Pentoxifylline improves insulin action limiting skeletal muscle catabolism after infection

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Abstract

We investigated the ability of pentoxifylline (PTX) to modulate protein synthesis and degradation in the presence and absence of insulin during incubation of epitrochlearis muscle, 2 or 6 days after injection of *Escherichia coli*. On days 2 and 6 after infection, protein synthesis was inhibited by 25%, whereas proteolysis was enhanced by 75%. Insulin (2 nM) *in vitro* stimulated protein synthesis in muscles from infected rats to the same extent as in controls. The ability of insulin to limit protein degradation was severely blunted 48 h after infection. On day 6 after infection, insulin inhibited proteolysis to a greater extent than on day 2. PTX suppressed the increase in plasma

concentrations of tumor necrosis factor more than 600-fold after injection of bacteria, and partially prevented the inhibition of protein synthesis and stimulation of protein degradation during sepsis. Moreover, PTX administration maintained the responsiveness of protein degradation to insulin during sepsis. Thus cytokines may influence skeletal muscle protein metabolism during sepsis, both indirectly through inhibition of the effects of insulin on proteolysis, and directly on the protein synthesis and degradation machinery.

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Introduction

Insulin resistance in skeletal muscle characterizes the host's response to severe infection. Insulin regulates both glucose and protein metabolism in skeletal muscle. Both these metabolic processes show a resistance to the anabolic actions of insulin during sepsis. With respect to protein metabolism, proteolysis in skeletal muscle consistently shows a relative resistance to inhibition by insulin during the period immediately after a trauma (Tischler & Fagan 1983) or during acute (16 h) peritonitis (Hasselgren *et al.* 1987, 1989). In contrast, the ability of insulin to enhance protein synthesis is variable in skeletal muscle during sepsis. During the period immediately after infection (up to 48 h), insulin stimulates protein synthesis (Tischler & Fagan 1983, Hasselgren *et al.* 1987, 1989). However, during chronic (5-day) intra-abdominal sepsis, insulin was without effect in augmenting skeletal muscle protein synthesis (Jurasinski *et al.* 1995a).

The intracellular mechanisms responsible for the development of insulin resistance have not been fully elucidated. The most likely explanation is a sepsis-induced block in cellular proteins associated with downstream effector molecules of insulin action. Insulin action is

initiated by binding of the hormone to its high-affinity receptor located on the cell membrane. The binding of insulin to its receptor induces a conformational change in the receptor resulting in autophosphorylation of tyrosine residues of the β -subunit of the insulin receptor, and subsequent activation of tyrosine kinase activity. The tyrosine receptor kinase phosphorylates non-receptor intracellular proteins, including insulin receptor subunit (IRS)-1. Phosphorylation of IRS-1 on tyrosine residues provides a docking molecule for proteins involved in the signal transduction pathway for insulin (for review see White & Kahn 1994, Avruch 1998).

Changes in the cellular abundance or phosphorylation state of the proteins involved in the signal transduction of insulin occur in other insulin-resistant states. In skeletal muscle, we (Vary *et al.* 1995) and others (Fan *et al.* 1996) have shown that neither the number of insulin receptors nor the binding of insulin to its receptor is responsible for the insulin resistance in sepsis. However, there is a marked decrease in the extent of tyrosine phosphorylation of the insulin receptor after *in vivo* insulin stimulation in endotoxin-treated rats (Fan *et al.* 1996). Likewise, the extent of phosphorylation of the IRS-1 in response to insulin is not increased. Thus the septic process affects

proteins associated with the initial steps in the signal transduction pathway. Eventually, changes in the secondary signaling elements modulate effector molecules, thereby limiting the metabolic response to insulin.

With regard to the potential mediators responsible for the sepsis-induced insulin resistance, several lines of evidence indicate a potential link between tumor necrosis factor (TNF) and insulin resistance. First, plasma concentrations of TNF α are increased after infection (Chang & Bistrian 1998). Secondly, TNF blocks the action of insulin through its ability to inhibit insulin receptor tyrosine kinase activity (Feinstein *et al.* 1993, Kroder *et al.* 1996, Peraldi *et al.* 1996, Peraldi & Spiegelman 1998). Thirdly, obesity-induced insulin resistance is prevented in mice lacking a functional TNF response (Uysal *et al.* 1997). However, the ability of TNF to diminish the responsiveness of protein metabolism to insulin during sepsis remains unresolved.

