Leupeptin Inhibits Adrenocorticotropic Hormone-induced Protein Breakdown in the Conscious Dog

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ABSTRACT To elucidate the role of proteinase inhibitors in the regulation of protein breakdown in vivo, we measured the effect of leupeptin on the rate of appearance of leucine in the plasma compartment in overnight-fasted conscious dogs. Two groups of dogs were studied. The control group (I) received saline infusion, and the experimental group (II) was rendered hypercatabolic with daily administration of adrenocorticotropic hormone (ACTH) (500 U/d) for 4 d.

ACTH treatment increased plasma cortisol from 2±0.4 to 17±2 μg/dl ($P < 0.005$). It raised plasma leucine levels (μmol/liter) from 123±6 in I to 206±5 in II ($P < 0.01$) and its rate of appearance into the plasma compartment (micromoles per kilogram per minute) from 3.1±0.1 in I to 4.6±0.3 in II ($P < 0.01$). Whole blood alanine concentration (micromoles per liter) increased by 50% (from 387±31 to 577±53, $P < 0.01$) and whole blood glutamine concentration (micromoles per liter) increased from 653±51 to 917±93 ($P < 0.01$). Leupeptin infusion in the ACTH-treated group significantly decreased both the concentration of plasma leucine and its rate of appearance. Blood glutamine declined by 30% ($P < 0.05$) after leupeptin, but no effect on blood alanine was observed. Leupeptin had no effect on the saline control group.

These data indicate that leupeptin decreases the accelerated rate of protein breakdown induced by cortisol excess. The fact that it did not affect protein degradation in controls may indicate that control of protein breakdown in the postabsorptive state may differ from that during accelerated turnover. Thus, the antibiotic proteinase enzyme inhibitors may be potentially useful in treating conditions of inappropriate protein breakdown.

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INTRODUCTION

Protein degradation is a selective, energy-requiring process necessary for basic cellular function. It provides amino acids for synthesis of new proteins and clears the cells of abnormal proteins (1). Most evidence suggests that the majority of long-lived intracellular proteins are degraded within lysosomes. This degradation is subject to hormonal and nutritional control. In contrast, the degradation of short-lived and abnormal proteins occurs in nonlysosomal fractions and is unresponsive to physiologic regulators (2).

Recent reports have shown that the use of microbial proteinase inhibitors decreases the degradation of long-lived proteins in cultured hepatocytes (3) and in perfused rat livers deprived of substrates or insulin (2). They have also been shown to inhibit protein breakdown in isolated rat skeletal and heart muscle (4), and can slow the accelerated muscle proteolysis seen in dystrophic chicks (5).

This study was performed to test the possibility of using microbial proteinase inhibitors to decrease the accelerated catabolism that accompanies various clinical conditions. We measured the effect of leupeptin on qualitative rates of protein breakdown in the con-

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TABLE I

*Plasma Insulin, Glucagon, Cortisol, and Blood Alanine and Glutamine Levels in Response to ACTH and Leupeptin in Conscious Overnight-fasted Dogs**

	Saline controls			ACTH-treated		
		Experimental			Experimental	
	Basal	(−) Leupeptin	(+)	Basal	(−) Leupeptin	(+)
Insulin (*μU/ml*)	11±3	11±2	12±2	11±2	13±2	12±2
Glucagon (*pg/ml*)	92±4	98±11	85±9	81±6	78±7	84±6
Cortisol (*μg/dl*)	2±0.4	2±0.5	3±0.4	17±2	18±2‡	19±3‡
Alanine (*μmol/liter*)	387±31	298±49	343±39	591±64§	577±53	512±61
Glutamine (*μmol/liter*)	653±51	691±59	627±86	917±93§	1,018±124	689±98‡

* The values during the basal period represent an average of four samples per dog and the values during the experimental period represent an average of 5–7 samples per dog for the hormones and four samples per dog for the last 2 h of the study for blood alanine and glutamine. (−) denotes absence and (+) denotes presence of leupeptin.

‡ Significance from corresponding basal period, $P < 0.01$.

