The vascular actions of insulin may contribute to the increase in glucose uptake by skeletal muscle. We have recently shown that when capillary recruitment by insulin is blocked in vivo, an acute state of insulin resistance is induced. Another agent that may have vascular effects is the inflammatory cytokine tumor necrosis factor-α (TNF-α), which has been reported to play an important role in the insulin resistance of obesity, type 2 diabetes, and sepsis in both animals and humans. Thus, in the present study, we have investigated the effect of an intravenous 3-h TNF treatment (0.5 µg · h⁻¹ · kg⁻¹) in control and euglycemic-hyperinsulinemic–clamped (10 mU · min⁻¹ · kg⁻¹ for 2 h) anesthetized rats. Hind-leg glucose uptake, muscle uptake of 2-deoxyglucose (2-DG), femoral blood flow (FBF), vascular resistance (VR), and capillary recruitment as measured by metabolism of infused 1-methylxanthine (1-MX) were assessed. Insulin alone caused a significant (P < 0.05) increase in FBF (1.7-fold) and capillary recruitment (2.5-fold), with a significant decrease in VR. In addition, hind-leg glucose uptake was increased (fourfold), as was 2-DG uptake in the soleus and plantaris muscles. TNF completely prevented the insulin-mediated increase in glucose uptake by skeletal muscle (19–21). At the isolated cellular level, it has been shown that 3–5 days exposure of 3T3-L1 or 3T3-F442A adipocytes to TNF causes reductions in insulin receptor and insulin receptor substrate-1 tyrosine phosphorylation in response to a maximal dose of insulin (10,11). Yet other researchers have shown that 3–4 days of exposure of 3T3-L1 adipocytes to TNF gives rise to large decreases in GLUT4, insulin receptor, and insulin receptor substrate-1 mRNA and protein (12,13). Shorter exposures have also been claimed to decrease insulin-stimulated tyrosine phosphorylation of the insulin receptor and insulin receptor substrate-1 in Fao hepatoma cells (14,15) and NIH3T3 fibroblasts (16), as well as insulin-stimulated glucose transport in L6 myocytes (17). However, direct effects of TNF on muscle rather than cell lines are less certain. Recently, Nolte et al. (18) showed that exposure of isolated soleus muscles to 6 nmol/l TNF for 45 min had no effect on insulin-stimulated tyrosine phosphorylation of the insulin receptor or insulin receptor substrate-1 or on phosphatidylinositol 3-kinase association with the insulin receptor substrate-1. More importantly, incubation of the epitrochlearis and soleus muscles with 6 nmol/l TNF for 45 min or 4 h or incubation of the epitrochlearis muscles with 2 nmol/l for 8 h had no effect on insulin-stimulated 2-deoxyglucose uptake.

Attempts to induce muscle insulin resistance by TNF administration have led to mixed success, and the mechanism by which TNF may cause insulin resistance is not clear. Administration of TNF over 3 h to anesthetized rats under clamp conditions markedly reduced insulin-mediated uptake of 2-deoxyglucose by muscle (9). At the isolated cellular level, it has been shown that 3–5 days exposure of 3T3-L1 or 3T3-F442A adipocytes to TNF causes reductions in insulin receptor and insulin receptor substrate-1 tyrosine phosphorylation in response to a maximal dose of insulin (10,11). Yet other researchers have shown that 3–4 days of exposure of 3T3-L1 adipocytes to TNF gives rise to large decreases in GLUT4, insulin receptor, and insulin receptor substrate-1 mRNA and protein (12,13). Shorter exposures have also been claimed to decrease insulin-stimulated tyrosine phosphorylation of the insulin receptor and insulin receptor substrate-1 in Fao hepatoma cells (14,15) and NIH3T3 fibroblasts (16), as well as insulin-stimulated glucose transport in L6 myocytes (17). However, direct effects of TNF on muscle rather than cell lines are less certain. Recently, Nolte et al. (18) showed that exposure of isolated soleus muscles to 6 nmol/l TNF for 45 min had no effect on insulin-stimulated tyrosine phosphorylation of the insulin receptor or insulin receptor substrate-1 or on phosphatidylinositol 3-kinase association with the insulin receptor substrate-1. More importantly, incubation of the epitrochlearis and soleus muscles with 6 nmol/l TNF for 45 min or 4 h or incubation of the epitrochlearis muscles with 2 nmol/l for 8 h had no effect on insulin-stimulated 2-deoxyglucose uptake.

