Insulin's Effect on Leucine Turnover Changes During Early Fasting in the Conscious Dog

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SUMMARY

To study the effects of insulin on leucine turnover during fasting, acute insulin deficiency was induced by the simultaneous infusion of somatostatin and glucagon in conscious dogs fasted 18 h (N = 10) and 48 h (N = 11). Insulin levels during the basal period (before hormone perturbation) were similar in both groups of dogs (12 ± 3 versus 10 ± 3 μU/ml, respectively). Glucagon levels were similar in the two groups (94 ± 9 versus 106 ± 19 pg/ml). Leucine levels rose from 118 ± 9 μmol/L to 155 ± 12 μmol/L as fasting progressed ($P < 0.005$). Its rate of appearance also increased by 30% ($P < 0.005$) from 3.4 ± 0.3 to 4.3 ± 0.4 μmol/kg/min ($P < 0.005$), while its clearance remained unchanged. Acute insulin deficiency caused an increase in leucine levels in both 18-h and 48-h-fasted dogs by 55% (to 181 ± 10 μmol/L) and 45% (to 225 ± 20 μmol/L), respectively ($P < 0.005$). However, while the rate of appearance of leucine remained unchanged in dogs fasted overnight, it rose to 5.1 ± 0.3 μmol/kg/min ($P < 0.01$) in those fasted 48 h. The metabolic clearance rate fell in both groups, although this drop was twice as great in the 18-h group (from 28 ± 3 to 17 ± 3 ml/kg/min, $P < 0.005$) as in the 48-h group (from 28 ± 3 to 23 ± 2 ml/kg/min, $P < 0.005$).

We conclude that insulin has disparate effects on protein turnover as fasting becomes more prolonged. Its acute absence results in enhanced proteolysis in the 2-day-fasted, but not in the overnight-fasted dog. These data suggest that the response of protein turnover to fasting is quite complex and involves more than the effects of changing insulin concentrations.

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Metabolic adaptation to early fasting involves increased rates of proteolysis in skeletal muscle[1–4] and liver tissues.[4] It has been frequently shown that early starvation in man is accompanied by a decline in circulating insulin levels. This led to the suggestion that this fall is responsible for initiating the observed protein loss.[5] The effect of insulin on protein turnover has received much investigative attention in recent years.[6] In vitro studies have shown that insulin can influence both formation and breakdown of tissue protein. In rat skeletal[1–3] and cardiac muscles,[1] insulin stimulates protein synthesis at the step of peptide chain initiation, and inhibits proteolysis by a mechanism that remains unclear. In the liver, insulin has been shown to stimulate protein synthesis[7] and inhibit protein breakdown,[4] and simultaneously decrease urea production and the conversion of amino acids to glucose.[4] Its effect on protein synthesis is cAMP dependent but its effect on breakdown is non-cAMP dependent and requires the internalization of the insulin receptor complex,[8] which leads, by a yet unknown mechanism, to the stabilization of lysosomal structure and consequently to a decrease in lysosomal autophagy.[4] In vivo studies have produced less definitive data, since they for the most part used nitrogen balance techniques. These studies documented increased nitrogen excretion in states of insulin deficiency but did not elucidate whether the protein loss is caused by enhanced proteolysis or diminished synthesis. We recently showed that, in the intact dog, insulin has different effects on leucine turnover depending on the dose used.[9] Acute insulin withdrawal in the postabsorptive state caused diminished clearance of leucine from the plasma compartment without affecting its rate of appearance, indicating that the net effect of basal insulin deficiency was to maintain protein synthesis. At twice basal levels, insulin inhibited leucine's rate of appearance and stimulated its metabolic clearance, suggesting that protein degradation was inhibited, while synthesis was most likely increased.[9]

This study examines the effects of insulin on leucine turnover during progressive fasting in the conscious dog. It dem-

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onstrates that acute insulin deficiency has different effects on leucine metabolism in 18- and 48-h-fasted dogs. The role this hormone plays in modulating leucine turnover changes as fasting progresses.

