Factors Affecting Secretory Protein Production in Primary Cultures of Rat Hepatocytes (43570)

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Abstract. **Previous studies suggest that protein synthesis in the liver may be influenced by alterations in hepatic proteolysis and gluconeogenesis. Since proteolysis and gluconeogenesis are accelerated in acute stress states (especially when associated with nutrient deprivation), these alterations may substantially affect hepatic protein synthesis, the integrity of which is important for host survival. In the present study, we have investigated albumin secretion and glucose production in primary cultures of rat hepatocytes in response to nutrient-limiting conditions, including amino acid depletion, proteolysis inhibition, and augmented gluconeogenesis. In nonlimiting nutrient culture medium containing 10 times the normal plasma amino acid concentrations, hepatocytes produced 8.05 ± 1.62 μg/plate-hr of albumin. Short-term (5 hr) inhibition of cellular protein degradation with the lysosomal protease inhibitor leupeptin did not influence albumin production, but caused a profound reduction (17–41%) when amino acid supply was reduced to the physiologic range (1.5–0.5 times, respectively). This indicates the need for active proteolysis for the maintenance of secretory protein production during nutrient limitation. Similarly, leupeptin inhibited glucose production by 22–30% at physiologic (1.5 times and 0.5 times, respectively) amino acid concentrations. Additionally, hepatocyte glucose production could be augmented 168% by epinephrine (2 μM) in 10 times medium, but this response was markedly depressed by leupeptin. Similar catecholamine-mediated effects, but of a smaller magnitude, were noted at lower medium amino acid concentrations. These findings indicate that hepatocyte albumin and glucose production are associated with the common factor of active cellular proteolysis, probably through the regulation of amino acid supply. However, protein synthesis exhibits a higher priority, since stimulated hepatocyte glucose production did not substantially alter albumin secretion.** [P.S.E.B.M. 1993, Vol 203]

Active hepatic protein synthesis, which is pivotal for survival after traumatic stress and nutrient deprivation, may be jeopardized when increased demands are place upon the liver by other metabolic processes. An understanding of the regulatory mechanisms controlling hepatic protein production is important if therapeutic support of the liver is to be successful under conditions of increased metabolic stress (1). The list of regulatory factors that are believed to modulate hepatic protein turnover includes availability of nutrients such as amino acids (2, 3), insulin (4), stress hormones (catecholamines, glucagon, glucocorticoids) (5, 6), growth factors (7), redox state (6), and cytokines (8). In addition, the influence of any specific factor is usually dependent upon the physiologic state of the host. For example, the fed state is associated with an increased amino acid supply and insulin concentration, both of which are stimulatory for hepatic protein synthesis and inhibitory for general liver protein degradation (2). In contrast, stress states are usually accompanied by elevations of the counterregulatory hormones and a limitation of nutrient availability with an associated stimulation of the hepatocellular proteolytic response (5, 6).

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Previous studies have noted that alterations in gluconeogenesis and cellular proteolysis may be two processes that significantly influence hepatic protein turnover (9, 10). Under appropriate conditions, the glucogenic hormone glucagon is known to stimulate hepatic protein degradation and mildly inhibit protein synthesis (11). The proteolytic effect is believed to be mediated in part through a reduction of intracellular concentrations of glucogenic amino acids, possibly due to their increased consumption for the purpose of glucose synthesis (12, 13). Consequently an inhibitory effect upon protein synthesis may arise if the extracellular amino acid supply or the associated proteolysis does not sufficiently restore cellular amino acid concentrations toward normal. Therefore, under conditions of augmented glucogenesis, a phenomenon that is commonly observed in stress states, including trauma and systemic inflammation, it appears reasonable to expect that the net effects of proteolysis and glucose production will exert a regulatory influence upon hepatic protein synthesis.

In this report, we have used primary cultures of rat hepatocytes to study albumin secretion and glucose production in response to a hormonal milieu that mimics posttraumatic states. Also, in order to investigate the influence of protein degradation upon albumin secretion, the effects of lysosomal proteolysis inhibition were studied. Albumin was selected as an index protein since it is the major secretory protein product of the liver accounting for approximately 50% of exported proteins, and its rate of secretion parallels the rate of total cellular protein production (2). Additionally, the relative role of metabolic regulators may vary in different regions of the hepatic acinar unit, since it has been established that a metabolic heterogeneity of hepatocytes exists in the periportal and perivenous zones (14). Gluconeogenesis occurs predominantly in the periportal regions and glycolysis occurs in the perivenous zones of the hepatic acinus (15). This location-dependent metabolism may also apply to secretory protein synthesis (16). However, our understanding of factors that affect the distribution of plasma protein synthesis in sublobular hepatic regions is very limited. The present study was also directed at investigating the albumin secretory response of hepatocytes to graded changes in amino acid concentrations in order to simulate the declining blood contents of amino acids along the course of the liver acinus, especially during periods of limited dietary intake.