§ Significance from basal period of saline controls, $P < 0.05$.

scious dog. Dogs were studied in the postabsorptive state and after stimulation of protein degradation by the administration of pharmacologic doses of adrenocorticotropic hormone (ACTH).[1]

METHODS

Experiments were carried out on 18 conscious dogs (18–23 kg) of either sex with silastic catheters chronically implanted (17–21 d) in the femoral artery (6). On the day of the study the catheters were removed from the subcutaneous pockets through skin incisions made under local anesthesia. Angiocatheters (18 gauge) were inserted percutaneously into the two cephalic veins. These were used for isotope, hormone, or saline infusions. After this preparation, the conscious dogs were then placed in a Pavlov harness and allowed to rest for ~1 h before beginning the intravenous infusion of the [^{3}H]leucine.

Experimental design. Two types of studies were performed. One group of animals (n = 10) received a 4-d pretreatment with ACTH (500 U i.m./d), while the rest (n = 8) received saline injections. After an 18-h overnight fast, all animals received a 7.5 h infusion of L-4,5,[^{3}H]leucine for the measurement of plasma leucine kinetics. After a 3-h isotopic equilibration period and a 0.5-h basal period, five ACTH-treated and four untreated dogs received a continuous intravenous infusion of leupeptin (a mixture of propionyl- and acetyl- leucyl-leucyl-arginal) at 4.3 mg/min for a 4-h experimental period. The rest received saline. Blood samples were obtained at 10-min intervals during the basal period and at 30-min intervals during the experimental period. The collection and immediate processing of blood samples have been previously described (6). Plasma immunoreactive glucagon was assayed with Unger's 30,000-mol wt antibody (7), insulin with the Sephadex-bound antibody procedure (Pharmacia Fine Chemicals, Piscataway, NJ) (8), and cortisol with radioimmunoassay (9). The radioactivity and concentration of plasma leucine were determined by rapid column chromatography (6). The specific radioactivity of [^{3}H]leucine in the infusate solution was checked in each experiment in a similar fashion to the plasma samples. The coefficient of variation in duplicate pairs (n = 16) was 2.1% for arterial plasma concentration and 2.8% for leucine-specific activity (6). Blood alanine and glutamine were determined enzymatically (10, 11).

Tracer methods and calculations. At steady-state conditions, the rate of appearance of leucine into the plasma compartment was estimated by dividing the rate of isotope infusion (disintegrations per minute per kilogram) by the plateau arterial specific activity (disintegrations per minute per micromole per 10^3). During nonsteady-state conditions, calculation of the rate was carried out according to the method of Wall et al. (12), used for determination of glucose production. This model assumes that rapid changes in leucine-specific activity and concentration do not occur uniformly within the entire pool. It also assumes that plasma reentry of isotope from the protein pool is negligible during the infusion period. Since the studies were done in the postabsorptive period, in the absence of exogenous leucine, the rate of appearance of this essential amino acid reflects the rate of protein breakdown (6). Since the intracellular specific activity of [^{3}H]leucine is lower than that in plasma (13), a rate of appearance calculated using plasma specific activity will underestimate the total rate of protein degradation. Despite these limitations, changes in leucine rate of appearance in the plasma compartment should reflect any increase or decrease in the rate of protein breakdown (6).

RESULTS

Table I summarizes the mean hormonal values in the four groups. ACTH treatment resulted in a ninefold elevation in plasma cortisol ($P < 0.001$) without a change in plasma insulin or glucagon. Leupeptin exerted no effect on the hormonal levels throughout the study period.

The rate of appearance of leucine during the post-

[1] *Abbreviation used in this paper*: ACTH, adrenocorticotropic hormone.