MATERIALS AND METHODS

Experiments were carried out on 35 mongrel dogs of either sex weighing 15–25 kg, which had been fed a regular dog chow diet (Wayne Dog Food, Allied Mill, Inc., Chicago, Illinois). Seventeen days before the study, silastic catheters were placed in the femoral artery and splenic vein under general anesthesia as previously described.[9] The catheters were filled with heparinized saline (200 U/ml, Abbott Diagnostics, North Chicago, Illinois) and the free ends were knotted and placed in subcutaneous (s.c.) pouches so that closure of the skin incision was possible.

Sixteen days after surgery, blood was drawn from each animal for complete blood count. Only those dogs with hematocrit >38%, leukocyte count <15,000/mm^3, good appetite, and having normal stools were used. Eighteen of the dogs were fasted overnight (18 h) and 17 were fasted 48 h before study. On the day of the study, the catheters were removed from their s.c. pockets using local anesthesia (1% lidocaine, Astra Pharmaceutical Products, Inc., Worcester, Massachusetts). The contents of each catheter were aspirated, and saline (0.9%) was slowly infused until the experiments began. Femoral artery catheters were used for blood sampling, while the splenic vein catheters were used for hormone infusion. Angiocatheters (18 gauge) were inserted percutaneously into both cephalic veins and were used for the constant infusion of L-4,5-^{3}H-leucine, somatostatin, or saline. After completion of these procedures, the conscious dogs were placed in Pavlov harnesses and were allowed to rest 1 h before starting the tracer infusion.

Experimental design. All experiments consisted of a 3-h tracer equilibration period, followed by a 30-min basal period and a 4-h period of hormonal perturbation. During the experimental period, ten 18-h-fasted (group 1) and eleven 48-h-fasted (group 2) dogs received a constant infusion of somatostatin (SRIF) at 0.8 μg/mg/min. To create a selective insulin deficiency, basal glucagon was replaced intraportally at 0.65 ng/kg/min in the 18-h-fasted group and at 0.55 ng/kg/min in the 48-h-fasted group. Controls within each group (8 from group 1 and 6 from group 2) received a saline infusion throughout the study.

Processing of blood samples. Blood samples were collected every 10 min during the basal period and every 30 min thereafter. Immediate processing of blood samples was performed as previously described.[9] Plasma immunoreactive glucagon was determined using Unger's 30K antibody,[10] obtained from the University of Texas, Southwestern Medical School, and insulin was determined by the Sephadex-bound antibody procedure (Pharmacia Fine Chemicals, Piscataway, New Jersey).[11] The radioactivity and concentration of ^{3}H-leucine in plasma samples was determined by rapid column chromatography as previously described.[9]

Materials. L-4,5-^{3}H-Leucine (New England Nuclear, Boston, Massachusetts) was used at 55 μCi/μmol. The purity of the tracer was determined for each stock of ^{3}H-leucine employed; 98–99% of the infused radioactivity was found in leucine. Glucagon was obtained from Eli Lilly and Company (Indianapolis, Indiana) and trasylol from FBA Pharmaceuticals (New York, New York). Phadebas insulin radioimmunoassay kits were purchased from Pharmacia Fine Chemicals. All hormone solutions were prepared with saline and 0.3% bovine albumin.

Calculations. At steady-state conditions, the rate of appearance (Ra) of leucine into the plasma compartment is equal to its rate of disappearance (Rd) and is calculated by dividing the rate of isotope infusion (dpm/kg/min) by the "plateau" plasma specific activity (dpm/μmol/10^3). During nonsteady-state conditions, calculations were performed using the method of Wall et al.[12] as modified by DeBodo et al.[13] Clearance of leucine from the plasma compartment (ml/kg/min) was determined by dividing Rd by plasma leucine concentrations. All values in the text and figures are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using Student's paired and unpaired *t*-tests.