One approach to understanding the role of TNF in initiating the metabolic response to infection is to inhibit its release or biological action, or both, during the bacterial insult. Administration of phosphodiesterase inhibitors, such as pentoxifylline (PTX) or amrinone, decreases the serum TNF α concentration in endotoxin-treated mice, rats and humans, and after induction of sepsis in rats (Doherty *et al.* 1991, Giroir & Beutler 1992, Breuille *et al.* 1993, Jurasinski *et al.* 1995b). The ability of amrinone to abrogate sepsis-induced inhibition of protein synthesis (Jurasinski *et al.* 1995b) is similar to the effect of a specific TNF binding protein (Cooney *et al.* 1999) to do the same, suggesting that TNF may be the common mediator affected by both treatment modalities in a chronic intraperitoneal model of sepsis. The purpose of the present study was to examine whether or not suppression of TNF α secretion by PTX could enhance the ability of insulin to improve skeletal muscle protein balance during the anorexic (2 days after infection) and hypermetabolic (6 days after infection) phases after injection of bacteria. To assess the role of insulin independent of other hormones or cytokines in modulating protein metabolism during sepsis, we used epitrochlearis muscles incubated *in vitro*. We conclude that the improvement of insulin action on skeletal muscle protein metabolism after treatment with PTX is mediated primarily through modulation of proteolysis, rather than protein synthesis, after infection.

Materials and Methods

Animals

Four groups of animals were used to investigate the effect of infection or pentoxifylline treatment on protein metabolism in skeletal muscle: control, control treated with pentoxifylline (control+PTX), infected, and infected treated with pentoxifylline (infected+PTX). Male Sprague–Dawley rats (250–300 g) were individually

housed in wire-bottom cages in a temperature-controlled environment (22–23 °C) with a 12-h light : 12-h darkness cycle. The infected group was produced by an injection of live *Escherichia coli* ($0.6\text{--}0.9 \times 10^9$ colony forming units/rat) into the tail vein as described previously (Breuille *et al.* 1993, Voisin *et al.* 1996, Vary *et al.* 1998). Control rats received an equal volume of saline. After such injection of bacteria, a sepsis-like condition develops, as indicated by leukocytosis, an acute phase response, and sustained muscle catabolism (Breuille *et al.* 1993, Voisin *et al.* 1996, Vary *et al.* 1998); bacteremia is maintained for at least 48 h after injection of *E. coli* (Voisin *et al.* 1996).

Inhibition of TNF production after injection of bacteria was accomplished by pretreatment of infected rats with PTX (Doherty *et al.* 1991, Breuille *et al.* 1993). PTX (100 mg/kg body weight) was injected intraperitoneally 1 h before the administration of *E. coli* (Breuille *et al.* 1993). Both control and infected rats received an equal volume of saline injected intraperitoneally at the time of the injection of PTX into infected rats. Blood samples (0.2 ml) were withdrawn via the tail vein 90 min after injection of the *E. coli* or saline, for measurement of plasma TNF α concentrations. Plasma was separated from other cells by centrifugation and stored at -80 °C until required for analysis for TNF α .

During a 6-day period of acclimatization before injection of bacteria, all rats had access to water and food *ad libitum*. The diet consisted of a semisynthetic chow containing 12% protein, which has previously been shown to sustain normal growth (Breuille *et al.* 1993). Injection of live *E. coli* causes anorexia in this model (Breuille *et al.* 1993, Voisin *et al.* 1996, Vary *et al.* 1998). Therefore, control, control+PTX and infected+PTX rats were paired with respect to the untreated infected rats by estimating food intake based on previous studies and correcting the daily intake with the actual food consumption of the untreated infected group (Vary *et al.* 1998). Animals were offered food twice daily, at 0900 h and 1700 h. All rats were fasted overnight before samples of muscles were taken for *in vitro* incubations. Animals were weighed on a daily basis. The experiments were carried out with the approval of animal care and use committees at both Institutions.

Incubations of epitrochlearis muscles

Epitrochlearis muscles were incubated *in vitro* as described previously (Stirewalt & Low 1983, Stirewalt *et al.* 1985, Dardevet *et al.* 1994, 1996, 1998, Voisin *et al.* 1996, Vary *et al.* 1998). The epitrochlearis was chosen to examine protein turnover after injection of bacteria because previous reports have provided evidence that sepsis preferentially affects protein metabolism in muscles composed of mixed fast-twitch fibers (Hasselgren *et al.* 1989, Vary & Kimball 1992, Cooney *et al.* 1994) and because its size

renders it suitable for *in vitro* incubation. On the day of the experiment, rats were anesthetized with pentobarbital (50 mg/kg body weight). The skin on each of the forelegs was removed. The epitrochlearis muscles were excised intact and immediately placed in Krebs–Henseleit bicarbonate buffer. The muscles were quickly rinsed and transferred to plastic tubes containing 2 ml buffer. The tubes were capped and immediately oxygenated. One muscle from each rat was incubated under basal conditions (no hormones), while the contralateral muscle was incubated in the presence of insulin. Because incubations were performed on different days, muscles from control and infected rats incubated under basal conditions were always included, to allow for comparisons from one experiment to the next.

