Infusion of Tumor Necrosis Factor/Cachectin Promotes Muscle Catabolism in the Rat

A Synergistic Effect with Interleukin 1

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Abstract

To improve our understanding of the metabolic role of cytokines in protein wasting, we estimated the rates of protein synthesis and degradation in muscle and liver tissues in intact rats treated with several doses of recombinant IL 1 and/or tumor necrosis factor (TNF)/cachectin. Protein breakdown in muscle and liver were derived in vivo from the relationship between [^{14}C]leucine distribution and tissue dilution in reference to circulating leucine. Synthesis was derived from the relationship between [^{14}C]leucine appearance in the protein-bound and free-tissue leucine pools. To specifically relate changes in leucine tracer metabolism to protein dynamics, we separately measured the effect of these cytokines on blood flow to different tissues. The increase in dilution of the tissue-free [^{14}C]leucine by TNF and TNF/IL 1 mixture, but not by IL 1 alone, could not be explained by a hemodynamic effect of these cytokines. Rather, this finding indicated that muscle proteolysis is enhanced by TNF and synergistically augmented by the addition of IL 1. Compatible with these data was the finding that more prolonged infusions of recombinant TNF/cachectin and the combination with IL 1 increased urinary nitrogen excretion. Changes in [^{14}C]leucine dilution in the liver were less pronounced than those in skeletal muscle and consistent with net anabolic effect of TNF on liver protein. We conclude that rats exposed systemically to sublethal doses of TNF respond with increasing muscle and decreasing liver proteolysis, similar to that observed in inflammation and in cancer.

Introduction

The metabolic response to sepsis, trauma, and malignancy includes weight loss, muscle wasting, increased urinary nitrogen excretion, as well as synthesis of acute-phase proteins (1). Although several aspects of this catabolic response may be mimicked by administration of hormones (2, 3), recent observations suggest a metabolic regulatory role for cytokines (4, 5). Macrophage-derived, purified IL 1 has been shown to induce fever, leukocytosis with neutrophilia, changes in circulating heavy metal concentrations, hormonal changes, synthesis of acute-phase proteins, and loss of nitrogen (6, 7). These effects have been compared with the response to live endotoxin in experimental animals (8).

It is generally thought that wasting of lean tissues and loss of nitrogen during infections and malignancy are caused by an increase in protein breakdown in skeletal muscle. Although purified IL 1 has been shown to increase plasma leucine flux in rats, thus indicating enhanced whole-body proteolysis (6, 7), it was not possible to demonstrate a similar response to recombinant IL 1 (9, 10). This observation suggested that the proteolytic effect of macrophage-derived IL 1 could possibly be attributed to other mediators concomitantly present in the purified cytokine preparation.

Tumor necrosis factor alpha (TNF)[1]/cachectin is another protein secreted by circulating, as well as tissue, mononuclear cells and has several biological activities that overlap with IL 1 (11). The hemorrhagic necrosis caused by this factor in growing tumorous tissue is responsible for its name. Recent evidence has further linked TNF with wasting characteristic of patients with cancer cachexia thus accounting for the alternative name. Whereas an effect of TNF on lipid metabolism has been documented (12, 13), it has also been hypothesized that muscle catabolism is mediated by this factor (14). However, recent studies have failed to consistently reveal any increase in protein breakdown in skeletal muscle tissues incubated in vitro with recombinant TNF (15–18). On the other hand, studies involving administration of TNF in vivo have shown an increase in nitrogen efflux from skeletal muscle in humans (19) and loss of body protein in growing rats (14). This net catabolic effect could be related to changes in either protein synthesis, protein breakdown, or both.

In this study, we have characterized the effect of intravenous administration of recombinant IL 1 and TNF, either alone or in combination, on rat protein metabolism in vivo. We used a tracer model based on infusion of L-[1-^{14}C]leucine and measurement of uptake and dilution of this essential amino acid in plasma and tissues. This model allows simultaneous estimation of the rates of protein synthesis and breakdown in different tissues if the distribution of the tracer amino acid to these tissues is known. Assuming that this may be approximated by perfusion (20, 21), we have separately measured the effect of recombinant IL 1 and TNF cytokines on blood flow to muscle and liver to further relate changes in leucine kinetics to protein metabolism. Additionally, we have measured the effects of repeated infusions of these recombinant cytokines on urinary nitrogen excretion at several dose levels.

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1. *Abbreviations used in this paper:* TNF, tumor necrosis factor.

Methods

Materials

Recombinant human TNF-alpha (cachectin), containing < 200 pg of endotoxin/mg of protein, was kindly provided by Genentech, Inc., South San Francisco, CA. Details of its purification are described elsewhere (22). Recombinant generated human pI 7 (beta) IL 1 (ROOGT 17 kD) was cloned as previously described (23). IL 1 ROOGT contains amino acids 112–269 of the precursor polypeptide with endotoxin concentration of 60 pg/mg of recombinant IL 1 protein, as determined by *Limulus* lysate assay. The identity of this cytokine was confirmed by amino acid composition and the sequence of the amino terminal heptapeptide.

Radiolabeled L-[1-^{14}C]leucine (50 μCi/mmol sp act) was purchased from ICN Radiochemicals, Irvine, CA. Hyamine hydroxide was obtained from Packard Instruments, Downer's Grove, IL. 0.5 N quaternary amonium hydroxide (BTS-450; Beckman Instruments, Fullerton, CA) and scintillant (Ready-Solv-MP; Beckman Instruments, Fullerton, CA) were used for tissue solubilization and counting, respectively. The scintillant Betafluor was purchased from National Diagnostics, Somerville, NJ, and used for counting of aqueous solutions. Urinary nitrogen was measured using a heating unit (BD-40; Technicon Instruments Corp., Tarrytown, NY).