Materials and Methods

Hepatocytes were obtained from male Sprague-Dawley rats weighing 160–180 g using a modification of the perfusion procedure described by Berry and Friend (17). Briefly, after the administration of anesthesia with sodium pentobarbital (50 mg/kg), the abdomen was opened and the liver was perfused *in situ* using a calcium-free Krebs-Henseleit bicarbonate buffer containing 17.5 mmol/liter of glucose and washed bovine red cells at a hematocrit of approximately 25% Collagenase (Boehringer Mannheim; 20–30 mg/100 ml, collagenase A) was then added to the perfusion circuit and the perfusion was continued for 30 min, at which time the liver was removed and the hepatocytes were dispersed. Isolation of hepatocytes was accomplished using multiple washes with serum-free culture medium followed by Percoll (Sigma) gradient centrifugation. Hepatocytes were then plated at a cell density of 2×10^6 cells/dish on 60-mm collagen-coated culture dishes. The culture medium was a 1:1 mixture of Ham's F12:Dulbecco's modified Eagle's medium supplemented with dexamethasone (0.1 μM), insulin (10 nM), glucagon (10 nM), and amino acids to a concentration approximating 10 times that of normal rat arterial plasma (4). The medium was changed at 4 hr and every morning thereafter. Prior to study, the cells were depleted of glycogen (in order to avoid studying glycogenolysis) by withdrawing insulin and reducing the medium glucose content to 5 mM on the morning of the fourth day of culture (18). Experiments were conducted on the morning of the fifth day of culture, when the rate of albumin secretion was in a steady state under control conditions. Upon beginning an experiment, plates were washed and fresh glucose-free medium was added. Substrate for these studies was either 5 mM lactate or alanine. On the experimental day, the medium amino acid concentrations were made 10 times, 1.5 times, or 0.5 times the normal rat arterial plasma amino acid concentration in order to simulate nonlimiting, periportal, or physiologic perivenous blood amino acid concentrations, respectively. Cultures were maintained for 5 hr. Leupeptin (0.25 mM), a specific inhibitor of lysosomal proteolysis (19), and/or epinephrine (2 μM), a potent stimulator of gluconeogenesis, were added to some plates. Leupeptin was utilized because of previous reports indicating that it exhibits no apparent cellular toxicity based upon morphologic and protein synthetic studies (19). Glucogenesis was augmented using epinephrine added to the glucose-free medium containing glucagon and dexamethasone, since medical and surgical stress is associated with the combined elevation of the counterregulatory hormones glucagon, a glucocorticoid, *and* epinephrine. Additionally, it has been observed experimentally that each individual hormone exerts only a transient glucogenic effect, whereas their combined administration effects a synergistic and sustained glucose overproduction (20). Epinephrine was increased to 4 μM in the 0.5 times amino acid medium experiments because of a poor glucogenic response at the lower dose. During the study, epinephrine was added repeatedly every hour, assuming that the previous dose had been metabolized entirely.

Analytical Procedures

Albumin secretion into the culture medium was measured every 24 hr and over a 5-hr period on the final study day using rocket immunoelectrophoresis (21). The degree of hepatocellular proteolysis inhibition by leupeption (0.25 m*M*) was established by prelabeling cells for 24 hr with [^{3}H]valine (30 μCi/ml) on culture Day 4. Protein degradation was measured on Day 5 as the rate of [^{3}H]valine released into the medium containing 5 m*M* lactate and 10 times, 1.5 times, or 0.5 times amino acids, except valine, which was 15 m*M* to prevent tracer reincorporation. Cellular glycogen depletion was verified by extracting cells with 1 ml of 0.6 *N* perchloric acid/plate for 30 min. Neutralized aliquots (100 μl) of these extracts were incubated with an equal volume of amyloglucosidase (Sigma; 1 mg enzyme/ml, 2 *M* sodium acetate buffer, pH 5.0) and the released glucose was assayed using a glucose-oxidase-based coupled chromogenic assay (Trinder assay, Sigma). The amount of glycogen was determined using rabbit liver glycogen standards (Sigma) (18). Urea production was measured in some culture aliquots using a urease-based assay. Released ammonia was coupled with a chromogenic substrate (phenol) based upon the Berthelot reaction (Sigma). Average hepatocyte protein contents were determined by digesting cells with 0.2 *N* NaOH and assaying aliquots using the procedure of Lowry *et al.* (22).