Epitrochlearis muscles were first preincubated for 30 min. After the preincubation period, muscles were transferred to fresh media (2 ml) and incubated for a further 180 min, with a change of buffer every 60 min. During the final 60 min of the incubation period, the buffer was supplemented with 0.5 mM L-[¹⁴C]phenylalanine (0.15 µCi/ml). At the end of the incubation, muscles were removed from the incubation buffer, trimmed of connective tissue, immersed in 2 ml ice-cold 10% (wt/vol) trichloroacetic acid (TCA), and weighed. The incubation medium was frozen and stored at –20 °C until required for analysis of tyrosine and the specific radioactivity of phenylalanine.

The Krebs–Henseleit bicarbonate buffer consisted of 120 mM NaCl, 4.8 mM KCl, 25 mM NaHCO₃, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄ (pH 7.4) supplemented with 5 mM glucose, 5 mM 4-(2-hydroxyethyl)-1-piperineethanesulfonic acid, 0.1% (wt/vol) BSA, 0.17 mM leucine, 0.20 mM valine, 0.10 mM isoleucine. Muscles were incubated at 37 °C under an atmosphere of 95% O₂–5% CO₂. In some experiments, 2 nM insulin (Novo Pharmaceuticals, Bagsvaerd, Denmark) was added; we had previously established that 2 nM was the minimum concentration of insulin that elicited a maximal stimulation of protein synthesis and an inhibition of proteolysis (Vary *et al.* 1998). Fresh dilutions of insulin were made on the day of the experiment, using Krebs–Henseleit buffer.

Protein synthesis

Rates of protein synthesis were estimated by the incorporation of radioactive phenylalanine into muscle protein. Muscles were homogenized in 2 ml 10% TCA using a Polytron PT10 set at 60% of maximal speed. The homogenate was centrifuged at 10 000 *g* for 10 min at 4 °C. The supernatant was decanted and the pellet washed three additional times with 10% TCA to remove any acid-soluble radioactivity. The resulting pellet was dissolved in 1 M NaOH and incubated at 37 °C for a minimum of 30 min. Aliquots were assayed for protein using the

bicinchoninic acid procedure (BCA, Pierce Chemicals, Rockford, IL, USA) according to the manufacturer's procedure, with crystalline BSA as a protein standard. Another aliquot was assayed for radioactivity by liquid scintillation spectrophotometry, using corrections for quenching (disintegrations/min). Rates of protein synthesis, expressed as nmol phenylalanine incorporated per h per mg protein, were calculated by dividing the amount of radioactivity incorporated into muscle protein over a 1-h period by the specific radioactivity of the phenylalanine in the incubation medium.

Protein degradation

Total protein degradation was estimated simultaneously with the rate of protein synthesis as the sum of the accumulation of tyrosine in the incubation buffer over a 1-h period plus the amount of tyrosine equivalents incorporated into protein via protein synthesis during the same time interval as described previously (Tischler & Fagan 1983, Hasselgren *et al.* 1987, 1989, Dardevet *et al.* 1994, 1996, 1998, Voisin *et al.* 1996, Vary *et al.* 1998). Because tyrosine is neither synthesized nor metabolized by muscle, except for use by protein synthesis, the release of tyrosine from muscle into the incubation medium reflects net protein balance. To obtain the amount of tyrosine incorporated into mixed muscle proteins, we multiplied the values for incorporation of radioactive phenylalanine into protein by the molar ratio (0.77) of tyrosine to phenylalanine in mixed proteins from skeletal muscle (Tischler & Fagan 1983, Dardevet *et al.* 1994, 1996, 1998). Thus values for tyrosine equivalents incorporated into mixed muscle protein could be estimated for each individual muscle. Tyrosine in the incubation medium was measured fluorimetrically as described previously (Dardevet *et al.* 1994, 1996).

Substrate assays

TNFα in plasma samples was assayed with an ELISA kit (Genzyme, Boston, MA, USA) according to the manufacturer's instructions. Plasma samples were thawed only once, at the time of the assay for TNFα. Duplicate determinations were performed for each plasma sample measurement. The sensitivity of detection for TNFα in rat plasma was 0.015 ng/ml.