Radioactive ^{85}Sr microspheres (NEN-TRAC; 16.5 μm±0.1 diameter, density 3.04×10^5 microspheres per mg, suspended in 10% dextran) were purchased from New England Nuclear, Boston, MA, and used for measurement of tissue perfusion.

Rat body temperatures were monitored with minimitters (Model V) purchased from Minimitter Co., Inc., Sun River, OR. These devices were implanted subcutaneously in the rats receiving cytokine infusions and undergoing protein turnover measurements (experiments 1 and 2, below).

Experimental protocols

Animals and catheterization. Male Sprague-Dawley rats (n = 150, Taconic Farms, Germantown, NY) were housed in individual cages in a light-controlled room at a temperature 26–28°C. All rats were maintained on regular laboratory chow diet ad lib. until they achieved a weight between 200 and 250 g. All animals underwent internal jugular vein catheterization under general diethyl ether anesthesia and were allowed 48 h for recovery. Rats in experiment 1 were also implanted subcutaneously with Minimitters for measurement of body temperature, as previously described (7, 9).

During recovery from surgery, patency of the catheters was maintained by constant infusion of isotonic saline. Rats in experiments 1 and 2 were fasted the night before and throughout the 6-h administration of cytokine infusions. As explained below, rats in experiment 4 received two cytokine infusions on consecutive days during which they did not have access to food.

Experimental design. Four experiments were conducted to measure the influence of intravenous cytokine administration on muscle and liver protein metabolism in the rat. In the first two experiments L-[$^{14}C_1$]leucine was infused over 6 h at a constant rate, and its distribution in plasma and tissues (free and protein-bound pools) was measured. Protein synthesis and degradation rates were estimated from the rate of [^{14}C]leucine appearance in tissue proteins and dilution in the free tissue amino acid pool, respectively. Because the dilution of radioactive leucine in tissues is a function of perfusion as well as protein breakdown (20, 21), separate experiments were conducted to measure the influence of cytokine infusions on blood flow distribution. In a fourth set of experiments, total urinary nitrogen excretion was measured at increasing infusion dosage of TNF, IL 1, and TNF/IL 1 mixture. A summary of all experimental groups is given in Table I.

Table I. Summary of Experimental Design and Groups

Group	Number	Infusion	Purpose
1a	6	Saline	Leucine turnover
1b	7	IL 1 (*20 μg/kg*)	Leucine turnover
1c	8	TNF (*20 μg/kg*)	Leucine turnover
1d	7	TNF/IL 1 (*20 μg/kg*)	Leucine turnover
2a	6	Saline	Leucine turnover
2b	6	IL 1 (*100 μg/kg*)	Leucine turnover
2c	7	TNF (*100 μg/kg*)	Leucine turnover
2d	5	TNF/IL 1 (*100 μg/kg*)	Leucine turnover
3a	12	Saline	Cardiac output and perfusion at 3 and 6 h
3b	6	IL 1 (*20 μg/kg*)	Muscle perfusion
3c	6	TNF (*20 μg/kg*)	Muscle perfusion
3d	6	TNF/IL 1 (*20 μg/kg*)	Muscle perfusion
3e	12	TNF (*100 μg/kg*)	Cardiac output and perfusion at 3 and 6 h
4	56	Saline, IL 1, TNF, and IL 1/TNF (*0.2, 2.0, 20.0, and 200 μg/kg*)	24-h urine

(i) EXPERIMENTS 1 AND 2

After surgery for catheter and Mini-Mitter placement, the rats were allowed to recover for 2 d. They were then randomly assigned to the following groups, depending upon the dose and type of cytokine infusions. Group 1a received saline; group 1b received IL 1 at a total dose of 20 μg/Kg; group 1c received TNF at 20 μg/Kg, and group 1d received TNF/IL 1 mixture at the same total dose. Experiment 2 consisted of groups 2a–2d, which corresponded to the first experiment except that the cytokine dose was increased to 100 μg/kg. Experiments 1 and 2 were conducted at different times and had separate control groups.

At the time of infusion, the rats were transferred to individual plexiglass cages that allow free movement of the animal and collection of expired gas. All recombinant cytokine infusions were freshly prepared in normal saline solution containing 0.1% albumin. Half of the dose was given as an intravenous bolus and half was infused at a constant rate over 6 h. Simultaneously with the cytokine infusion, L-[1-^{14}C]leucine was administered at a constant rate of 1.2 μCi/h, also for a period of 6 h. Measurement of the rate of $^{14}CO_2$ appearance in breath, as well as CO_2 production and O_2 consumption rates were made at half-hour intervals, as described elsewhere (6, 7). At termination of the infusion, the rats were decapitated and blood, liver, and muscle tissues were collected for measurement of leucine-specific activity, as previously described (24).

(ii) EXPERIMENT 3

This set of experiments was designed to measure changes in blood flow distribution associated with systemic cytokine infusions. Catheterization was done under general ether anesthesia, as in the first two experiments; a catheter in the internal jugular vein was used for infusion of cytokines. Another catheter was placed in the right carotid artery and advanced into the left ventricle for injection of labeled microspheres and blood flow determinations. Treatment groups in experiment 3 included saline (group 3a); IL 1 at a dose of 20 μg/kg (group 3b); TNF at a dose of 20 μg/kg (group 3c); and group 3d, which received a 1:1 combination of TNF and IL 1, also at a total cytokine dose of 20 μg/kg. All microsphere injections were made after 6 h of cytokine infusions. Additionally, blood flow distribution and cardiac output measurements were made in groups 3a (saline controls) and 3e (TNF, 100 μg/Kg) at 3 and 6 h after starting the intravenous infusion. In these groups, an additional polyethylene (PE-50) catheter was placed in the left carotid artery for withdrawal of reference blood and estimation of the cardiac output.

