TNF-α acutely inhibits vascular effects of physiological but not high insulin or contraction

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Submitted 20 March 2003; accepted in final form 11 May 2003

Zhang, Lei, Catherine M. Wheatley, Stephen M. Richards, Eugene J. Barrett, Michael G. Clark, and Stephen Rattigan. TNF-α acutely inhibits vascular effects of physiological but not high insulin or contraction. Am J Physiol Endocrinol Metab 285: E654–E660, 2003. First published May 20, 2003; 10.1152/ajpendo.00119.2003.—TNF-α is elevated in many states of insulin resistance, and acutely administered TNF-α in vivo inhibits insulin-mediated hemodynamic effects and glucose uptake in muscle. In this study, we assess whether the inhibitory effects of TNF-α are affected by insulin dose or muscle contraction. Whole body glucose infusion rate (GIR), femoral blood flow (FBF), hindleg vascular resistance, hindleg glucose uptake (HGU), 2-deoxyglucose uptake into muscles of the lower leg (Rg) and hindleg metabolism of infused 1-methylxanthine (1-MX), a measure of capillary recruitment, were determined. Three groups were studied with and without infusion of TNF-α: euglycemic insulin-clamped, one-leg field-stimulated (2 Hz, 0.1 ms at 30 V), and saline-infused control anesthetized rats. Insulin infusions were 3, 10, or 30 mU·kg⁻¹·min⁻¹ for 2 h. 1-MX metabolism was maximally increased by all three doses of insulin. GIR, HGU, and Rg were maximal at 10 mU and FBF was maximal at 30 mU of insulin. Contraction increased FBF, HGU, and 1-MX. TNF-α (0.5 μg·kg⁻¹·h⁻¹) totally blocked the 3 and 10 mU insulin-mediated increases in FBF and 1-MX, and partly blocked GIR, HGU, and Rg. None of the increases due to twitch contraction was affected by TNF-α, and only the increase in FBF due to 30 mU of insulin was partly affected. We conclude that muscle capillary recruitment and glucose uptake due to high levels of insulin or contraction were 3, 10, or 30 mU insulin-clamped, one-leg field-stimulated (2 Hz, 0.1 ms at 30 V), and partly blocked GIR, HGU, and Rg. These findings may have implications for ameliorating muscle insulin resistance resulting from increased plasma TNF-α and for the differing mechanisms by which contraction and insulin recruit capillary flow in muscle.

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sponses, including capillary recruitment and increases in total limb blood flow. Taken together, this raises the interesting possibility that, although muscle cell lines respond acutely to TNF-α in vitro, the vasculature in vivo may be an important target for TNF-α. The loss of the hemodynamic responses may limit insulin and/or glucose access and account for inhibition of ~50% of the insulin-stimulated glucose uptake by muscle (29). Such a loss would be apparent only in vivo and thus be consistent with the negative outcomes of using TNF-α in isolated incubated muscles, as found by Nolte et al. (22).

The mechanism for capillary recruitment by insulin is an early (27) and nitric oxide (NO)-dependent (28) process, likely to be mediated at the endothelial cells. Brief treatment by TNF-α inhibits the insulin signaling in endothelium that leads to NO production (19). Because capillary recruitment in muscle can also be stimulated by muscle contraction and there is evidence that TNF-α and insulin oppose each other in a dose-dependent manner (9), we now explore whether TNF-α inhibitory effects on capillary recruitment are opposed by high doses of insulin or by muscle contraction.

METHODS

Animals. Male hooded Wistar rats weighing 245 ± 3 (insulin/TNF-α) or 220 ± 1 g (contraction) were raised on a commercial diet (Pivot, Launceston, Australia) containing 21.4% protein, 4.6% lipid, 68% carbohydrate, and 6% crude fiber with added vitamins and minerals together with water ad libitum. Rats were housed at a constant temperature of 21 ± 1°C on a 12:12-h light-dark cycle. All procedures adopted and experiments undertaken were approved by the University of Tasmania Animal Ethics Committee.