Statistics

The experimental data for each condition are expressed as means ± s.e. for each group. The statistical evaluation of the data was performed using analysis of variance (ANOVA) to test for overall differences among groups, followed by the Sidak test for multiple comparisons to determine significance between means, only when the ANOVA indicated a significant difference among the

group means. Differences among means were considered significant when $P < 0.05$.

Results

Effect of injection of PTX on protein synthesis and degradation in control rats

Before assessing the effects of treatment of infected rats with PTX, it was important to establish that PTX did not significantly alter protein synthesis or degradation in muscles from control rats. In these experiments, control rats were injected with either PTX or an equal volume of saline via the tail vein, as described in Materials and Methods. Food was restricted equally in both groups, to values obtained for infected rats (Breuille *et al.* 1993). Muscles were excised and incubated 2 or 6 days after injection of PTX. Rates of protein synthesis were lower in rats 2 days after injection of PTX (Fig. 1, top). By six days, rates of protein synthesis were not significantly different from each other in the basal condition between the groups (Fig. 1, top). Insulin (2 nM) caused a similar stimulation of protein synthesis in each group, such that there were no significant differences between saline- and PTX-treated rats on either day. There were no significant differences in protein degradation in the basal condition between the two groups at either time point (Fig. 1, bottom). In saline-treated rats, insulin significantly ($P < 0.05$) depressed proteolysis, by 20%, on day 2. Insulin limited proteolysis in PTX-treated rats by 24%, although the decrease did not reach statistical significance ($P < 0.1$). However, by day 6, insulin significantly inhibited protein degradation to the same extent in saline- and PTX-treated control rats. Therefore, treatment of control rats with PTX caused only minor effects with respect to protein synthesis or proteolysis, either under basal conditions or after incubation with insulin, compared with saline-treated rats.

Effect of infection and treatment with PTX on plasma TNF α concentrations

In control rats, the plasma concentration of TNF α averaged 0.087 ± 0.063 ng/ml ($n=4$). Ninety minutes after injection of bacteria, the plasma concentration of TNF α increased more than 600-fold, to 55 ± 6 ng/ml ($n=10$; $P < 0.001$ compared with control). Plasma TNF α concentrations in untreated infected rats were similar to those observed in previous studies using this model of sepsis (Breuille *et al.* 1993). Administration of PTX before injection of bacteria suppressed the increase in plasma TNF α concentrations (0.78 ± 0.05 ng/ml, $n=5$; $P < 0.001$ compared with non-treated infected rats). The mortality rate in untreated infected rats was approximately 15% and was significantly reduced, to 5% (Fisher's exact test: $P < 0.05$ compared with untreated infected rats) by pretreatment with PTX before injection of bacteria.

Figure 1 Effects of PTX on protein synthesis and proteolysis in control rats. Epitrochlearis muscles from control rats pair-fed to match the food consumption of infected rats were excised and incubated *in vitro* either 2 or 6 days after initiation of the feeding regimen in the presence or absence of insulin. In some animals, control rats were injected with PTX. Insulin was added to give a final concentration of 2 nM in the incubation medium. Rates of protein synthesis and protein degradation were calculated as described in Materials and Methods. Values shown are means $\pm$ S.E. for 3–14 muscles in each group. Protein synthesis Day 2 (ANOVA $F=16.73$, $P < 0.001$), Day 6 (ANOVA $F=5.68$, $P < 0.005$), $\dagger P < 0.001$ vs control, $* P < 0.05$ vs basal condition of same group-insulin, $** P < 0.01$ vs PTX. Proteolysis Day 2 (ANOVA $F=3.89$, $P < 0.05$), Day 6 (ANOVA $F=15.92$, $P < 0.001$), $* P < 0.05$ vs control, $\dagger P < 0.001$ vs control, $** P < 0.05$ vs PTX.