At the time of blood flow measurements, a dose ranging between 500,000 and 1,000,000 ^{85}Sr-microspheres suspended in 10% dextran with 0.01% Tween 80 was prepared for each rat. Immediately before

injection into the left ventricle, the microspheres were sonicated in warm water for 20 min and vortexed repeatedly. An aliquot was taken for measurement of the syringe content, and the microsphere dose was then injected into the left ventricle over 20 s. 2 min later, the rats were killed by an overdose of ketamine hydrochloride (50 mg/ml; Parke, Davis & Co., Detroit, MI). Tissues were then sampled, weighed, and counted in a gamma counter (model 5500; Beckman Instruments) for ^{85}Sr content. Residual radioactivity remaining in the injection syringe and silastic tubing was also counted and subtracted from the total counts in the syringe.

In addition to this procedure, rats in groups 3a and 3e had their left carotid line attached to a syringe withdrawal pump (Harvard Instruments, S. Natick, MA) which was activated at a rate of 0.76±0.03 ml/min starting 10 s before the injection. Withdrawal of the reference blood continued for 2 min.

(iii) EXPERIMENT 4

This experiment was designed to determine whether the changes in plasma and tissue leucine kinetics in experiments 1 and 2, showing net muscle catabolism, are also associated with increased urinary nitrogen excretion. Rats were randomized to receive infusions of IL-1, TNF, IL 1/TNF mixture, and saline over four logarithmic doses ranging between 0.2 and 200 μg/kg. Surgical procedures for catheter placement and postoperative care of these rats were identical to those of experiment 1. To maximize the likelihood of detecting a difference in nitrogen excretion, two 6-h infusions were administered during the 24-h period. The rats remained fasted and urine was collected for measurement of total nitrogen excretion.

Analytical methods

Serum leucine-specific activity was measured as previously described (21). Briefly, 1-ml aliquots were deproteinized with 30% sulfosalicylic acid, and the supernatant was analyzed for leucine concentration with HPLC (Waters Associates, Milford, MA) using precolumn *o*-phthaldialdehyde derivatization and fluorescence detection by μBondapak C18 column. Total radioactivity was measured by liquid scintillation spectrometry, after treatment with H_2O_2 at 37°C to remove radioactivity in the form of α-ketoisocaproate (24). Ready Solv-MP (Beckman Instruments) was used as scintillant with external standards for efficiency determination. All samples were counted to a SE of < 2%.

Tissue leucine pools were separated into free (acid-soluble) and protein-bound (acid-insoluble) fractions by homogenization in 10% sulfosalicylic acid (Polytron homogenizer; Brinkmann Instruments, Westbury, NY). Measurement of free tissue leucine-specific activity from leucine counts and concentration was made as described above for plasma samples. Protein-bound leucine specific activity was measured in the acid-insoluble fraction after drying and solubilization with 0.5 N quaternary ammonium hydroxide. Tissue and total urinary nitrogen were measured spectrophotometrically after micro-Kjeldahl digestion as previously described in detail elsewhere (24).

Serum levels of iron and zinc were measured with an atomic absorption spectrophotometer (Perkin-Elmer Corp, Norwalk, CT) in aliquots after deproteinization with 2 vol of 1.8 N perchloric acid (9).

Calculations

PLASMA LEUCINE KINETICS

Total flux of leucine into plasma was calculated from [$^{14}C_1$]leucine dilution at isotopic steady state, according to the following equation:

$$Q = I/Sp \tag{1}$$

where Q is plasma leucine flux in micromoles per hour, I is the rate of radioactive tracer infusion in disintegrations per minute per hour and Sp is leucine-specific activity in plasma at isotopic steady state, measured after 6 h of constant tracer administration. Total plasma leucine flux is derived from breakdown of endogenous proteins since the rats were kept fasted. Furthermore, because plasma leucine is either oxidized or used in protein synthesis, the following relationship exists (21, 26):

$$B = Q = S + O \tag{2}$$

where B is leucine derived from body protein breakdown; S and O are the rates of plasma leucine utilization in protein synthesis and oxidation, respectively.

The rate of plasma leucine oxidation was calculated from the product of plasma flux, Q, and the percent of infused tracer appearing in breath. The latter was measured according to the following equation:

$$\%\ \text{leucine oxidized} = \frac{V_{CO_2} \times SA(^{14}CO_2)}{0.8 \times I} \tag{3}$$

Where V_{CO_2} and $SA(^{14}CO_2)$ are the rate of CO_2 production and its steady-state specific activity in breath, respectively, measured by indirect calorimetry and analysis of expired $^{14}CO_2$ in hyamine hydroxide. The term I is the rate of infusion of [$^{14}C_1$]leucine; 0.8 is a factor that accounts for oxidized leucine not appearing in breath (6).

TISSUE LEUCINE KINETICS

The amount of radioactive leucine taken up in liver and muscle proteins was measured and expressed as leucine-specific activity, *Sb*. Assuming that free tissue leucine, which may be represented by the specific activity *Si*, is the precursor of protein-bound leucine, then the following relationship between the rate of protein synthesis and the ratio Sb/Si has been derived (21, 26):

$$\frac{Sb}{Si} = \frac{\lambda_i}{\lambda_i - K_s} \cdot \frac{(1 - e^{-K_s t})}{(1 - e^{-\lambda_i t})} - \frac{K_s}{(\lambda_i - K_s)} \tag{4}$$

where Ks is fractional protein synthesis rate (FSR); t is the duration of isotope infusion, and λ_i is the rate of increase in tissue leucine radioactivity.

Release of unlabeled leucine from tissue protein breakdown results in dilution of specific activity of free tissue leucine relative to circulating leucine. If the contribution of plasma to this pool is known, then protein breakdown can be quantified according to the following equation, which was derived elsewhere (21):

$$Tb = \frac{qI(Sp - Si)}{Si \cdot Sp} \tag{5}$$

where Tb is the contribution of tissue breakdown to free leucine; I is the rate of tracer infusion; q is the rate of tracer distribution to the tissue; and Si and Sp are the specific activities of free leucine in plasma and tissue, respectively.