Each nutrient condition studied (amino acid concentration levels times 10, 1.5, and 0.5) utilized cells from a different liver preparation and all studies represent the average of three cultures. Results are presented as the mean value ± SE. Multiple group comparisons were made using one-way analysis of variance and the method of least significant difference. Significance was accepted at $P < 0.05$.

Results

The pattern of albumin secretion for primary cultures of rat hepatocytes maintained under the conditions described herein is shown in Figure 1. A steady state rate of secretion was reached on approximately Day 5 of culture (15.7 ± 0.3 μg/plate-hr) and all subsequent studies were performed on this day. The cultures represented by the data shown in Figure 1 were supplemented with insulin each morning. Since it was the aim of this project to study factors other than insulin (i.e., the fasted state), this hormone was omitted in subsequent experiments beginning on Day 4. In conjunction with a reduction in medium glucose concentration to 5 m*M*, insulin withdrawal resulted in depletion of cellular glycogen, a commonly observed state after acute stress events. The mean hepatocyte glycogen content in undepleted (+insulin) and depleted (−insulin) cells was 371 ± 25 μg/plate and 75 ± 9 μg/plate, respectively, which represents an 80% reduction in cellular glycogen. In order to allow these data to be normalized to cellular protein content, the average cellular protein content per plate was determined to be 2.1 ± 0.1 mg.

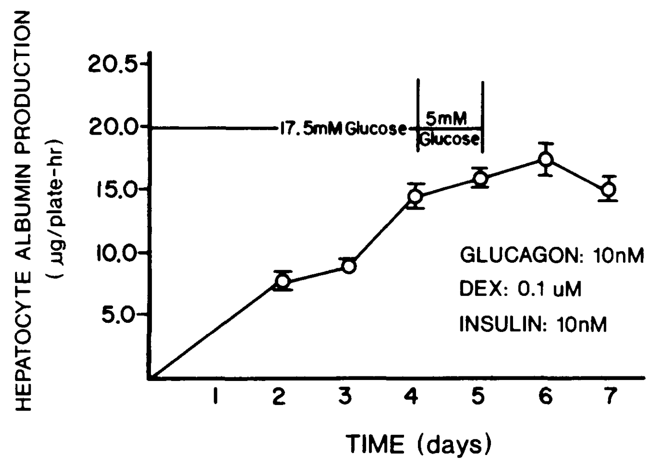

Figure 1. The albumin secretory response of rat hepatocytes to serum-free medium containing glucagon, insulin, and dexamethasone is indicated. Amino acids are present at approximately 10 times the normal rat plasma amino acid concentration. In subsequent experiments, insulin was withdrawn and glucose was reduced to 5 m*M* on the fourth culture day in order to deplete cells of glycogen. (Amino acid composition of 10 times culture media [m*M*]: Asp, 0.36; Thr, 0.99; Ser, 0.99; Asn, 0.83; Gln, 3.44; Pro, 2.01; Glu, 0.80; Gly, 3.97; Ala, 1.78; Cys, 0.41; Met, 0.41; Ile, 0.81; Leu, 1.50; Val, 0.95; Tyr, 0.82; Phe, 0.76; Lys, 2.45; His, 0.51; Trp, 0.69; Arg, 1.29).

The response of the albumin secretion rate to various substrates and amino acid concentrations on culture Day 5 is shown in Table I. Albumin secretion was independent of the culture substrate (lactate or alanine) and the rate of albumin secretion at 10 times amino acids was markedly reduced compared with the same time period in cultures containing insulin (Fig. 1). This stresses the importance of insulin as an anabolic agent in maintaining hepatic protein production. Table I also shows that hepatocyte albumin secretion is only mildly sensitive to a 20-fold range in medium amino acid concentrations. Although amino acid availability is considered an important regulator of hepatic albumin production (4), in the absence of insulin, its effect appears to be minor.

The interrelationships of hepatocyte gluconeogenesis and proteolysis upon albumin production were examined utilizing epinephrine and leupeptin. With lactate as substrate in medium already containing the glucogenic hormones glucagon and dexamethasone, the addition of epinephrine did not alter albumin secretion significantly under any nutrient condition (Fig. 2). However, leupeptin inhibited both cellular proteolysis and albumin synthesis under similar culture conditions. Figure 3 indicates that leupeptin significantly inhibited proteolysis (measured as the rate of release of [^{3}H]valine from prelabeled cells) by 34% and 38% in the 1.5 times and 0.5 times cultures, respectively, as compared with

Table I. Hepatocyte Albumin Production

Culture amino acid concentration	Albumin secretion rate[a] (μg/plate-hr)		
	10 × Normal amino acids	1.5 × Normal amino acids	0.5 × Normal amino acids
Substrate concentration			
Lactate (5 m*M*)	8.05 ± 1.62	6.74 ± 0.26	7.03 ± 0.37
Alanine (5 m*M*)	7.34 ± 1.49	6.40 ± 0.30	ND

[a] Hepatocyte albumin production was determined at different media amino acid concentrations ranging from 10 times to 0.5 times the normal rat plasma amino acid concentration. Lactate (5 m*M*) or alanine (5 m*M*) was used as substrate during these culture experiments. No statistical difference is noted among these groups. Each value is the mean of three cultures. ND, not done.