Food consumption and whole-body weight changes after infection

Treatment of infected rats with PTX enhances food consumption after injection of bacteria (Breuille *et al.* 1993). However, protein metabolism is sensitive to nutritional status. Thus the possibility existed that the previous

Figure 2 Food consumption in control, infected, and PTX-treated infected rats. Food consumption in control (open bars), infected (solid bars), and infected+PTX (striped bars) was measured during the first 5 days after injection of bacteria. Values shown are means $\pm$ S.E. for 4–15 animals in each group at each time point.

reports demonstrating an improvement in protein synthesis in infected rats treated with PTX could have resulted merely from increased food consumption (Breuille *et al.* 1993). To eliminate any alterations in protein metabolism resulting from a differential food intake between the experimental groups, all animals received the same amount of food. Hence, both control and infected rats treated with PTX were pair-fed with respect to the untreated infected rats. Figure 2 shows the food consumption in control, untreated infected, and infected rats treated with PTX. Essentially, all animals received the same nutrition throughout the course of the experiment. Figure 3 shows the effect of pair-feeding control and infected rats treated with PTX on the weight loss after injection of bacteria. When animals were pair-fed with respect to the untreated infected rats, no differences in the body weights were observed during the 4 days after infection between pair-fed control, pair-fed PTX-treated infected rats, and untreated infected rats. On day 5, however, the control rats did show a gain in weight that was not evident in the infected rats treated with PTX.

Figure 3 Cumulative weight changes in control, infected, and PTX-treated infected rats. The weight of rats from pair-fed control and infected groups described in Fig. 2 were measured daily. The differences in weight from day 0 are plotted. Values shown are means $\pm$ S.E. for 5–15 animals in each group at each time point.

Table 1 Effect of food consumption and pentoxifylline on weight of epitrochlearis muscle after infection. Values are means $\pm$ S.E.M. for 4–18 observations for each group at each day after infection

	Muscle weight (mg)		
	Control	Infected	Infected+ pentoxifylline
Days after infection			
2	56 $\pm$ 3	44 $\pm$ 2*	50 $\pm$ 2
6	53 $\pm$ 4	36 $\pm$ 2*	52 $\pm$ 2

ANOVA Day 2: $F=5.28$, $P<0.005$. ANOVA Day 6: $F=25.89$, $P<0.001$:

* $P<0.05$ compared with Control or Infected+pentoxifylline for the same day after infection.

Effect of PTX on infection-induced reductions in epitrochlearis weights

The mass of the epitrochlearis in untreated infected rats was significantly reduced, by approximately 20%, relative to pair-fed control rats 2 days after infection (Table 1). Pretreatment of infected rats with PTX prevented the loss of muscle mass. Likewise, the weight of the muscle from untreated infected rats was significantly reduced relative to

control (31%) or PTX-treated infected rats (31%) on day 6 after infection. There were no significant differences in the weights of the epitrochlearis between infected rats treated with PTX and controls at either time. To understand better the mechanisms responsible for the alterations in epitrochlearis weights, we measured rates of protein synthesis and degradation.

Effect of PTX on protein synthesis during infection: modulation by insulin

Rates of protein synthesis in control, infected, and PTX-treated infected rats at 2 and 6 days after the injection of bacteria are shown in Fig. 4. On day 2 after infection, the rate of protein synthesis in muscles from untreated infected rats was significantly reduced, by 17% compared with controls (0.17 ± 0.01 nmol Phe/mg protein/h, $n=12$ compared with 0.205 ± 0.009 nmol Phe/mg protein/h, $n=9$; $P<0.05$). Administration of PTX prevented the inhibition of protein synthesis observed in untreated infected rats (0.216 ± 0.01 nmol Phe/mg protein/h, $n=13$ compared with 0.17 ± 0.01 nmol Phe/mg protein/h, $n=12$; $P<0.05$). Addition of insulin (2 nM) to the incubation medium stimulated protein synthesis in all the groups (control without insulin 0.205 ± 0.009 nmol Phe/mg protein/h, $n=9$ compared with control+insulin 0.354 ± 0.019 nmol Phe/mg protein/h, $n=11$, $P<0.001$; infected without insulin 0.17 ± 0.01 nmol Phe/mg protein/h, $n=12$ compared with infected+insulin 0.315 ± 0.022 nmol Phe/mg protein/h, $n=10$, $P<0.001$; infected+PTX 0.216 ± 0.01 nmol Phe/mg protein/h, $n=13$ compared with infected+PTX+insulin 0.345 ± 0.017 nmol Phe/mg protein/h, $n=8$, $P<0.001$). The magnitude of the stimulation of protein synthesis in muscles incubated with insulin was the same in each of the groups examined.