TISSUE PERFUSION AND BLOOD FLOW

Tissue perfusion, expressed as a fraction of the cardiac output, was calculated from the following relationship:

$$\text{Tissue perfusion} = \frac{\text{tissue } ^{85}\text{Sr counts}}{\text{microsphere dose}} \tag{6}$$

Estimates of the cardiac output in groups 3a and 3e, in which a reference arterial blood sample was withdrawn at a constant rate, were made according to the following relationship (27, 28):

$$\frac{CO}{dose} = \frac{\text{rate of withdrawal of reference blood}}{\text{activity in reference blood}} \tag{7}$$

where CO is cardiac output, and dose is the total ^{85}Sr counts of the injected microspheres.

Statistical analysis

Statistical significance was tested separately in each experiment by analysis of variance (ANOVA), using a computer program (BMDP statistical package, UCLA, CA). Comparison of the treatment groups within each experiment was achieved by means of Bonfferoni intervals at a level of significance of $P < 0.05$.

Statistical analysis in experiment 4, consisting of comparison of several cytokine treatments at four different dosages, was made by two-way ANOVA using the same statistical package.

Results

Experiments 1 and 2: protein turnover

Infusion of either TNF, IL 1, or coinfusion of both cytokines resulted in a significant increase in body temperature compared with the groups infused with saline. The increase in temperature, which was comparable for all the cytokines, averaged half a degree °C, starting at ~ 30 min after the infusion. At 4 h of infusion, the average increase in body temperature was ~ 1.8°C and remained constant until the end of the experiment. Serum concentrations of zinc and iron were 152.9 (SE 4.9) and 128.3 (SE 7.2) μg/dl, respectively in the rats infused with saline. At 6 h of recombinant IL 1, TNF, IL 1/TNF infusions, these concentrations were significantly reduced by ~ 40% in all the rats in groups 1b, 1c, 1d, 2b, 2c, and 2d. There were no differences in trace metal concentrations related to either the type or dose of cytokine, because both IL 1 and TNF, or combination resulted in equal changes.

Rats treated with recombinant cytokines also had significant neutrophilia with 42.8 (SE 3.4), 44.6 (SE 2.2), and 55.7% (SE 3.4) of the cells as immature neutrophils (bands) in the IL 1, TNF, and coinfusion groups, respectively. The increase in number of bands in the coinfusion group was significantly larger than in either cytokine infusion separately ($P < 0.05$). Simultaneously, there was a significant ($P < 0.05$) reduction of the relative number of lymphocytes in the coinfusion group.

Results of dilution of [^{14}C]leucine in plasma and breath CO_2 are summarized in Table II and expressed as total plasma leucine flux, oxidation, and rate of utilization in protein synthesis per 100 g body weight. Plasma leucine flux, which is contributed by endogenous protein breakdown in the postabsorptive rat, tended to be larger in the animals receiving 20-μg/kg cytokine infusions (experiment 1), including the saline controls (group 1a). However, there were no significant differences related to cytokine treatment regardless of the infused dose. The percent of plasma leucine oxidized was slightly increased in the rats infused with TNF at 100 μg/kg ($P < 0.05$, by t test). However the rate of plasma leucine oxidation, expressed in micromoles per hour, was not statistically different between the treatment groups in experiments 1 and 2.

Estimates of the rates of protein synthesis in muscle and liver are summarized in Tables III and IV. When expressed as fractional synthetic rates (FSR), these estimates reflect the rate of appearance of radioactive leucine in tissue protein relative to free tissue [^{14}C]leucine specific activity (Si). Infusion of recombinant cytokines were not associated with differences in the rates of fractional protein synthesis in skeletal muscle. Similarly, there were no significant differences in the percent protein content of the muscle tissues analyzed, even though muscle protein content tended to be numerically larger in the IL 1-treated rats. Total leucine utilization in muscle protein synthesis (Ts in Table III), estimated from FSR and muscle leucine content was significantly larger ($P < 0.05$, by ANOVA) in the IL 1 group receiving 20 μg/kg but not in the rats infused with 100 μg/kg. Although it is difficult to interpret the significance of this observation, it is interesting to note that 20-μg/kg dose of recombinant IL 1 was also associated with lower percent plasma leucine oxidation. These findings are consistent with those of Canalis (29) showing increased [^{3}H]proline uptake in cultured rat calvariae incubated with IL 1.

In contrast to protein synthesis, estimates of muscle protein breakdown were significantly increased in the rats receiving TNF alone or in combination with IL 1, as summarized in Table III. The increase in proteolysis was larger in the rats treated with cytokine mixture. These results were based on dilution of free radioactive leucine in muscle relative to plasma, according to Eq. 5. Values for the parameter q in this equation were derived from measurements of tissue perfusion in experiment 3, and will be presented below. In making estimates of total muscle protein degradation in the whole rat, it was also assumed that muscle mass accounts for 40% of body weight.

Estimates of protein synthesis and degradation in the liver are summarized in Table IV. Infusions of 100 μg/kg of either recombinant IL 1, TNF, or IL 1/TNF were not associated with any significant change in fractional or total liver protein synthesis rates. Protein degradation rates, calculated from dilution of free liver leucine, also were not affected by treatment with recombinant cytokines at a statistically significant level. An infusion dose of 100 μg/kg recombinant TNF, either alone or in 1:1 mixture with IL 1, was associated with a decrease in liver protein breakdown and an increase in liver protein mass. Even though these results were not individually significant, comparison of the ratio of protein synthesis to breakdown within each group in experiment 2 reveals a net catabolic condition in the saline control group ($P < 0.05$ for synthesis vs. breakdown in