Surgery. Rats were anesthetized using Nembutal (50 mg/kg body wt) 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 intravenous 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 overlying the femoral vessels of both legs, and the femoral artery was separated from the femoral vein and saphenous nerve. Unless indicated otherwise, the epigastric vessels were then ligated, and an ultrasonic flow probe (VB series, 0.5 mm; Transonic Systems) was positioned around the femoral artery of the right leg just distal to the rectus abdominis 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 flowmeter (model T106 ultrasonic volume flowmeter; Transonic Systems). This was in turn interfaced with an IBM-compatible PC computer, which acquired the data (at a sampling frequency of 100 Hz) for femoral blood flow (FFB), heart rate, and blood pressure by use of WINDAQ data acquisition software (DATAQ Instruments). The surgical procedure generally lasted ~30 min, and then the animals were maintained under anesthesia for the duration of the experiment by means of a continual infusion of Nembutal (0.6 mg·kg−1·min−1) via the left jugular cannula. For clamps, the femoral vein of the left leg was used for venous sampling by use of an insulin syringe with an attached 29-gauge 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 hindlimb 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.

For animals undergoing twitch contraction studies in vivo, one leg was prepared for field stimulation (2 Hz, 0.1 ms at 30 V) and blood sampling. A platinum electrode was placed under the skin at the proximal end of the upper surface of the thigh and another at the Achilles tendon. Femoral blood was sampled at the end of the contraction period (10 or 60 min) from a cannula positioned in the epigastric vein. Blood samples were assayed for glucose (60 min) and 1-methylxanthine (1-MX; 10 min).

Experimental procedures. Once the surgery was completed, a 45- to 60-min equilibration period was allowed so FBF and blood pressure could become stable and constant. Rats were then allocated into either protocol A (Fig. 1), where animals were infused with saline or TNF-α for 3 h and underwent
euglycemic insulin clamp (3, 10, or 30 mU·kg⁻¹·min⁻¹ Humulin R; Eli Lilly, Indianapolis, IN) or saline alone for the final 2 h, or protocol B (Fig. 1), where animals were infused with saline or TNF-α for 2 h and underwent muscle contraction by electrical field stimulation (n = 6 in each group) for the last 1 h (2-deoxyglucose and glucose uptake) or the last 10 min (1-MX metabolism). Before the start of these experiments, a force-tension curve was constructed to optimize stimulation parameters.

TNF-α (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 (23). To do this, an injection of a specific xanthine oxidase inhibitor, allopurinol (6) (10 mg·kg⁻¹·h⁻¹), was administered as a bolus dose 5 min before commencing the 1-MX infusion (0.5 mg·kg⁻¹·min⁻¹ for 1 h; Fig. 1). This allowed constant arterial concentrations of 1-MX to be maintained throughout the experiment.

At 45 min before the completion of each experiment, a 50-μCi bolus of 2-deoxy-α-[2,6-¹³C]glucose (2-DG, specific activity = 1 Ci/mmol; Amersham Life Science) was administered. Plasma samples (25 μl) were collected at 5, 10, 15, 30, and 45 min to determine plasma clearance of the radioactivity. At the conclusion of the experiment, the soleus, plantaris, gastrocnemius white, gastrocnemius red, extensor digitorum longus, and tibialis muscles were removed, clamp frozen in liquid nitrogen, and stored at −20°C until assayed for 2-DG 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 for by the volume of infused saline.

Duplicate arterial and venous samples (300 μl) were taken at the end of the experiment (total time 180 min) and placed on ice. These blood samples were immediately centrifuged, and 100 μl of plasma were mixed with 20 μl of 2 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, insulin, and TNF-α analysis.

Analytical methods. A glucose analyzer (model 2300 Stat plus, Yellow Springs Instruments) was used to determine whole blood glucose (by the glucose oxidase method) during the insulin clamp. A blood sample of 25 μl was required for each determination. Human insulin levels at the end of the euglycemic insulin clamp were determined from arterial plasma samples by ELISA (Dako Diagnostics, Ely, UK), using human insulin standards. Plasma TNF-α levels were also determined using an ELISA based on mouse TNF-α (Pierce Endogen). Perchloric acid-treated plasma samples were centrifuged for 10 min, and the supernatant was used to determine 1-MX, allopurinol, and oxypurinol concentrations by reverse-phase HPLC as previously described (23, 24).