Studies in this laboratory, as well as others, have shown that in addition to its many direct metabolic actions on skeletal muscle, insulin also has hemodynamic effects that may increase access of insulin and glucose to muscle (19–21).
Insulin’s hemodynamic effects comprise two components. One is concerned with increasing the total blood flow to skeletal muscle via a nitric oxide–dependent vasodilatation (22). Thus, when a nitric oxide synthase inhibitor was present, insulin-mediated glucose uptake was blocked by ~30% (19). The second component involves an increased capillary recruitment (or nutritive flow) within skeletal muscle (21), which has now been detected using different methodologies in anesthetized rats (1-methylxanthine [1-MX] metabolism) (21) and human forearm (micro-bubbles and contrast enhanced ultrasound) (23) and may be independent of the first (21, 23).

Measurement of capillary exposure (or nutritive flow) in anesthetized rats was assessed using 1-MX metabolism. Metabolism of this exogenously added substrate for capillary endothelial xanthine oxidase was shown to increase in the presence of insulin (21). In addition, if α-methyl serotonin (α-met5HT), an agent that prevented capillary recruitment, was administered, the ability of insulin to increase either total blood flow or capillary recruitment was markedly impaired and insulin-mediated glucose uptake was blocked by 60% (24).

Thus, mindful on the one hand of the strong association of TNF with insulin resistance in vivo, and on the other with the failure of TNF to cause insulin resistance when incubated with isolated muscles, we undertook the present study to assess whether TNF could induce insulin resistance in vivo by influencing hemodynamic parameters.

**RESEARCH DESIGN AND METHODS**

**Animals.** Male hooded Wistar rats weighing 245 ± 3 g were raised on a commercial diet (Gibsons, Hobart, Australia) containing 21.4% protein, 4.6% lipid, 68% carbohydrate, and 6% crude fiber with added vitamin and minerals together with water ad libitum. Rats were housed at a constant temperature of 21 ± 1°C in a 12 h light/dark cycle. All procedures adopted and experiments undertaken were approved by the University of Tasmania Ethics Committee.

**Surgery.** Rats were anesthetized using Nembutal (50 mg/kg body weight) and had polyethylene cannulas (PE-50, Intramedic) surgically implanted into the carotid artery for arterial sampling and measurement of blood pressure (pressure transducer Transpac IV, Abbott Critical Systems) and into both jugular veins for continuous administration of anesthetic and other intra-venous infusions. A tracheotomy tube was inserted, and the animal was allowed to spontaneously breathe room air throughout the course of the experiment. Small incisions (1.5 cm) were made in the skin overlaying the femoral vessels and epigastric vessels were ligated, and an ultrasonic flow probe (Transonic Systems, VB series 0.5 mm) was positioned around the femoral artery of the right leg just distal to the rectus abdominus muscle. The cavity in the leg surrounding the flow probe was filled with lubricating jelly (H-R; Mohawk Medical Supply, Utica, NY) to provide acoustic coupling to the probe. The probe was then connected to the flow meter (Model T186 ultrasonic volume flow meter, Transonic Systems). This was in turn interfaced with an IBM-compatible computer, which acquired the data (at a sampling frequency of 100 Hz) for femoral blood flow, heart rate, and blood pressure using WINDAQ data acquisition software (DATALOG Instruments). The surgical procedure generally lasted ~30 min, and then the animals were maintained under anesthesia for the duration of the experiment using a continual infusion of Nembutal (0.0 mg · min⁻¹ · kg⁻¹) via the left jugular cannula. The femoral vein of the left leg was used for venous sampling, using an insulin syringe with an attached 29G needle (Becton Dickinson). A duplicate venous sample was taken only on completion of the experiment (120 min) to prevent alteration of the blood flow from the hind limb due to sampling and to minimize the effects of blood loss. The body temperature was maintained using a water-jacketed platform and a heating lamp positioned above the rat.