Table II. Estimates of Plasma Leucine Flux and Whole Body Protein Metabolism

Group	Treatment	Flux	Oxidation	Synthesis	Oxidized
			μmol Leu/h · 100 g		%
1a	Saline	28.7 + 0.9	7.8 + 0.4	20.9 + 0.7	27.2 + 1.1
1b	IL 1 (*20 μg/kg*)	25.9 + 1.2	5.7 + 0.6	20.1 + 0.8	21.7 + 0.8*
1c	TNF (*20 μg/kg*)	27.6 + 1.2	7.7 + 0.7	19.8 + 1.0	26.4 + 0.9
1d	TNF/IL 1 (*20 μg/kg*)	25.0 + 1.8	6.9 + 0.7	18.0 + 1.4	27.9 + 1.9
2a	Saline	20.8 + 1.4	4.5 + 2.1	16.2 + 1.1	21.7 + 1.3
2b	IL 1 (*100 μg/kg*)	20.6 + 0.7	5.2 + 0.5	13.7 + 2.5	25.6 + 2.8
2c	TNF (*100 μg/kg*)	19.4 + 0.9	4.8 + 0.4	14.6 + 0.6	26.4 + 0.9*
2d	TNF/IL 1 (*100 μg/kg*)	18.2 + 1.9	4.2 + 1.2	14.0 + 1.2	22.4 + 1.4

Data are mean + SE. * $P < 0.05$ vs. control by t test.

Table III. Estimates of Leucine Kinetics in Skeletal Muscle

Group	Treatment	FSR*	Synthesis (*Ts*)	Breakdown (*Tb*)	P‡	B/F§
		%/day	*μmol/h*		*%*	
1a	Saline	2.6 + 0.2	11.0 + 0.9	11.8 + 0.9	17.4 + 1.2	505 + 57
1b	IL 1 (*20 μg/kg*)	2.9 + 0.4	17.3 + 2.2‖	14.4 + 1.3	21.3 + 1.1	589 + 33
1c	TNF (*20 μg/kg*)	2.4 + 0.2	10.9 + 1.1	14.0 + 0.5‖	18.6 + 2.0	447 + 46
1d	TNF/IL 1 (*20 μg/kg*)	2.0 + 0.2	12.8 + 1.8	20.8 + 2.9¶	18.0 + 3.2	NA
2a	Saline	2.4 + 0.3	10.5 + 1.1	10.6 + 0.7	17.4 + 0.7	525 + 38
2b	IL 1 (*100 μg/kg*)	2.9 + 0.2	13.1 + 1.1	13.7 + 1.0	17.4 + 0.5	542 + 28
2c	TNF (*100 μg/kg*)	2.4 + 0.1	11.3 + 0.8	14.5 + 0.6¶	18.0 + 0.8	509 + 45
2d	TNF/IL 1 (*100 μg/kg*)	2.4 + 0.1	9.4 + 0.3	16.3 + 1.5**	15.5 + 0.3	466 + 27

Data are mean + SE; NA, not available. * FSR, fractional protein synthesis rate. ‡ % protein composition of muscle sample. § Ratio of bound/free leucine in muscle tissue. ‖ $P = 0.05$ vs. saline control. ¶ $P < 0.01$ vs. saline control. ** $P < 0.02$ vs. saline control.

group 2a) but not in the rats treated with either TNF or a mixture of TNF and IL 1 (2c and 2d).

Experiment 3: hemodynamic changes

Regional blood flow distribution was significantly changed after 3 h of TNF infusion at a dose of 100 μg/kg. As shown in Fig. 1, portal blood flow significantly increased from 9.3 (SE 1.0) to 15.5% (SE 1.7) of cardiac output ($P < 0.01$). Relative kidney perfusion also increased from 3.9 (SE 0.7) to 5.9% (SE 0.5) of cardiac output ($P < 0.025$).

Summary of blood flow measurements in experiment 2 is presented in Figs. 1 and 2 showing the effect of 6-h infusions of recombinant TNF and IL 1 on muscle and liver perfusion. In addition, the effect of 100 μg/kg TNF on muscle perfusion and cardiac output was measured after 3 and 6 h of infusion. Each of these treatment groups had a separate saline-infused control.

Significant hemodynamic changes were seen after 3 h of 100 μg/kg TNF administration. A drop in cardiac output from 92 (SE 8) to 62 ml/min (SE 14) was associated with a significant redistribution of blood flow away from skeletal muscle towards splanchnic organs (liver, gastrointestinal tract, kidneys). However, at 6 h of the same intravenous TNF infusion, there was an increase in cardiac output back to the control level. Blood flow distribution to skeletal muscle, expressed as a percent of total cardiac output, was equal in the saline and 100 μg/kg TNF-infused groups.

Results of regional blood flow distribution to skeletal muscle after 6 h of TNF, IL 1, and TNF/IL 1 infusions at a dose of 20 μg/kg are summarized in Fig. 2 and compared with the corresponding saline control group. No significant differences related to treatment with recombinant cytokines were noted in this experiment.

Experiment 4: nitrogen excretion

To confirm the catabolic effect of recombinant TNF and IL 1/TNF infusions on protein metabolism, a dose-response experiment was conducted at infusion levels of 0.2, 2, 20, and 200 μg/kg. The infusion was given twice in 24 h during which the rats were kept fasted. The results of this experiment are presented in Table V showing 10–25% increase in total urinary nitrogen excretion ($P < 0.01$, by ANOVA) as a result of two 6-h infusions of recombinant TNF and IL 1/TNF at dose levels of 20 and 200 μg/kg. Two-way ANOVA showed that the effect of cytokine treatment was independent of the total infused dose. Therefore, these results were pooled for all the dosages within each cytokine infusion group and summarized in Fig. 3, which shows a significant effect for TNF and IL