On day 6 after infection, the rate of protein synthesis remained depressed in untreated infected rats compared with controls (0.170 ± 0.007 nmol Phe/mg protein/h, $n=13$ compared with 0.229 ± 0.009 nmol Phe/mg protein/h, $n=1$, $P<0.001$; Fig. 4). Protein synthesis under basal conditions was not significantly increased in muscles of infected rats treated with PTX compared with untreated septic rats (0.170 ± 0.007 nmol Phe/mg protein/h, $n=13$ compared with 0.208 ± 0.008 nmol Phe/mg protein/h, $n=12$). Addition of insulin (2 nM) to the incubation medium stimulated protein synthesis in all the groups (control without insulin 0.229 ± 0.009 nmol Phe/mg protein/h, $n=10$ compared with control+insulin 0.359 ± 0.019 nmol Phe/mg protein/h, $n=11$, $P<0.001$; infected without insulin 0.170 ± 0.007 nmol Phe/mg protein/h, $n=13$ compared with infected+insulin 0.314 ± 0.009 nmol Phe/mg protein/h, $n=10$, $P<0.001$; infected+PTX 0.208 ± 0.008 nmol Phe/mg protein/h, $n=12$ compared with infected+PTX+insulin 0.294 ± 0.012 nmol Phe/mg protein/h, $n=6$, $P<0.001$). The

Figure 4 Rates of protein synthesis in control, infected, and PTX-treated infected rats on day 2 and day 6 after infection: effects of insulin. Epitrochlearis muscles from infected and pair-fed control and PTX-treated infected rats were excised and incubated *in vitro* 2 days or 6 days after injection of bacteria as described in Fig. 1, in the presence or absence of insulin. Insulin was added to give a final concentration of 2 nM in the incubation medium. Rates of protein synthesis were calculated as described in Fig. 1. Values shown are means $\pm$ S.E. for 6–13 muscles in each group. Day 2 (ANOVA $F=25.2$; $P<0.001$): * $P<0.001$ compared with same group under basal incubation conditions; † $P<0.05$ compared with control or infected+PTX under basal conditions. Day 6 (ANOVA $F=88.97$; $P<0.001$): * $P<0.001$ compared with same group under basal incubation conditions; † $P<0.01$ compared with control under basal conditions.

maximal rate of protein synthesis in muscles incubated with insulin was the same in each of the groups examined.

Effect of PTX on protein degradation during infection: modulation by insulin

Under basal conditions, protein degradation was enhanced by 50% in infected rats on day 2 after infection compared

Figure 5 Rates of protein degradation in control, infected, and PTX-treated infected rats on day 2 or day 6 after infection: effects of insulin. Epitrochlearis muscles from infected and pair-fed control and PTX-treated infected rats were excised and incubated *in vitro* 2 days or 6 days after injection of bacteria as described in Fig. 4, in the presence or absence of insulin. Insulin was added to give a final concentration of 2 nM in the incubation medium. Rates of protein synthesis were calculated as described in Fig. 1. Values shown are means $\pm$ S.E. for 4–14 muscles in each group. Day 2 (ANOVA $F=14.70$; $P<0.001$): * $P<0.005$ compared with control basal conditions; ** $P<0.05$ compared with infected+PTX basal conditions or control+insulin; † $P<0.005$ compared with control or infected+PTX, basal conditions; ‡ $P<0.005$ compared with control+insulin or infected+PTX+insulin. Day 6 (ANOVA $F=24.01$; $P<0.001$): * $P<0.01$ compared with same group under basal incubation conditions; † $P<0.001$ compared with control or infected+PTX, basal conditions; ‡ $P<0.01$ compared with control+insulin or infected+PTX+insulin.

with controls (2.07 ± 0.014 nmol Tyr/mg protein/h, $n=12$ compared with 1.29 ± 0.06 nmol Tyr/mg protein/h, $n=8$, $P<0.001$; Fig. 5). The rate of protein degradation was significantly reduced in PTX-treated infected rats compared with untreated infected rats

controls (1.47 ± 0.005 nmol Tyr/mg protein/h, $n=13$ compared with 2.07 ± 0.014 nmol Tyr/mg protein/h, $n=12$; $P<0.001$). There were no significant differences in protein degradation between control and PTX-treated rats.