**Experimental procedures.** Once the surgery was completed, a 60-min equilibration period was allowed so leg blood flow and blood pressure could become stable and constant. Rats were then allocated into either the control group (saline or TNF alone) as shown in Protocol A (Fig. 1) or the euglycemic insulin clamp group (insulin alone or TNF + insulin) as shown in Protocol B (Fig. 1) (n = 6–10 in each group). Saline and TNF infusions in the control groups were matched to the volumes of insulin (Humulin R; Eli Lilly, Indianapolis, IN) and glucose infused in the euglycemic insulin clamp. TNF (mouse recombinant, Sigma Aldrich) was dissolved in saline and 0.1% bovine serum albumin. Because 1-MX (Sigma Aldrich) clearance was very rapid, it was necessary to partially inhibit the activity of xanthine oxidase (21). To do this, an injection of a specific xanthine oxidase inhibitor, allopurinol (25) (10 µmol/kg), was administered as a bolus dose 5 min before commencing the 1-MX infusion (0.4 mg · min⁻¹ · kg⁻¹) (Fig. 1). This allowed constant arterial concentrations of 1-MX to be maintained throughout the experiment.

At 45 min before the completion of the experiment, a 50 µCi bolus of 2-deoxy-O-[125I]-Glucose (2DG) (specific activity = 44.0 Ci/mmol, Amersham Life Science) in saline was administered. Plasma samples (20 µl) were collected at 15, 30, and 45 min to determine plasma clearance of the radioactive. At the conclusion of the experiment, the soleus and plantaris muscles were removed, clamp-frozen in liquid nitrogen, and stored at –80°C until assayed for [3H]-2DG uptake.

The total blood volume withdrawn from the animals before the final arterial and venous samples did not exceed 1.5 ml and was easily compensated by the volume of fluid infused.

Duplicate arterial and venous samples (300 µl) were taken at the end of the experiment (180 min) and placed on ice. These blood samples were immediately centrifuged, and 100 µl of plasma was mixed with 20 µl of 0.1 M perchloric acid. The perchloric acid–treated samples were then stored at –20°C until assayed for 1-MX. The rest of the plasma was used for plasma glucose analysis and plasma insulin analysis.

**Analytical methods.** A glucose analyzer (Model 2300 Stat plus; Yellow Springs Instruments, Yellow Springs, OH) was used to determine whole blood glucose (by the glucose oxidase method) during the insulin clamp. A blood sample was required for each determination. Human insulin levels at the end of the euglycemic insulin clamp were determined from arterial plasma samples by ELISA assay (Dako Diagnostics, U.K.), using human insulin standards. Perchloric acid–treated plasma samples were centrifuged for 10 min and the supernatant used to determine 1-MX, allopurinol, and oxypurinol concentrations by reverse-phase high-performance liquid chromatography (HPLC) as previously described (21, 24).

**2-DG uptake assay.** The frozen soleus and plantaris muscles were ground under liquid nitrogen and homogenized using an Ultra Turrax. Free and phosphorylated [3H]-2DG were separated by ion exchange chromatography using an anion exchange resin (AG1-X8) (26, 27). Biodegradable Counting Scintillant-BCA (Amersham, Arlington Heights, IL) was added to each radioactive sample and radioactivity determined using a scintillation counter (Beckman LS8801). From this measurement and a knowledge of plasma glucose and the time course of plasma 2-DG disappearance, R’g, which reflects glucose uptake into the muscle, was calculated as previously described by others (26, 27).

**Data analysis.** All data are expressed as means ± SE. Mean femoral blood flow, mean heart rate, and mean arterial blood pressure were calculated from 5-s subsamples of the data, representing ~500 flow and pressure measurements every 15 min. Vascular resistance in the hind leg was calculated as mean arterial blood pressure in millimeters of mercury divided by femoral blood flow in milliliters per minute and expressed as resistance units (RUs).