Table IV. Estimates of Leucine Kinetics in the Liver

Group	Treatment	FSR	Synthesis (*Ts*)	Breakdown (*Tb*)	P*	B/F‡
		%/day	*μmol/h*		*g*	
1a	Saline	55.2 + 10.7	21.3 + 5.5	27.3 + 8.2	1.29 + .15	187 + 46
1b	IL 1 (*20 μg/kg*)	52.9 + 6.2	22.7 + 2.6	21.8 + 1.8	1.70 + .19	277 + 71
1c	TNF (*20 μg/kg*)	49.2 + 6.2	22.5 + 3.2	22.6 + 4.1	1.54 + .09	177 + 42
1d	TNF/IL 1 (*20 μg/kg*)	57.2 + 5.6	21.8 + 1.8	31.4 + 4.9	1.72 + .10	NA
2a	Saline	91.1 + 18.6	32.1 + 4.7	50.7 + 9.6	1.12 + .12	82 + 21
2b	IL 1 (*100 μg/kg*)	81.0 + 19.6	28.3 + 3.7	53.6 + 14.0	1.26 + .17	112 + 30
2c	TNF (*100 μg/kg*)	69.5 + 16.1	24.3 + 3.7	37.1 + 8.4	1.46 + .14	137 + 26
2d	TNF/IL 1 (*100 μg/kg*)	51.2 + 4.6	23.6 + 3.3	25.4 + 2.1	1.57 + .18	198 + 28

Data are mean + SE; NA, not available. * Total liver protein in grams. ‡ Ratio of bound/free leucine in liver.

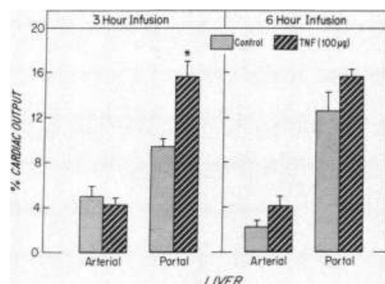

Figure 1. Blood flow distribution to the liver in saline- and TNF-treated rats. Blood flow distribution (mean±SE), expressed as percent of cardiac output, was measured by radioactive microspheres. Measurements were made after 3 and 6 h of infusion of saline and recombinant TNF at a dose of 100 µg/kg. Portal blood flow distribution was significantly increased only after 3 h of cytokine infusion.

1/TNF mixture ($P < 0.01$, and < 0.05, respectively by ANOVA and Bonferroni confidence intervals). When given separately, IL 1 did not change urinary nitrogen excretion. These results are consistent with leucine kinetic data in experiments 1 and 2.

Discussion

Increased muscle catabolism is an important component of the metabolic response to acute and chronic inflammatory processes accompanying infections, trauma, tissue injury, and cancer cachexia (14, 30, 31). Although this has been linked to changes in several hormones, such as corticosteroids (32), catecholamines (3), and growth and thyroid hormones (33), emphasis has recently been placed on secretory peptides derived from inflammatory cells. Initially, systemic infusions of purified circulating peptide factors, collectively known as "leukocyte endogenous mediators," were shown to induce several metabolic effects associated with inflammation. Experiments in our laboratory thus demonstrated an increase in plasma leucine flux in rats infused with biologically derived, purified IL 1 (6, 7), suggesting an increase in body protein breakdown. Other metabolic effects such as changes in circulating trace mineral concentrations and acute-phase proteins, as well as fever, were also shown to result from infusion of the purified IL 1 (6, 7). Furthermore, stimulation of PGE2 production has been associated with the increase in muscle catabolism initially thought to be caused by purified IL 1 (34). The availability of recombinant technology has opened an opportunity for further investigation of the role of cytokines in metabolic regulation. It has already been shown that systemic infusion of recombinant cytokines can induce fever in experimental animals (22).

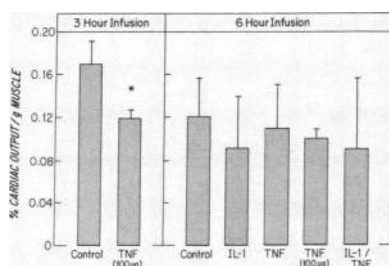

Figure 2. Blood flow distribution to skeletal muscle in saline-, IL 1-, TNF-, and IL 1/TNF-treated rats. Blood flow distribution to rectus abdominis muscle, expressed as percent of cardiac output per gram tissue. Measurements were made with labeled microspheres at 3 h of infusion of saline and 100 µg/kg of TNF and at 6 h of infusion of saline, 20 µg/kg of IL 1, 20 µg/kg of TNF, 100 µg/kg of TNF, and 20 µg/kg of TNF/IL 1 mixture. Reduction of blood flow distribution was significant only at 3 h of administration of 100 µg/kg of TNF. Data are mean±SE.

Table V. Summary of Urinary Nitrogen Excretion

	Dose (µg)			
Treatment*	0.2	2.0	20	200
IL 1	79.9 + 3.5	86.9 + 2.0	73.5 + 6.9	86.5 + 0.8
TNF	87.5 + 1.9	94.1 + 8.8	102.4 + 9.5‡	100.5 + 9.4‡
TNF/IL 1	93.7 + 8.8	76.9 + 3.9	94.8 + 4.1‡	108.4 + 2.6‡
Saline	76.7 + 3.0	76.7 + 3.0	76.7 + 3.0	76.7 + 3.0

Data are mean + SE, expressed as milligrams nitrogen /100 grams rat per 24 hours. Dose expressed as micrograms/kilograms.
* $P < 0.01$ by two-way ANOVA (treatment, dose).
‡ $P < 0.05$ vs. saline (Bonferroni confidence intervals).