RESULTS

Insulin and glucagon (Figure 1). During the basal period, plasma insulin (12 ± 3 versus 10 ± 3 μU/ml) and glucagon

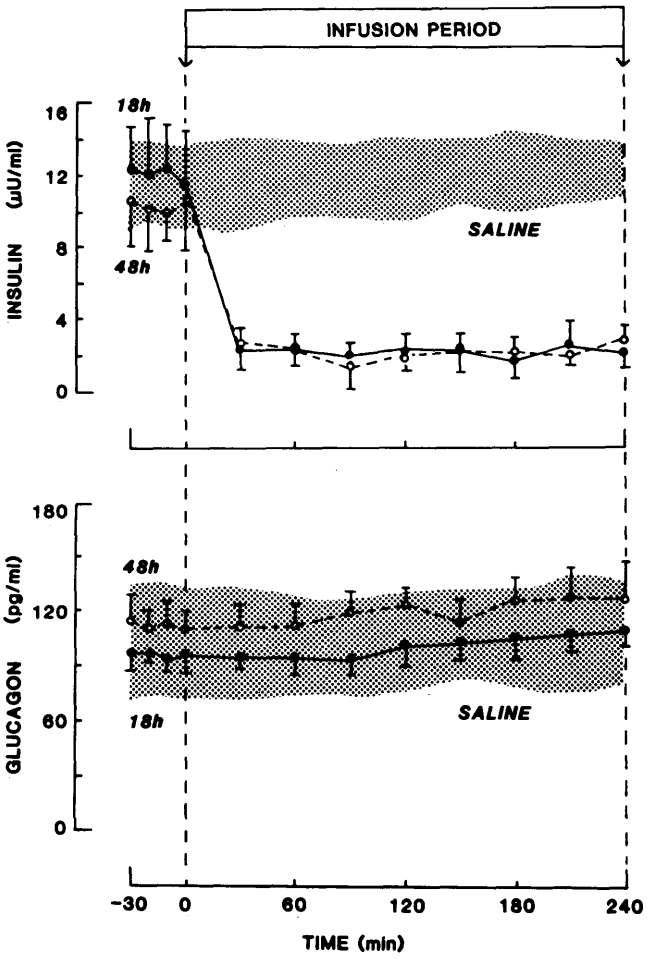

FIGURE 1. Plasma insulin (top panel) and glucagon (lower panel) levels in conscious dogs fasted for 18 h and 48 h. Lines (– insulin) denote levels during the peripheral infusion of somatostatin (0.8 μg/kg/min) and intraportal replacement of basal glucagon. Shaded areas represent values during saline infusion. Values are expressed as mean ± SEM.

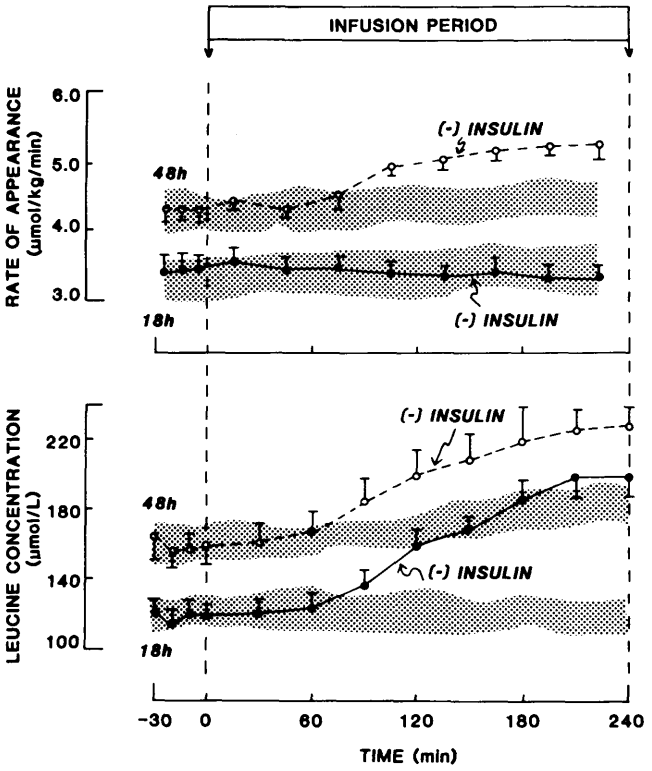

FIGURE 2. Leucine levels (lower panel) and rates of appearance into the plasma compartment (upper panel) in conscious 18-h- and 48-h-fasted dogs. Lines (−insulin) denote values obtained during the infusion of somatostatin and intraportal replacement of basal glucagon. Shaded areas represent values obtained with saline infusion. Values are expressed as mean ± SEM.