Glucose uptake in the hind limb was calculated from arterial-venous (A-V) glucose difference, multiplied by femoral blood flow (corrected for the volume accessible to 1-MX, 0.871, determined from plasma concentrations obtained after additions of standard 1-MX to whole rat blood), and expressed as nanomoles per minute.

**Statistical analysis.** To ascertain differences between treatment groups at the end of the experiment (120 min), one-way analysis of variance was used. When a significant difference (P < 0.05) was found, Dunnett’s test was used to ascertain which times were significantly different from saline control (for femoral blood flow, arterial blood pressure, femoral vascular resistance, arterial glucose and 1-MX, hind-leg glucose extraction and uptake, and hind-leg 1-MX extraction and disappearance). Pair-wise comparisons were made using the Student-Newman-Keuls method. An unpaired Student’s t test was used to determine whether there was a significant difference (P < 0.05) between the glucose infusion rates at the conclusion of the experiments. All tests were performed using the SigmaStat statistical program (Jandel Software).

**RESULTS**

**Hemodynamic effects.** Figure 2 shows the femoral blood flow, mean arterial blood pressure, and hind-leg vascular resistance following saline or TNF infusions and following insulin or TNF plus insulin infusions at the completion of the experiment (120 min). TNF infusion alone had no significant effect.
on any of these hemodynamic parameters, although it did significantly \( (P < 0.05) \) decrease heart rate compared with saline infusion \((350 \pm 8 \text{ vs. } 391 \pm 13 \text{ bpm})\). Insulin infusion alone caused a significant increase \((70\%)\) in femoral blood flow \((0.91 \pm 0.11 \text{ to } 1.51 \pm 0.14 \text{ ml/min})\) by the end of the experiment. Because blood pressure was unchanged, this increase corresponded to a 35% decrease in hind-leg vascular resistance. The insulin-mediated increase in femoral blood flow and decrease in hind-leg vascular resistance was completely prevented during infusion with TNF.

**Glucose metabolism.** There was no significant difference in arterial blood glucose concentration between any of the treatment groups at either the beginning of the experiment \((\text{time } = 0 \text{ min})\) or at the end \((\text{time } = 120 \text{ min})\). During the euglycemic insulin-clamp experiments, arterial blood glucose was maintained at or above basal values by infusion of glucose. At the conclusion of the experiment, the whole-body glucose infusion rate required to maintain euglycemia was significantly higher \((17\%) \ (P < 0.001)\) during the insulin-alone infusions \((22.5 \pm 0.4 \text{ mg } \cdot \text{min}^{-1} \cdot \text{kg}^{-1})\) than during the TNF + insulin infusions \((18.6 \pm 0.4 \text{ mg } \cdot \text{min}^{-1} \cdot \text{kg}^{-1})\).

Arterial plasma insulin concentrations in the insulin-treated animals \((1,226 \pm 118 \text{ pmol/l})\) were not significantly different from the insulin + TNF \((1,388 \pm 107 \text{ pmol/l})\) infused rats at the end of the clamp.

Hind-leg glucose extraction and uptake were significantly increased during the euglycemic insulin clamps \((\text{Fig. 3})\). TNF infusion alone had no effect on hind-leg glucose extraction or uptake, but it significantly decreased both the insulin-mediated hind-leg glucose extraction \((\text{by } 37\%)\) and uptake \((\text{by } 61\%)\). This latter effect may be even greater because there was no significant difference between the hind-leg glucose uptake during the TNF + insulin infusions and the saline or TNF alone experiments; TNF-blocked extraction and flow were each contributory.