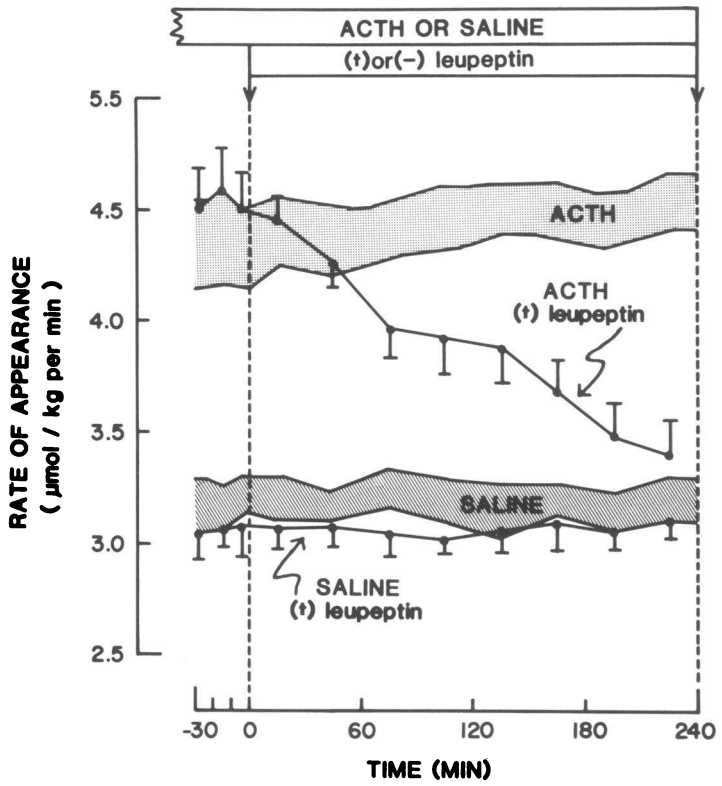

FIGURE 1 The effect of leupeptin and ACTH on the rate of appearance (Ra) of leucine in the plasma compartment in overnight-fasted conscious dogs. One group (n = 10) received daily intramuscular injections of ACTH (500 U/d) for 4 d, while the other (n = 8) received daily injections of saline. On day 5, and after an 18 h of overnight fast, the ACTH-treated group received a constant intravenous infusion of ACTH (1 U/min) for 7.5 h, while the others received saline. During the experimental period (0–240 min) four dogs of the saline-infused group (saline + leupeptin) and five dogs of the ACTH-treated group (ACTH + leupeptin) received a continuous infusion of leupeptin (4.3 mg/min), while the other dogs served as controls. Analyses of variance during the basal period showed that ACTH treatment resulted in significantly higher leucine rates of appearance than in saline group ($P < 0.01$). Leupeptin resulted in significant reduction in the rate of appearance only in the ACTH-treated group ($P < 0.01$).

absorptive control period was 70% greater in the ACTH-treated dogs than in untreated dogs (Fig. 1). This increased rate of appearance was associated with a 68% increase in the plasma concentration of leucine (Fig. 2). In response to ACTH treatment, blood alanine rose by 53% (from 387±31 to 591±64 μmol/liter, $P < 0.05$) and glutamine rose by 40% (from 653±51 to 917±93 μmol/liter, $P < 0.05$).

In ACTH-treated animals, leupeptin caused a prompt and progressive fall in the rate of appearance of leucine, so that by the end of the experiment it did not differ significantly from that of the control animals. The plasma concentration of leucine fell concurrently, although it remained significantly elevated after 240 min. Leupeptin decreased blood glutamine by 30% (to 689±98 μmol/liter, $P < 0.05$), as shown in Table I. In sharp contrast, leupeptin had no effect on untreated animals. Both leucine rate of appearance and plasma concentration as well as blood glutamine and alanine remained constant throughout the entire 4-h period.

DISCUSSION

It is well known that glucocorticoid hormones promote the net release of amino acids from muscle by decreasing protein synthesis (14, 15). Their effect on protein breakdown, however, remains controversial. Hopgood et al. (16) have recently shown that glucocorticoids enhance degradation by 10% as a result of increased autophagic activity in hepatocyte monolayers. Goldberg (17) and Tomas et al. (18) demonstrated an increase in the breakdown of myofibrillar and soluble proteins from muscle tissues of rats receiving high