This study demonstrates similar results for IL 1 and TNF, and also shows an effect on circulating levels of trace minerals, as previously demonstrated for infusion of purified endogenous mediator (6, 7). On the other hand, the effect of recombinant TNF and IL 1 on protein metabolism, particularly in skeletal muscle, has not been consistently documented. Goldberg et al. thus have recently incubated skeletal muscle fragments with recombinant TNF, separately and in combination with other recombinant cytokines, but were unable to detect a significant proteolytic effect as measured by the release of tyrosine in vitro (16). In addition, bolus intraperitoneal administration of recombinant TNF in rats was not associated with a significant increase in muscle proteolysis measured in vitro 4 h later (17). Using similar methodology, Moldawer et al. (18) also concluded that recombinant TNF did not regulate protein metabolism in skeletal muscle. Based on these results, it was hypothesized that the putative proteolytic effect of purified endogenous mediator is probably distinct from either IL 1 and TNF (16–18). On the other hand, recent studies have shown an increase in nitrogen efflux from skeletal muscle in patients treated with TNF (19), as well as a higher rate of loss of body protein in rats treated with recombinant TNF and pair-fed to controls (14). Although the origin of this discrepancy is not very clear, it represents methodological differences in estimating the rate of protein breakdown. Incubation of skeletal muscle segments in vitro consistently results in net release of amino acids to the extent that estimates of total muscle protein degradation in the unstimulated state exceed the rate of syn-

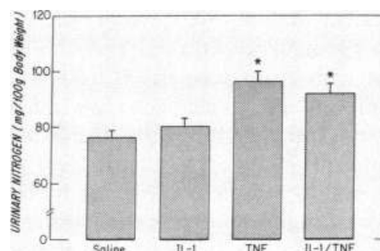

Figure 3. Urinary nitrogen excretion in saline, IL 1-, TNF-, and IL 1/TNF-treated rats. Total 24-h urinary nitrogen in mg/100 g body weight after two administrations of saline and cytokines. Two-way ANOVA showed a significant effect for treatment but not for dose of cytokine. Results shown here are mean values (±SE) of four dosages in each treatment group.

thesis by a factor of two (16–18). This nonphysiological increase in muscle proteolysis has been attributed to tissue hypoxia, lack of normal nutrient supply, as well as cofactor depletion and enzyme denaturation. Therefore, if an effect of TNF on protein degradation is exerted through the same pathway as that activated by tissue isolation, then this effect may not be detectable in vitro.

In the method used here, protein synthesis and degradation rates are estimated simultaneously in the intact animal under physiological conditions. The rate of [^{14}C]leucine incorporation into tissue proteins is expressed relative to the specific activity of free leucine in the precursor pool and this ratio is used for derivation of the synthetic rate (21). It is noted that the specific activity of free leucine in the homogenate (*Si*, Eq. 4) represents a mixture of intracellular and extracellular amino acids. Although the actual intracellular leucine specific activity may be estimated by subtracting the extracellular contribution, it has been shown that amino acyl-tRNA charging is affected by both intracellular and extracellular amino acid pool (35, 36). Thus, the specific activity of free leucine in tissue homogenate, although not identical to that of leucyl-tRNA, is a better representative of the true precursor pool than intracellular leucine (37). This assumption is not directly tested in this study. However, Tables III and IV show that estimates of muscle protein synthesis in the control rats (1a and 2a), as well as liver protein synthesis in group 1a, are very close to estimates of breakdown separately derived from [^{14}C]leucine tissue dilution. This observation suggests that synthetic rates based on the specific activity of leucine in tissue homogenate are consistent with a steady state in skeletal muscle of control rats. It will be shown below that a steady-state range (synthesis = breakdown) for [^{14}C]leucine dilution may be defined in relationship to protein-bound uptake of the amino acid tracer. Therefore, in as much as estimates of protein synthesis vary according to choice of the precursor pool specific activity, results of this study are also supportive of the assumption that total tissue homogenate is a better representative of the precursor amino acid pool than the actual intracellular pool.

Calculation of the rate of protein degradation is independent of the estimates of protein synthesis because it is derived from the dilution of free tissue [^{14}C]leucine relative to plasma leucine. It is assumed that total tissue distribution of tracer leucine molecules (intracellular as well as extracellular) is directly proportional to perfusion, and may be approximated by the distribution of microspheres administered into the left ventricle. Furthermore, because specific activity of free leucine in the total homogenate, rather than the actual intracellular specific activity, is used in these calculations, the degree of tissue/plasma dilution of [^{14}C]leucine in Eq. 5 is related only to the perfusion of that tissue and is independent of intracellular leucine transport or compartmentation. We have previously shown that estimates of liver protein breakdown based on Eq. 5 were in close agreement with estimates derived from the difference between total synthesis and net change in liver mass (21). We have also shown in healthy adult rats that the distribution of systemically infused [^{14}C]leucine to the liver is closely similar to blood flow distribution as measured by labeled microspheres (20). Assuming that the difference between muscle protein synthesis and breakdown in the control groups of the current study is negligible, Eq. 5 may be evaluated for q when the synthetic rate is known. For the rats in groups 1a and 2a, the mean estimate of q per gram muscle tissue is 0.14% (SE 0.01), which is not statistically different from the experimental muscle perfusion measurement based upon the distribution of 15-μm microspheres (experiment 3, Fig. 2).

Because protein synthesis and degradation rates are separately made, it is possible to express tissue/plasma [^{14}C]leucine dilution as a function of the anabolic/catabolic status. Thus, Eq. 5 may be rearranged into the following form:

$$Si/Sp = \frac{q}{q + (Tb/Q)} \qquad (8)$$

where Q is equivalent to total plasma leucine flux ($Q = I/Sp$). Because protein synthesis is independent of tissue/plasma dilution, and denoting the ratio of protein breakdown to synthesis (Tb/Ts) by k, Eq. 6 is written as:

$$Si/Sp = \frac{q}{q + k(Ts/Q)} \qquad (9)$$

This equation shows that tissue [^{14}C]leucine dilution, expressed as the ratio of specific activities Si/Sp, is determined by tracer distribution to the tissue, the rate of relative protein synthesis relative (Ts/Q), as well as the catabolic/anabolic protein status (k). Fig. 4 shows the expected range of Si/Sp in skeletal muscle, taking into consideration the 95% confidence range for mean blood flow distribution in skeletal muscle as measured by microsphere distribution in experiment 3. Points falling in the shaded area satisfy the condition for $k = 1$, where protein synthesis and breakdown are not measurably different (basal turnover). On the other hand, Si/Sp values below the shaded area indicate more tissue dilution of radioactive leucine than can be accounted for by basal protein turnover. Therefore, intravenous treatment with TNF, and to a significantly larger extent treatment with a mixture recombinant TNF/IL 1, is associated with an increase in the rate of muscle proteolysis and a net catabolic state.