(89 ± 10 versus 106 ± 12 pg/ml) levels were similar in 18-h and 48-h-fasted dogs, respectively. Somatostatin infusion caused a marked drop in plasma insulin levels in both groups. The measured levels were <3.0 μU/ml for both groups, and these levels are below the limits of sensitivity of our assay. Glucagon was successfully replaced to basal levels as shown (102 ± 12 pg/ml in 18-h-fasted dogs and 114 ± 18 pg/ml in 48-h-fasted dogs). Saline infusion had no effect on any of the parameters studied.

Leucine kinetics. Leucine concentration increased as fasting was prolonged (Figure 2). Leucine levels were 118 ± 9 and 155 ± 12 μmol/L, respectively, after 18 and 48 h of fasting ($P < 0.005$). Leucine's rate of appearance also rose significantly from 3.4 ± 0.3 to 4.3 ± 0.4 μmol/kg/min ($P < 0.005$). Its clearance rate remained unchanged (28 ± 3 versus 29 ± 3 ml/kg/min).

Acute insulin deficiency resulted in increased leucine concentration in both groups of dogs. The levels reached were 181 ± 10 μmol/L in the overnight-fasted group and 225 ± 20 μmol/L in the 48-h-fasted group, the percent increase above basal being 53% and 45%, respectively ($P < 0.005$). Insulin deficiency had, however, disparate effects on leucine kinetics in the two groups. Leucine's rate of appearance did not change in dogs fasted overnight, while it increased significantly in those dogs fasted 48 h (from 4.3 ± 0.4 to 5.1 ± 0.3 μmol/kg/min, $P < 0.005$). The metabolic clearance rate fell in both groups, shown in Figure 3, although the magnitude of this drop was twice as great in the 18-h-fasted dogs. (In group 1, leucine clearance fell from 28 ± 2 to 16 ± 3 ml/kg/min, $P < 0.005$, while in group 2, it fell from 30 ± 4 to 24 ± 5 ml/kg/min, $P < 0.005$.)

DISCUSSION

In the present study, we examined the effect of insulin on leucine turnover during fasting in the intact, conscious dog. The tracer methodology used requires injection into and sampling from the plasma compartment. This technique can provide useful qualitative estimates of intracellular rates of formation or utilization of particular substrates. It can indicate the magnitude and direction of changes induced by metabolic or hormonal alterations. The measurements thus obtained, however, do not constitute an exact quantitation of intracellular rates of protein turnover.[14,15] For example, the rate of appearance of leucine (Ra) actually measures the rate at which unlabeled leucine enters the plasma compartment. Since leucine is an essential amino acid, its entry only occurs by exogenous supply or by proteolysis. In fasted dogs, leucine's rate of appearance into plasma can be correctly regarded as an estimate of the rate of protein breakdown. This measurement, however, underestimates the intracellular rate of appearance;[14,15] thus, the observed Ra is a minimal estimate of the rate of proteolysis. The corresponding changes in protein synthesis cannot be quantitated without an independent measure of leucine oxidation. Our data concerning the role of basal insulin on protein ki-