In controls, incubation of muscles with 2 nM insulin inhibited proteolysis by approximately 35% (0.763 ± 0.07 nmol Tyr/mg protein/h, $n=4$ compared with 1.29 ± 0.06 nmol Tyr/mg protein/h, $n=8$; $P<0.05$). Unlike its effect in control animals, insulin did not significantly reduce proteolysis in muscles from infected rats (1.82 ± 0.15 nmol Tyr/mg protein/h, $n=10$ compared with 2.07 ± 0.014 nmol Tyr/mg protein/h, $n=12$), indicating an insulin resistance. In the presence of insulin, proteolysis was increased approximately 2.4-fold in muscles from infected rats compared with controls (1.82 ± 0.15 nmol Tyr/mg protein/h, $n=10$ compared with 0.763 ± 0.07 nmol Tyr/mg protein/h, $n=4$; $P<0.001$). The ability of insulin to inhibit protein degradation was partially restored by treatment of infected rats with PTX. Incubation of muscles from PTX-treated infected rats with medium containing insulin resulted in a 25% inhibition in protein degradation (1.07 ± 0.05 nmol Tyr/mg protein/h, $n=7$ compared with 1.47 ± 0.05 nmol Tyr/mg protein/h; $P<0.001$). However, protein degradation in the presence of insulin remained significantly increased, by 40%, compared with controls (1.07 ± 0.05 nmol Tyr/mg protein/h, $n=7$ compared with 0.763 ± 0.07 nmol Tyr/mg protein/h; $P<0.05$).

The rate of protein degradation in epitrochlearis muscle from infected rats decreased on day 6 after infection relative to day 2 (1.69 ± 0.067 nmol Tyr/mg protein/h, $n=10$ compared with 2.07 ± 0.014 nmol Tyr/mg protein/h, $n=12$; $P<0.05$), but remained increased compared with controls under basal conditions on day 6 (1.69 ± 0.067 nmol Tyr/mg protein/h, $n=10$ compared with 1.15 ± 0.0068 nmol Tyr/mg protein/h, $n=8$, $P<0.001$; Fig. 5). Administration of PTX to infected rats significantly reduced protein degradation, by 35% compared with untreated infected rats (1.69 ± 0.067 nmol Tyr/mg protein/h, $n=10$ compared with 1.10 ± 0.0061 nmol Tyr/mg protein/h, $n=11$; $P<0.001$). There were no significant differences in proteolysis between PTX-treated infected rats and controls (1.15 ± 0.0068 nmol Tyr/mg protein/h, $n=8$ compared with 1.10 ± 0.0061 nmol Tyr/mg protein/h, $n=11$).

In controls, insulin reduced proteolysis compared with basal conditions (0.785 ± 0.049 nmol Tyr/mg protein/h, $n=14$ compared with 1.15 ± 0.068 nmol Tyr/mg protein/h, $n=8$; $P<0.001$). Unlike its effects on day 2, insulin significantly reduced protein degradation in muscles from rats 6 days after injection with bacteria (1.14 ± 0.07 nmol Tyr/mg protein/h, $n=10$ compared with 1.69 ± 0.067 nmol Tyr/mg protein/h, $n=10$; $P<0.001$). However, rates of protein degradation in infected rats remained more than 50% greater compared

with muscles from controls incubated in the presence of insulin (1.14 ± 0.07 nmol Tyr/mg protein/h, $n=10$ compared with 0.785 ± 0.049 nmol Tyr/mg protein/h, $n=14$; $P<0.005$). Incubation of muscles from PTX-treated infected rats with medium containing insulin resulted in a 50% inhibition in protein degradation (0.617 ± 0.099 nmol Tyr/mg protein/h, $n=6$ compared with 1.10 ± 0.061 nmol Tyr/mg protein/h, $n=11$; $P<0.001$). Unlike on day 2, on day 6 there were no significant differences between infected rats treated with PTX and controls in the presence of insulin (0.617 ± 0.099 nmol Tyr/mg protein/h, $n=6$ compared with 0.785 ± 0.049 nmol Tyr/mg protein/h, $n=14$).

Conclusions

Insulin resistance is defined as a smaller than normal response to a given dose of insulin. In the present study, inhibition of proteolysis by insulin was reduced in skeletal muscle after infection, indicating an insulin resistance. However, knowledge of the basis of the insulin resistance after infection is very incomplete. By analogy with obesity, cytokines such as TNF may limit the ability of insulin to modulate cellular metabolism (Hotamisligil & Spiegelman 1994). Likewise, there is evidence that TNF participates in the regulation of protein turnover in skeletal muscle *in vivo* (Cooney *et al.* 1999) or human myoblasts in culture (Frost *et al.* 1997). In the present set of investigations, we sought to determine the effect of limiting TNF α expression on the ability of insulin to modulate protein metabolism. Pretreatment of rats injected with live bacteria with PTX suppressed the appearance of TNF in the plasma by 98%. Reducing plasma TNF concentrations improved the sensitivity of proteolysis to insulin after infection compared with untreated infected rats. Rates of protein degradation decreased by more than 50% after addition of insulin to the incubation medium in septic rats treated with PTX, whereas muscles from untreated infected rats were relatively unresponsive to the inhibitory effects of insulin. Therefore, administration of PTX reduced the development of insulin resistance with respect to protein degradation after infection. The association of reduced TNF concentrations coupled with an enhanced ability of insulin to inhibit proteolysis suggests that TNF has a central role in the development of insulin resistance after infection. However, we cannot exclude the possibility that PTX modulates protein metabolism in septic rats by some mechanism unrelated to inhibition of TNF α expression.