**2-DG uptake.** 2-DG was administered for the final 45 min of each experiment. Figure 4 shows uptake values for soleus and plantaris muscles removed at completion. TNF alone tended to cause a small decrease in 2-DG uptake in both the soleus and plantaris muscles, but this was not significant. Insulin infusion alone resulted in a marked increase in 2-DG uptake in both the soleus \((5.7\text{-fold}; \text{from } 1.9 \pm 0.4 \text{ to } 11.0 \pm 0.4 \text{ µg } \cdot \text{g}^{-1} \cdot \text{min}^{-1}; P < 0.001)\) and plantaris \((6.3\text{-fold}; \text{from } 1.3 \pm 0.1 \text{ to } 7.6 \pm 0.6 \text{ µg } \cdot \text{g}^{-1} \cdot \text{min}^{-1}; P < 0.01)\). However, when combined with TNF infusion, the insulin-mediated increase in 2-DG uptake by both soleus and plantaris muscles was significantly blocked \((52\text{ and } 68\%, \text{ respectively})\).
No significant difference was found between the experimental groups in arterial plasma concentrations of 1-MX (Fig. 5) or oxypurinol ($P = 0.51$ and $P = 0.41$, respectively), the metabolite of allopurinol and inhibitor of xanthine oxidase.

Insulin infusion alone significantly increased hind-leg 1-MX metabolism (Fig. 5). This resulted from the combined trend of insulin to increase 1-MX extraction and marked effect to increase femoral blood flow (Fig. 2). When TNF was combined with the insulin, the increase in 1-MX metabolism was completely abolished. TNF infusion alone did not affect 1-MX metabolism.

**DISCUSSION**

Two findings emerge from this study. First, acute administration of TNF led to marked insulin resistance with decreased insulin-mediated 2-DG uptake by individual muscles, decreased hind-limb glucose uptake, and decreased whole-body glucose infusion. Second, the inhibitory effect of TNF appears to be wholly hemodynamic in that insulin-mediated increases in femoral blood flow and capillary recruitment were totally blocked. Most striking was the effect of TNF on insulin-mediated increases in capillary recruitment as measured by 1-MX metabolism. This substrate has been used by us in two previous studies in vivo (21,24) as a marker for capillary endothelial xanthine oxidase. The experimental approach involves infusion of exogenous 1-MX during the clamp (or control protocols) with the intention of targeting capillary endothelial xanthine oxidase. The premise is based on observations that xanthine oxidase in skeletal muscle is concentr-
trated in endothelial cells (28,29), and thus any change in capillary flow (recruitment) will be reflected by changes in 1-MX metabolism. To support this notion, we have shown that in the constant flow perfused rat hind limb, 1-MX metabolism is decreased when the ratio of nutritive:nonnutritive flow is decreased pharmacologically (24) and metabolism is increased when the ratio is increased, for example, with exercise (30). In addition, we have shown in vivo that insulin increases the metabolism of 1-MX independently of changes in total flow, leading to the conclusion that insulin mediates an increase in capillary recruitment (presumably as a consequence of diminished nonnutritive flow) as part of its action to increase glucose uptake by muscle. Moreover, in a recent study, we have shown that the vasoconstrictor \(\alpha\)-methyl serotonin, which decreased the proportion of nutritive flow in perfused muscle, caused an acute state of insulin resistance in vivo. Thus, insulin-mediated increases in femoral blood flow, hind-leg glucose uptake, and hind-leg 1-MX disappearance were all markedly inhibited (24). Indeed, there are striking similarities between the effects of \(\alpha\)-methyl serotonin in that study and the effects of TNF in the present study, suggesting that the mechanisms may be similar. From isolated perfused hind-limb studies (31) and the increase in blood pressure in vivo (24), it would appear likely that \(\alpha\)-methyl serotonin acts in vivo to constrict vessels, preventing access to the nutritive capillaries and thereby preventing insulin from acting to recruit capillaries. TNF, however, does not increase blood pressure, and so its effects are unlikely to involve a redistribution of blood flow to the detriment of insulin's action to recruit capillaries. Two possibilities emerge. First, TNF may block the action of vasodilatory molecule released from the myocytes under the impetus of insulin. In this scenario, the hemodynamic changes may be a consequence rather than a regulator of changes in glucose metabolism in the muscle cell. Second, the effect of TNF directly or indirectly, may involve an inhibitory effect at the level of signal transduction. Candidate targets include tyrosine phosphorylation of the insulin receptor or insulin receptor substrate-1 and the association of phosphatidylinositol 3-phosphate kinase with phosphorylated insulin receptor substrate-1 (18). Most favored among these is the activation of the p55 and/or p75 TNF receptor, leading to insulin receptor substrate-1 serine phosphorylation that then blocks insulin signaling (6). What is not clear is whether the effects of TNF occur at the skeletal muscle cell, where a putative vasodilator capable of increasing capillary recruitment might be released, or at the vascular tissue, where insulin may act directly to enhance flow. These issues are beyond the scope of the present study and may only be resolved when tissue-specific receptor deleted animals are compared. It is unlikely that TNF directly inhibits xanthine oxidase because TNF had no effect on hind-leg 1-MX disappearance when added alone.