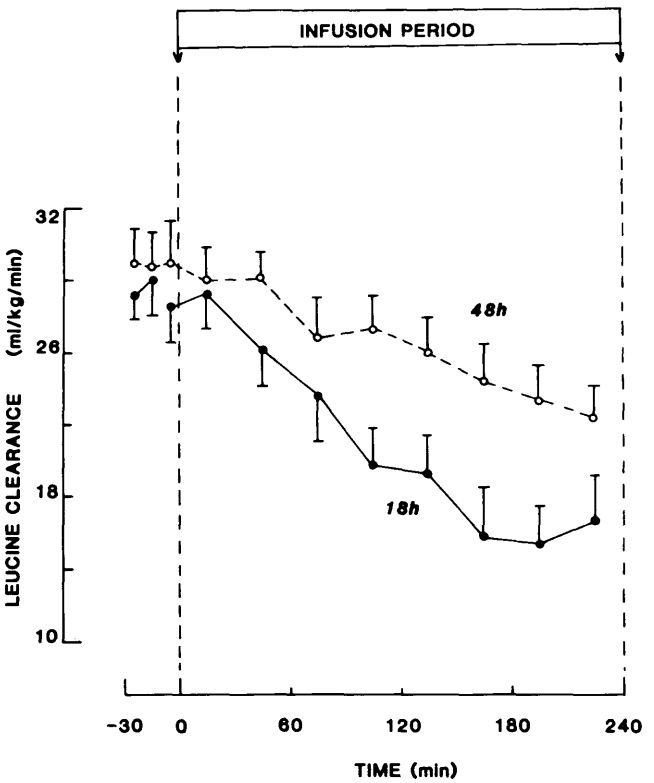

FIGURE 3. Calculated rates of leucine clearance from the plasma compartment in 18-h- and 48-h-fasted conscious dogs during a basal period (−30 to 0 min) and after the peripheral infusion of somatostatin and intraportal replacement of basal glucagon. Control values are not shown, but did not differ from the basal rates of both fasted groups. Values are expressed as mean ± SEM.

netics during progressive fasting will be discussed within these limitations.

Progressive fasting during the basal period (before hormone manipulation) increased plasma leucine concentration by increasing its rate of appearance. Insulin concentrations, as expected,[16] were similar in both groups of fasted dogs, indicating that factors other than insulin are important in regulating proteolysis during fasting. These may include substrates such as free fatty acids,[17] ketone bodies,[18] or branched chain amino[19] and keto acids,[20] or hormones such as catecholamines, glucocorticoids,[21,22] glucagon,[23] growth hormones, and others. Insulin might, however, interact with other regulatory factors to modulate their action.

To assess the role of ambient insulin concentration on leucine kinetics, this hormone was selectively removed by the simultaneous infusion of somatostatin and glucagon. Selective insulin removal was found to have disparate effects on protein degradation in the 18-h versus 48-h-fasted dogs. In both groups, leucine concentration rose and its clearance fell. However, while insulin withdrawal produced no change in the rate of leucine appearance in the postabsorptive period, it increased leucine Ra by 20% after a 48-h fast. These observations indicate that, after a 2-day fast, basal insulin serves to restrain proteolysis, whereas after an overnight fast it does not limit proteolysis but most likely exerts its effect on either protein synthesis and/or oxidation. In support of this hypothesis is the recent observation that acute selective insulin deficiency had dramatic effects on amino acid metabolism but did not increase the release of amino acids across the human forearm (ref. 24 and N. Abumrad, unpublished observations) or the hindlimb of a baboon,[25] when both were investigated after an overnight fast.

The mechanism for such a shift in insulin's action could involve a multitude of factors. It is possible that the effects of basal insulin on protein metabolism persist longer after an overnight fast that after a 2-day fast, which may relate to a more rapid degradation of the insulin-receptor complex. Alternatively, the organs that undergo most of the protein loss may change with fasting to those that are more sensitive to insulin action. There is no information in the literature, however, to support these hypotheses. It is also possible that a change in the sensitivity of tissue proteolytic enzymes to insulin could occur as fasting was prolonged. This is supported by recent findings that insulin inhibits proteolysis in hepatocytes obtained from overnight-fasted but not from 2-day-fasted rats.[26] The latter probably involves a greater participation of the lysosomal system, which is subject to regulation by insulin. Finally, since somatostatin is known to inhibit growth hormone release,[27] it is possible that these changes are totally unrelated to insulin and are modulated by changes in growth hormone levels. It is thus evident that the changes in protein turnover associated with fasting are not solely related to changes in insulin levels. This complexity in insulin interaction is also evident in situations of fasting and feeding, as has been emphasized recently by Garlick et al.[28] These authors showed that infusion of insulin to overnight-fasted rats to levels comparable to those seen with feeding failed to raise the rates of protein synthesis in gastrocnemius muscles to postprandial rates.

In summary, this study suggests that the negative nitrogen balance observed early in fasting is not solely due to a fall in circulating insulin. In the 48-h-fasted dogs, basal insulin helps restrain the rate of proteolysis, while after an overnight fast its role is to maintain protein synthesis, although an effect on protein breakdown or oxidation cannot be ruled out.

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