2-DG uptake assay. Individual frozen muscles from the clamps were ground under liquid nitrogen and homogenized using an Ultra Turrax. Free and phosphorylated parts of 2-DG were separated by ion exchange chromatography using an anion exchange resin (AG1-X8) (17, 20). Biodegradable Counting Scintillant-BCA (Amersham) was added to each radioactive sample and radioactivity determined using a scintillation counter (Beckman LS8001). From this measurement and a knowledge of plasma 2-DG clearance and the time course of plasma 2-DG disappearance, Rg, which reflects glucose uptake into the muscle, was calculated as previously described by others (17, 20).

Data analysis. All data are expressed as means ± SE. Mean FBF, mean heart rate, and mean arterial blood pressure were calculated from 5-s subsamples of the data, representing 400 flow and pressure measurements every 15 min. Vascular resistance in the hindleg 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 hindlimb was calculated from the arterial-venous (a-v) glucose difference and multiplied by FBF and expressed as micromoles per minute. The 1-MX metabolism was calculated from the a-v plasma 1-MX difference and multiplied by FBF (corrected for the volume accessible to 1-MX, 0.87, 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), oneway analysis of variance was used. When a significant difference (P < 0.05) was found, Dunnett’s test was used to determine which times were significantly different from saline control (for FBF, arterial blood pressure, femoral vascular resistance, arterial glucose, and 1-MX, hindleg glucose extraction and uptake, and hindleg 1-MX extraction and disappearance). Pairwise 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 of insulin. Figure 2 shows the change in PBF from the zero time point for three doses of insulin, 3, 10, and 30 mU·kg⁻¹·min⁻¹ with or without TNF-α, and the saline control. There was an increase commensurate with the increase in insulin dose. TNF-α (0.5 μg·kg⁻¹·h⁻¹) infusion for 1 h before and during the 2-h clamp completely inhibited the increase in FBF at 3 and 10 mU of insulin but only partly inhibited the increase due to the highest insulin dose of 30 mU·kg⁻¹·min⁻¹. Neither insulin nor TNF-α plus insulin had any effect on heart rate or mean arterial blood pressure (data not shown), and, as reported previously, TNF-α alone had no significant effect on these hemodynamic parameters (29). Because blood pressure was unchanged, there was a corresponding decrease in vascular resistance with insulin dose that was fully inhibited by TNF-α at 3 and 10, and only partly (50–60%) at 30 mU of insulin (data not shown).

Metabolic effects of insulin. There was no significant difference in the arterial blood glucose concentration between any of the treatments either at the beginning of the experiments (time = 0 min) or at the end (time = 120 min) (data not shown). During the euglycemic insulin clamp experiments (insulin alone or insulin + TNF-α), arterial blood glucose was maintained at basal values by infusion of glucose. Glucose infusion rates are shown in Fig. 3. Steady-state rates were 11.5 ± 0.4, 1.1 ± 0.8, and 25.5 ± 0.8 mg·kg⁻¹·min⁻¹ for 3, 10, and 30 mU of insulin, respectively. The constant infusion of TNF-α inhibited the glucose infusion rates for the two lower insulin doses of 3 and 10 mU by ~50 and 29%, respectively. TNF-α had no effect on the glucose infusion rate due to 30 mU of insulin (Fig. 3). End-of-
experiment (120 min) arterial plasma insulin concentrations (pmol/l) were 298 ± 33 for saline and 479 ± 50, 1,704 ± 152, and 7,391 ± 152 for the insulin infusion rates of 3, 10, and 30 mU·kg⁻¹·min⁻¹, respectively. None of these was affected by TNF-α infusion. End-of-experiment (120 min) arterial plasma TNF-α concentrations were 354 ± 65 pg/ml (n = 8).

Figure 4 shows data for hindleg glucose uptake for three doses of insulin with and without TNF-α. Hindleg glucose uptake (extraction / FBF) was stimulated by insulin 2.5-fold at 3 mU and 3.5-fold at 10 and 30 mU of insulin. TNF-α fully inhibited the increase due to insulin at 3 and 10 mU but was without effect at 30 mU of insulin.

