after (Post-Ex) 25 minutes of supine bicycle exercise at 60% $Vo_{2peak}$ in type 2 diabetic subjects (n = 9) and matched healthy control subjects (n = 8). †Significant difference ($P < .05$) between groups (values expressed as mean ± SE).

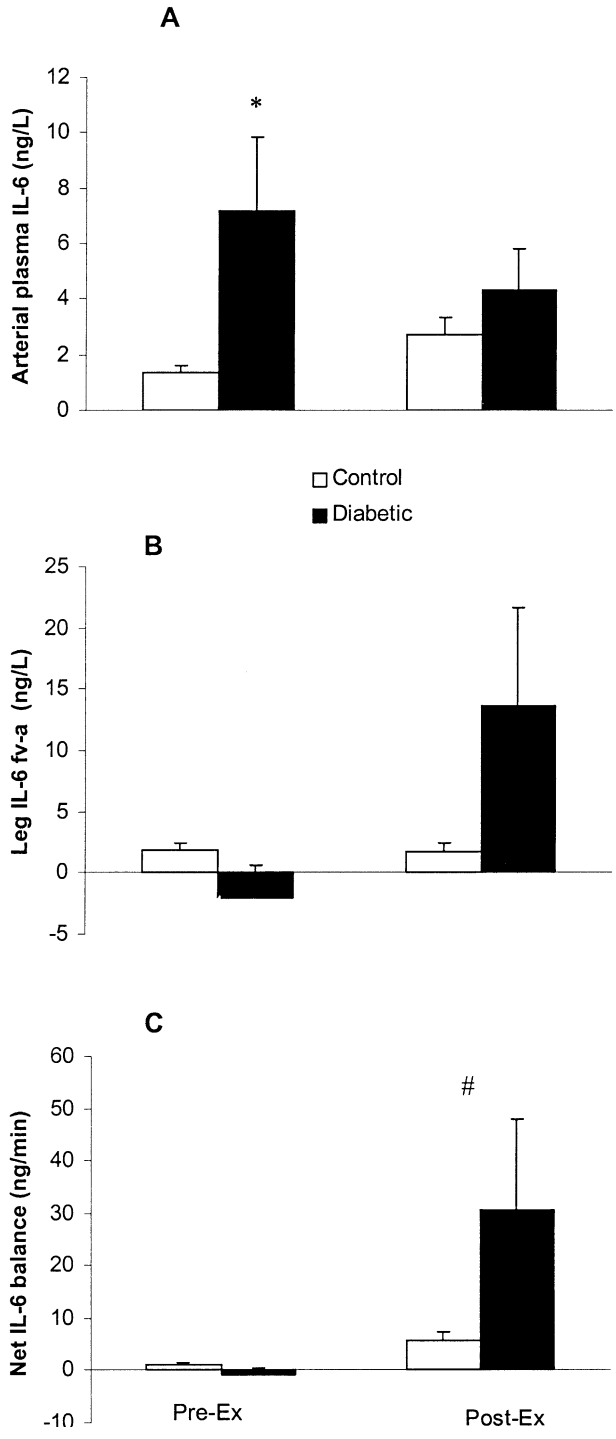


Fig 2. (A)Arterial interleukin (IL)-6, (B) leg femoral arteriovenous IL-6 difference, and (C) net leg IL-6 balance before (Pre-Ex) and after (Post-Ex) 25 minutes of supine bicycle exercise at 60% $Vo_{2peak}$ in type 2 diabetic subjects (n = 9) and matched healthy control subjects (n = 8). *Difference ($P < .05$) between groups Pre-Ex; #denotes denotes main effect ($P < .05$) for exercise (values expressed as mean ± SE).

release increased ($P < .05$) when comparing post- with pre-exercise levels in both groups. In addition, while not statistically significant, IL-6 release tended ($P = .1$) to be greater in D compared with C at the end of exercise (Fig 2). Arterial plasma TNF-$\alpha$ was not different when comparing D with C at rest. Furthermore, exercise did not alter arterial plasma TNF-$\alpha$ levels in either D nor C. There was no difference in leg a-v TNF-$\alpha$ difference when comparing D with C. As a consequence, TNF-$\alpha$ was not released across the leg at rest or after exercise in either D or C (Fig 3).