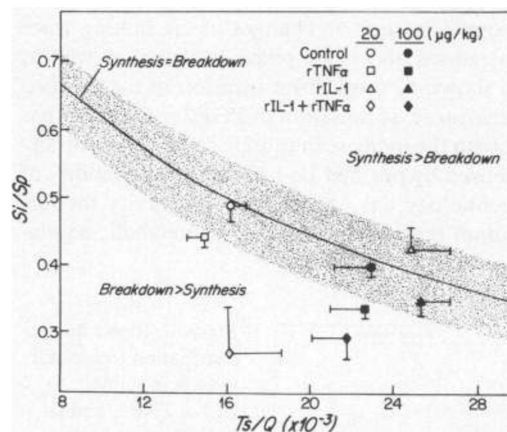

Figure 4. Dilution of free [^{14}C]leucine in muscle relative to plasma in saline-, IL 1-, TNF-, and IL 1/TNF-treated rats. Dilution of muscle free [^{14}C]leucine relative to circulating leucine, expressed as the ratio of tissue/plasma specific activities (Si/Sp), drawn along the y-axis. The x-axis is the ratio of leucine utilization in muscle protein synthesis (Ts) to plasma leucine flux (Q). This relationship is depicted by Eq. 9 in the text. Shaded area represents the 95% confidence range for blood flow distribution to 1 g of muscle (parameter q in Eq. 9), as measured by radioactive microspheres in experiment 3. Points within this area indicate steady state (synthesis = breakdown), while points below indicate net catabolism. Data are mean±SE.

Little is known about the effect of TNF and other cytokines on cardiovascular physiology. In view of the possible role of these agents in shock (38–40), experiment 3 was conducted to measure the hemodynamic changes associated with cytokine infusions shown to influence leucine kinetics in experiments 1 and 2. Although a dose of 100 μg/kg TNF significantly decreased cardiac output and resulted in a redistribution of blood from skeletal muscle into splanchnic organs, this effect was transient and could not possibly account for increased leucine dilution in skeletal muscle at 6 h of infusion. Systemic administrations at a dose level of 20 μg/kg were associated with smaller changes in muscle perfusion that did not achieve statistical significance by analysis of variance. It is also noted that, of the muscle perfusion changes in experiment 3, those associated with recombinant IL 1 were numerically the largest (P = NS). However, there were no significant differences in [^{14}C]leucine dilution in this group compared with the saline-treated controls. Therefore, we conclude that the effect of recombinant TNF and IL 1/TNF on leucine dilution in skeletal muscle represents a true increase in proteolysis rather than a decrease in perfusion. This is further substantiated by the increase in nitrogen excretion as a result of TNF and IL 1/TNF treatments.

Changes in [^{14}C]leucine dilution in the liver were less pronounced than those in skeletal muscle, especially in the 20 μg/kg dose infusion. Although it is difficult to interpret these results in terms of total liver protein synthesis and breakdown because secretory proteins were not isolated and measured separately, comparison of TNF and TNF/IL 1-treated groups with their respective control groups indicates a reduction of liver proteolysis. Table III shows that livers of saline-treated rats in experiment 2 (group 2a) were significantly catabolic. Assuming that this increase in basal liver catabolism has affected all the rats in experiment 2, possibly due to prolonged starvation or surgical stress, then infusion of recombinant TNF and IL 1/TNF at a dose of 100 μg/kg had an anabolic effect on liver protein, predominantly through reduction of proteolysis. This effect, which is larger in the cytokine coinfusion treatment group, is opposite to the effect on skeletal muscle, and is consistent with other recent observations of increased liver weight with multiple TNF administrations (14). These findings parallel the increase in liver size and circulating acute-phase proteins associated with sepsis (1). Further studies to characterize the pathophysiological significance of these observations are needed.

Finally, we note that, while this study demonstrates a catabolic effect for systemic infusions of recombinant TNF on muscle protein, it does not necessarily prove a causal relationship between this cytokine and metabolic derangements of sepsis and trauma. Although direct cellular injury has been demonstrated for TNF in the gastrointestinal tract (40), the possibility of involvement of other hormones and cytokine mediators, as well as the possibility of synergistic interaction of several cytokines have been suggested. That no clear dose effect can be demonstrated in this study indicates that regulation of protein metabolism in trauma, sepsis and malignancy is complex. Thus, whereas a small dose of IL 1 seems to enhance muscle protein synthesis, possibly through stimulation of insulin secretion (41), interaction with TNF is synergistic on proteolysis. This synergism, which has also been observed for other phenomena (39, 42–44), is best demonstrated by the ratio of muscle protein breakdown to synthesis being significantly larger in the cytokine coinfusion groups of experiments 1 and 2 ($P < 0.003$ and < 0.03, respectively, by ANOVA). It is also demonstrated in this study in the differential counts of circulating neutrophils and lymphocytes.

We conclude that systemic infusions of recombinant TNF in rats are associated with dilutional changes in tissue/plasma [^{14}C]leucine, as well as with increased urinary nitrogen excretion, consistent with enhanced proteolysis. Although these results are important for understanding of the metabolic effects of infusions, the exact regulatory role of these mediators in the pathophysiology of infection, trauma, and malignancy remains to be proven.

Acknowledgments

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