2-DG uptake. 2-DG was administered for the final 45 min of each experiment. Figure 4 shows combined uptake values for six lower leg muscles removed at completion. The response to insulin varied depending on the muscle (data not shown), but in general 3 mU of insulin led to a twofold increase, and maximal stimulation was reached at 10 mU, as reflected by the combined data (Fig. 4). The highest dose of insulin (30 mU) did not further increase R’g for individual muscles (not shown) or for the combination (Fig. 4). TNF-α alone, as reported previously (29), had little or no effect (data not shown). When combined with insulin, TNF-α fully inhibited the stimulation due to 3 mU of insulin, partly blocked that of 10 mU, but was without effect on 30 mU of insulin (Fig. 4).

1-MX metabolism. No significant difference was found between experimental groups in arterial plasma concentrations of 1-MX or oxypurinol (data not shown; P = 0.81 and P = 0.29, respectively). Figure 4 shows 1-MX metabolism (A-V extraction / FBF) for the three doses of insulin (3, 10, and 30 mU) with and without TNF-α. Neither insulin nor insulin plus TNF-α had any significant effect on 1-MX extraction (data not shown). However, insulin alone did significantly increase 1-MX metabolism at all three concentrations, and the increase compared with saline (control) was the same at all three doses of insulin (Fig. 4). TNF-α completely blocked the insulin-mediated increase in 1-MX metabolism at 3 and 10 mU of insulin but was without effect at the highest dose of 30 mU (Fig. 4).

Hemodynamic effects of contraction. Figure 5 shows FBF and calculated vascular resistance for the three 1-MX metabolism (A-V extraction / FBF) for the three doses of insulin (3, 10, and 30 mU) with and without TNF-α. Neither insulin nor insulin plus TNF-α had any significant effect on 1-MX extraction (data not shown). However, insulin alone did significantly increase 1-MX metabolism at all three concentrations, and the increase compared with saline (control) was the same at all three doses of insulin (Fig. 4). TNF-α completely blocked the insulin-mediated increase in 1-MX metabolism at 3 and 10 mU of insulin but was without effect at the highest dose of 30 mU (Fig. 4).
experimental conditions: saline infusion alone for 2 h, saline infusion for 2 h with electrical stimulation (2 Hz, 0.1 ms at 30 V) in the last 60 min (protocol B, Fig. 1), and TNF-α infusion for 2 h and electrical stimulation in the last 60 min (protocol B, Fig. 1). Electrical stimulation increased FBF approximately threefold and heart rate by approximately 14% (data not shown) but did not affect mean arterial pressure (not shown). Vascular resistance calculated from FBF and mean arterial pressure decreased from 150 to 61 RU, and this decrease was not affected by TNF-α. TNF-α concentrations were not affected by muscle contractions of either 10 min (246 ± 20 pg/ml, n = 5) or 60 min (411 ± 33 pg/ml, n = 5) duration, compared with noncontraction controls (354 ± 65 pg/ml, n = 8).

Metabolic effects of contraction. Blood glucose concentrations were constant for all three experimental conditions (data not shown). Because measurement of 2-DG uptake required 45 min, uptake was determined after 1 h of contraction. Measurement of hindleg glucose uptake was also determined at this time. Figure 6 shows that 60-min electrical stimulation increased R'g in plantaris, gastrocnemius red and white, extensor digitorum longus, and tibialis muscles 8- to 27-fold. Soleus was increased only approximately twofold. Combined uptake for the six muscles was 23-fold. TNF-α had no significant effect on individual or combined R'g (Fig. 6). Hindleg glucose uptake was increased approximately ninefold, and this was uninhibited by TNF-α (Fig. 6).

Contraction effects on 1-MX metabolism. Figure 6 shows 1-MX metabolism for saline control, contraction, and contraction plus TNF-α. Contraction had no effect on 1-MX extraction (data not shown) but increased 1-MX metabolism. TNF-α had no effect on extraction (not shown) or metabolism due to muscle contractions (Fig. 6).