## DISCUSSION

The results from this study demonstrate that when patients with type 2 diabetes are well matched to a control group basal IL-6, but not TNF-$\alpha$, levels are elevated in the patient population. Our data also provide evidence that skeletal muscle does not contribute to the circulating IL-6 or TNF-$\alpha$ at rest and, therefore, it is not the organ responsible for the elevated circulating IL-6 at rest in D. Furthermore, in contrast with IL-6, TNF-$\alpha$ is not released by skeletal muscle during exercise in either healthy subjects or patients with type 2 diabetes.

The higher arterial plasma IL-6 concentration in D compared with C is consistent with previous observations.[4,12,14] No data have previously examined basal IL-6 flux from skeletal muscle in diabetic patients, but because we observed no net leg release of IL-6 at rest in either group (Fig 2), our data suggest that the skeletal muscle is unlikely to contribute to the circulating IL-6 at rest or is it likely to be the organ responsible for the higher IL-6 in diabetic patients. Of note, arterial plasma IL-6 decreased during exercise in D, but increased in C (Fig 2). This was a somewhat surprising result, but may be explained in the following manner. Type 2 diabetes is associated with a marked increase in IL-6 secretion from adipose tissue.[4,14] However, Lyngsø et al[25] recently demonstrated that IL-6 secretion by adipose tissue is totally suppressed during exercise. In addition, IL-6 clearance by the hepatosplanchnic viscera is marked during exercise.[26] Therefore, in patients with type 2 diabetes, the reduction in adipose tissue IL-6 secretion and increase in IL-6 clearance that accompanies exercise may have a greater quantitative effect compared with the increase in net IL-6 release from the contracting limb.

Consistent with our previous observations,[16,17] we found that IL-6 was released by the leg in both D and C during exercise (Fig 2). In our previous studies, however, IL-6 was not released from skeletal muscle until subjects had exercised for at least 60 minutes.[16,17] In contrast, in the present study, we observed marked IL-6 release after only 25 minutes of exercise (Fig 2). An important difference between our previous[16,17] and current study is the mode of exercise. In our previous studies, we used the 2-legged knee extensor model, which recruits the knee extensor muscles alone. In the present study, subjects performed bicycle exercise, which results in the recruitment of most lower limb muscles. Hence, our data suggest that IL-6 production is related to the mass of muscle recruited. The fact that skeletal muscle releases IL-6 during contraction is a relatively recently observed phenomenon (for review, see Febbraio and Pedersen[1]) and the biologic significance of IL-6 production and release by skeletal muscle has not been fully elucidated.