The finding that TNF administration before and during the hyperinsulinemic-euglycemic clamp causes insulin resistance is not new and has been shown by others (9). In that study, TNF was administered initially as a 10 \(\mu\)g/kg bolus followed by a continuous infusion of 10 \(\mu\)g/kg over 3 h. Thus, a

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**FIG. 4.** \[^{3}H\]2-DG uptake values for soleus and plantaris muscles. Values are means ± SE for six animals in each group. S, saline; T, TNF-alone infusion, as shown in protocol A (Fig. 1). I, insulin infusion alone; IT, insulin + TNF as shown in protocol B (Fig. 1). *Significantly different (\(P < 0.05\)) from saline values; #significantly different from insulin alone.

**FIG. 5.** Systemic and hind-leg 1-MX values of control groups (saline or TNF alone) and euglycemic insulin clamp groups (insulin alone or TNF + insulin). Values are means ± SE for 6–10 animals in each group. S, saline; T, TNF-alone infusion, as shown in protocol A (Fig. 1). I, insulin infusion alone; IT, insulin + TNF as shown in protocol B (Fig. 1). *Significantly different (\(P < 0.05\)) from saline values.
total of 20 µg/kg was administered. In the present study, we infused 0.5 µg/kg per h over 3 h, a total of 1.5 µg. This is perhaps more physiological, given that mini-osmotic pump delivery of TNF at 0.5 µg/kg per h for 4–5 days has been shown to give a serum concentration of 300 ± 47 pg/ml (32), which compares favorably with serum levels of ~200 pg/ml for genetically obese insulin-resistant animals (33). In humans, the levels of TNF in serum are somewhat lower. For example, type 2 diabetic (90 ± 10 pg/ml) and obese (78 ± 12 pg/ml) patients and control subjects (20 ± 8 pg/ml) (34) have lower TNF serum levels, although levels in patients with peritoneal adhesions after abdominal surgery are as high as 261 ± 88 pg/ml (35).

At first glance, the present findings might seem at odds with the recent report by Nolte et al. (18) that exposure of isolated incubated muscles to TNF (up to 6 nmol/l or 102 ng/ml for 4 h) had no effect on insulin signaling or insulin-mediated glucose uptake. This may mean that the inhibitory effects of TNF on insulin action are targeted exclusively at the vascular tissue, which is a minor component of incubated muscles and if modified could not affect insulin action, which reaches the muscle by diffusion in this preparation. Also, there is the possibility that the muscle, when removed from the animal and because of the lack of vascular delivery, is in fact already partly insulin resistant.

In conclusion, acute administration of TNF causes insulin resistance and involves effects exerted directly or indirectly at the vascular level to prevent insulin action to increase total limb blood flow and to increase capillary recruitment. These two effects are likely to be separate because increasing limb blood flow, as we have shown previously (21), does not necessarily increase capillary (nutritive) flow. Finally, if the hemodynamic changes due to insulin are not a consequence of changes in glucose metabolism by muscle, then the findings suggest that at least 50% of the increase in muscle glucose uptake due to insulin is mediated by a hemodynamic contribution involving capillary recruitment.

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