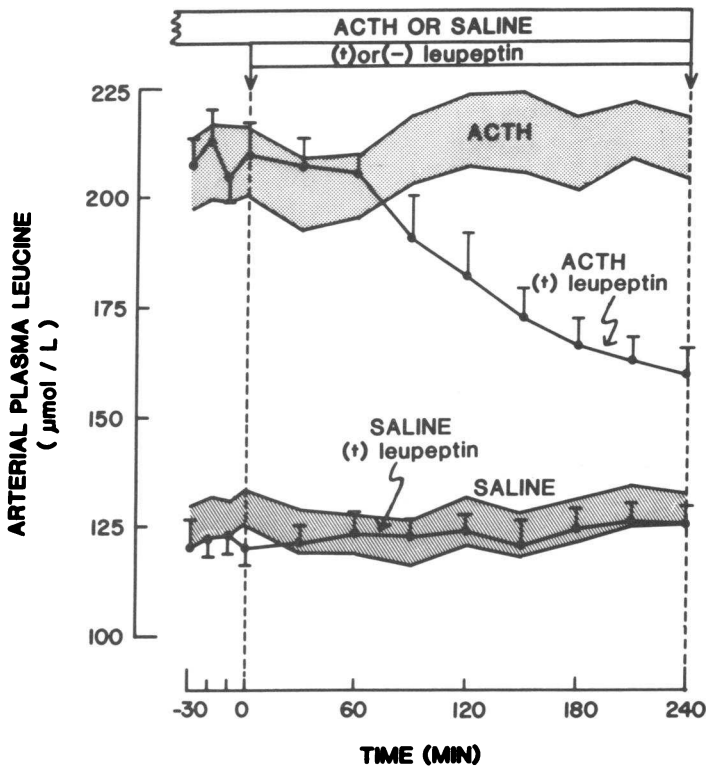

FIGURE 2 The effect of leupeptin and ACTH on arterial plasma leucine in overnight-fasted dogs. The experimental design is outlined in Fig. 1. Analyses of variance during the basal period showed that ACTH resulted in significantly higher leucine levels ($P < 0.01$). Leupeptin infusion resulted in marked reduction in leucine concentrations in the ACTH-treated group ($P < 0.01$). L, liter.

doses of glucocorticoids. Other investigators (14, 15) failed to show increased protein breakdown after glucocorticoids even though they observed depression of protein synthesis. In this study, the 70% increase in the rate of appearance of leucine in plasma represents a minimum estimate, since intracellular specific activity may be lower than plasma. Since the studies were performed in the absence of exogenous intake of leucine, the accelerated appearance must have been from protein degradation and indicates that proteolysis must have increased by at least 70%.

Protein breakdown can involve lysosomal and nonlysosomal mechanisms. Lysosomal involvement in certain aspects of protein breakdown has been documented. Using perfused rat liver, Mortimore (2) presented compelling evidence for lysosomal participation in deprivation-enhanced protein breakdown and to a lesser degree in basal or steady-state protein turnover. Nonlysosomal mechanisms of protein breakdown are not yet clearly defined. Reticulocytes possess a nonlysosomal ATP-dependent system for protein breakdown with several essential components including the universally occurring polypeptide, ubiquitin (19). This system is responsible for the rapid hydrolysis of abnormal hemoglobin (19). In other cells, the biologic function of nonlysosomal proteolysis is not well understood, but may be related to the degradation of the short-lived proteins (2).

Muscle preparations obtained from glucocorticoid-treated rats show dramatic increases in both cathepsin B and alkaline myofibrillar proteinase activities (20), implicating these enzymes in increased proteolysis. Leupeptin has been shown to exert 80–85% of its activity against the lysosomal cathepsins B_1, H, and L (21); it has also been shown to be effective against calcium-activated protease in cytoplasm of all cells (22). In vivo, leupeptin has been shown to inhibit accelerated muscle proteolysis in dystrophic chicks (5) and to delay denervation atrophy of chicken pectoralis muscles (5). Thus, the demonstration that leupeptin decreases total body protein breakdown in the ACTH-treated animals may signify that glucocorticoid excess enhances activity of the lysosomal and possibly nonlysosomal proteinases, specifically those sensitive to leupeptin.

Leupeptin's failure to affect protein degradation in

the untreated animals indicates that in the postabsorptive state, regulation of protein breakdown is different from that during states of accelerated protein loss. These findings agree with in vitro studies showing that such microbial inhibitors were only slightly effective in suppressing basal proteolysis in either perfused rat livers (2) or in cultured hepatocytes (3).

In summary, in vivo leupeptin infusion decreases the rate of appearance of plasma leucine during glucocorticoid excess, suggesting a decrease in proteolysis. Since many of the changes in protein turnover occurring with trauma, diabetes mellitus, infection, and starvation are similar to those observed with glucocorticoid excess, proteinase inhibitors might prevent protein breakdown in these catabolic situations, as was previously suggested (4).

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