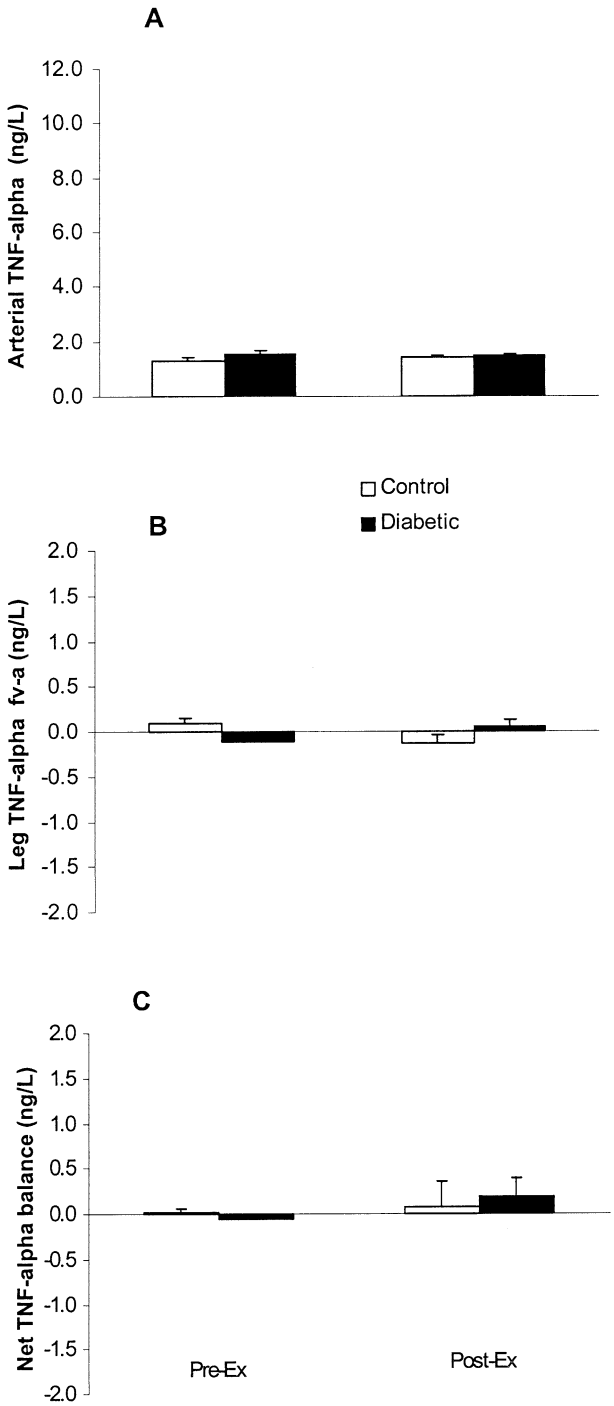


**Fig 3. (A) Arterial TNF-$\alpha$, (B) leg femoral arteriovenous TNF-$\alpha$ difference, and (C) net leg TNF-$\alpha$ balance before (Pre-Ex) and after (Post-Ex) 25 minutes of supine bicycle exercise at 60% $Vo_{2peak}$ in type 2 diabetic subjects (n = 9) and matched healthy control subjects (n = 8) (values expressed as mean ± SE).**

Although not statistically significant, an interesting observation was the trend ($P = .1$) for augmented IL-6 release after exercise in D compared with C (Fig 2). Despite the fact that mean net IL-6 release was more than 2-fold higher postexercise in D

compared with C, the lack of statistical significance was probably due to the fact that in D the range was very broad (1.5 to 141.2 ng · min$^{-1}$). This suggests that while some patients with type 2 diabetes have a marked IL-6 release during exercise, others have a normal response. The results from the present study can neither determine the mechanism nor the biologic significance of this observation. There is some evidence that IL-6 can increase whole body glucose disposal in humans[27] and glucose uptake in cultured adipocytes,[28] while an IL-6–deficient mouse becomes insulin resistant,[29] although the mechanisms for these findings have not been elucidated. The exercise-induced glucose uptake was normal when comparing D with C (Fig 1), a finding consistent with previous observations.[30] However, in a concurrent study in the same cohort of subjects, Kingwell et al[19] recently demonstrated that while the nitric oxide inhibitor L-NMMA decreased glucose uptake in both D and C, it did so to a greater extent in D, leading to the suggestion that type 2 diabetic muscle relies on nitric oxide for glucose uptake to a greater extent than does healthy muscle. Perhaps it may also rely more heavily on IL-6 in a similar manner. This suggestion is, however, speculative and warrants investigation.

A major finding from the present study was that resting arterial TNF-$\alpha$ concentrations were not different when comparing groups (Fig 3). This observation is in agreement with some,[4,9] but not other[6-8] previous studies. Of note, in the present study, we matched our subjects for most parameters, including BMI. Our data suggest, therefore, that in well-matched subjects, type 2 diabetes does not result in elevated systemic levels of TNF-$\alpha$. TNF-$\alpha$ was also not released by the leg at rest, suggesting that like IL-6, the muscle is unlikely to contribute to basal TNF-$\alpha$ levels in the circulation.

We have previously observed that in some circumstances exercise can result in an increase in circulating TNF-$\alpha$.[24] It is well known that cytokines are produced in many cells and/or tissues in response to stress and given that muscle contraction can markedly disrupt muscle cell homeostasis, it was possible that such cells may produce TNF-$\alpha$ during contraction. It is clear from this study that, unlike IL-6, TNF-$\alpha$ is not released by skeletal muscle in response to contraction in either healthy or type 2 diabetic muscle. Hence, our data provide evidence that the patterns of IL-6 and TNF-$\alpha$ release are very different during exercise.

In conclusion, this study demonstrates that when type 2 diabetes are well matched, they have higher circulating IL-6, but not TNF-$\alpha$, levels in the basal state. Our data also provide evidence that skeletal muscle does not contribute to the circulating IL-6 or TNF-$\alpha$ at rest and, therefore, it is not the organ responsible for the elevated circulating IL-6 at rest in D. Furthermore, in contrast with IL-6, TNF-$\alpha$ is not released by skeletal muscle during exercise.

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