DISCUSSION

In the present study, TNF-α completely blocked the hemodynamic responses due to physiological insulin (3 mU·kg⁻¹·min⁻¹) of capillary recruitment and increased FBF. In addition, at this dose, the whole body glucose infusion rate was inhibited 50%
and the R’g of individual muscles 80–90%. At higher doses of insulin, the effects became less such that, at 10 mU·kg⁻¹·min⁻¹, TNF-α blocked the whole body glucose infusion rate 25% and R’g of individual lower leg muscles 50%. There is evidence that the hemodynamic responses of FBF and capillary recruitment were more sensitive to the cytokine. Thus at 10 mU·kg⁻¹·min⁻¹ insulin, the outcome was the same as for physiological insulin, and these remained completely inhibited even though the metabolic responses were partly recovered. Only at the highest dose of insulin used of 30 mU·kg⁻¹·min⁻¹ were some of the hemodynamic responses to insulin recovered. An important finding emerging from this study was that twitch contraction-induced hemodynamic and metabolic changes were resistant to TNF-α in vivo at the same dose that had marked inhibitory effects against physiological insulin.

Current views on TNF-α and insulin resistance in rodents suggest a key role linking them with obesity (13). Thus overexpression of TNF-α by adipose tissue is frequently found in different rodent models of obesity (10, 15). In addition, acutely administered TNF-α during insulin clamps in vivo creates resistance to insulin, particularly evident as reduced muscle glucose uptake (29) and complete loss of insulin-mediated hemodynamic responses (29). However, the mechanism by which TNF-α exerts its effect on insulin action is not yet understood. Effects may be direct via TNF-α itself or via an intermediary that is released in vivo by the cytokine. An indirect effect via fatty acid-dependent activation of the serine kinase IKKβ, which plays a role in the pathogenesis of insulin resistance through tissue inflammation, is one possibility. In at least one system, there is evidence that TNF-α and insulin oppose each other (9), suggesting, as does the present study, that insulin’s action is most vulnerable to TNF-α-mediated inhibition when insulin levels are low and least vulnerable when levels are high. At the low insulin level, inhibition of insulin action appears to have occurred at both hemodynamic and metabolic sites. Insulin-mediated increases in FBF as well as 1-MX metabolism were completely blocked. Because it is unlikely that the hemodynamic responses account for more than 50% of the insulin-mediated glucose uptake by muscle in vivo, complete inhibition of hindleg glucose uptake suggests that insulin’s action at the myocyte is likely to have also been affected by TNF-α.

Exercise is now considered to be an insulin-independent mechanism for stimulating muscle glucose uptake. Exercise also has marked hemodynamic effects in vivo, including increases in limb blood flow (4) and capillary recruitment (11). Mechanisms for exercise-mediated glucose uptake by myocytes are currently under intense investigation and are quite distinct from the mechanism used by insulin. Possibilities include Ca²⁺, protein kinase C, AMP kinase, a combination of these, nitric oxide, or adenosine (18). Mechanisms to account for the hemodynamic responses are less well understood, but it is thought that a metabolic vasodilator released from the myocytes or a radiating membrane depolarization is responsible. To our knowledge, TNF-α has not been used against exercise previously. We now report that infused TNF-α at the same dose that markedly inhibited physiological insulin had no effect on either the hemodynamic or metabolic responses to electrical stimulation of the hindleg muscle in vivo. In particular, capillary recruitment, which was increased as a result of the exercise, was unaffected by the cytokine.

Together, the present findings suggest that insulin signaling at sites in the vasculature where capillary recruitment and total flow are controlled by insulin is particularly sensitive to inhibition by TNF-α. Furthermore, the findings imply that, if muscle insulin resistance results from the effects of TNF-α, these can be
circumvented by exogenous insulin, possibly by agents that enhance insulin action at endothelial cells or by exercise.

DISCLOSURES

This work was supported in part with grants from the National Health and Medical Research Council of Australia, National Heart Foundation of Australia, American Diabetes Association, and National Institute of Diabetes and Digestive and Kidney Diseases Grant RO1 DK-057878-01A1.

REFERENCES