The effect of PTX on the ability of insulin to limit protein degradation and stimulate protein synthesis after infection may result from a direct effect of TNF on skeletal muscle or indirectly via substances produced by other cells or tissues sensitive to TNF. These two possibilities are not mutually exclusive. The effect of PTX was observed after only a single dose of the compound, indicating that PTX

suppresses the compound(s) responsible for initiation of the insulin resistance during the period immediately after injection of the live bacteria. Treatment of control animals with PTX did not affect epitrochlearis weight, protein synthesis, or protein degradation appreciably, indicating that PTX appears not to exert a non-specific effect on skeletal muscle protein metabolism in the absence of infection. However, caution must be exercised in ascribing the metabolic effects of PTX on protein metabolism in muscle. TNF stimulates the secretion of other cytokines, including interleukins-1 and -6 and other inflammatory mediators. Furthermore, TNF often acts in synergy with other cytokines. However, our results are at least consistent with the hypothesis that modulation of TNF α by PTX may mediate changes in muscle protein metabolism after a bacterial challenge.

Pretreatment with PTX of rats injected with live bacteria prevented the loss of epitrochlearis mass on either day 2 or day 6 after infection. Presumably, alteration in the rates of protein synthesis or protein degradation were responsible for the increase in muscle weight in septic rats treated with PTX during sepsis. Rates of protein synthesis tended to be greater in PTX-treated septic rats, but were only significantly increased in muscles from infected rats treated with PTX on day 2 after infection. In contrast, PTX treatment prevented the stimulated proteolysis observed in infected rats on day 2 after infection. However, administration of PTX did not completely prevent the acceleration of protein degradation after infection. Zamir *et al.* (1992) reported that muscle protein breakdown was also reduced, but not normalized, after infusion of polyclonal antibodies raised against TNF into acutely infected rats. During the hypermetabolic phase of sepsis, PTX administration in infected rats completely prevented the enhanced proteolysis in muscle compared with untreated infected rats. These findings provide evidence that a decreased rate of protein degradation appeared primarily responsible for the maintenance of the muscle mass in septic rats treated with PTX during the anorexic and hypermetabolic period. Furthermore, a part of the protein-sparing effects of PTX results from its ability to abrogate the inhibitory effects of infection on protein synthesis and stimulatory effects on proteolysis.

Previous studies from this laboratory suggested the effects of PTX on protein synthesis measured *in vivo* 2 days after infection were independent of food consumption (Breuille *et al.* 1993). However, in those studies, infected rats treated with PTX were never pair-fed with respect to the non-treated infected rats. The nutritional status of animals and man has long been known to modulate both protein synthesis and protein degradation. Thus the possibility exists that the effects of PTX in the previous report (Breuille *et al.* 1993) were related simply to increased food consumption during periods immediately after the injection of bacteria. To eliminate the influence of increased food consumption in the present study, PTX-treated

infected rats were pair fed to the food consumption of the untreated septic rats. The results obtained from pair-feeding PTX-treated septic rats to the same level of food intake as untreated septic rats was essentially that observed when PTX-treated rats were allowed to feed *ad libitum*. Therefore, the ability of PTX to limit the inhibition of protein synthesis and stimulation of protein degradation during sepsis are independent of food intake over the first 5 days after infection. Thus the effect of PTX to partially prevent the alterations in the regulation of protein turnover during sepsis appears not to be dependent on food intake.

In summary, our study brings new insights into the relationship between TNF and the ability of insulin to promote anabolism in skeletal muscle after infection. Data from the present study indicate that infection produces fundamental defects in skeletal muscle protein synthesis and proteolysis that are independent of the food intake or nutritional state of the animal. Administration of PTX can partially prevent the inhibition of protein synthesis in infected rats during the anorexic phase of sepsis. Treatment of septic rats with PTX prevented the stimulation of proteolysis. Furthermore, PTX treatment abolished the insulin resistance with respect to proteolysis and restored the responsiveness of protein degradation to inhibition by insulin after infection. Because PTX administration suppressed the increase in plasma TNF α concentrations after infection, these data suggest that this cytokine has an important role in the regulation of protein turnover during sepsis.

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