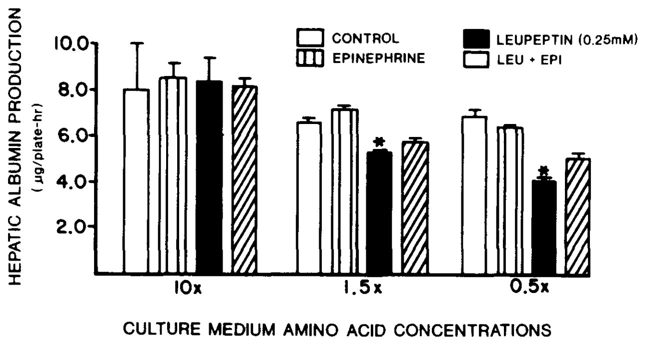

Figure 2. Hepatocyte albumin production in glycogen-depleted cells in the absence of insulin is indicated for various conditions. In addition to the additives indicated, all cultures contained glucagon (10 n*M*) and dexamethasone (0.1 μ*M*), and lactate (5 m*M*) was used as substrate for these cultures. Each value is the mean of three cultures (leupeptin [Leu], 0.25 m*M*; epinephrine [Epi], 2 μ*M*—except at 0.5 times amino acid concentration where Epi = 4 μ*M*); *$P < 0.05$ relative to control (no additives).

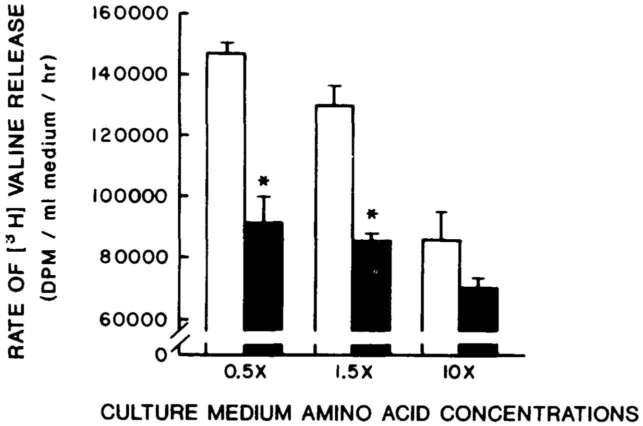

Figure 3. The response of hepatocyte protein degradation to leupeptin was measured as the rate of [^{3}H]valine release from prelabeled cells into the culture medium. Studies were performed on the fifth culture day in glucose-free medium containing 5 m*M* lactate and 0.5 times to 10 times the amino acid content of normal rat plasma. Each value is the mean of five cultures. Open bars represent cultures maintained in the absence of 0.25 m*M* leupeptin and solid bars represent cultures maintained in the presence of 0.25 m*M* leupeptin. *$P < 0.0005$ vs control (no leupeptin).

cells not treated with leupeptin. It also reduced proteolysis by 18% in the 10 times cultures, but the difference was not significant. Correspondingly, leupeptin significantly depressed albumin secretion only at both lower concentrations (1.5 times and 0.5 times) of medium amino acids by 17% and 41%, respectively, relative to their control values (Fig. 2). This indicates that intracellular proteolysis is an important regulatory mechanism for maintaining protein synthesis under deprivational states, since the inhibitory effects of leupeptin were not evident at 10 times amino acids. Enhanced proteolysis probably provides a source of intracellular amino acids which serve either a regulatory role or simply as increased substrate for secretory protein synthesis. The addition of epinephrine to cultures containing leupeptin returned low protein secretion rates toward control values (Fig. 2). A qualitatively similar response was noted when norepinephrine was substituted for epinephrine or alanine was substituted as the substrate (results not shown).

The gluconeogenic response of hepatocytes in culture largely resembled the protein secretory response pattern (Fig. 4). Using lactate as substrate (control), glucose production was similar at all medium amino acid concentrations, indicating that basal gluconeogenesis was independent of medium amino acid concentra-

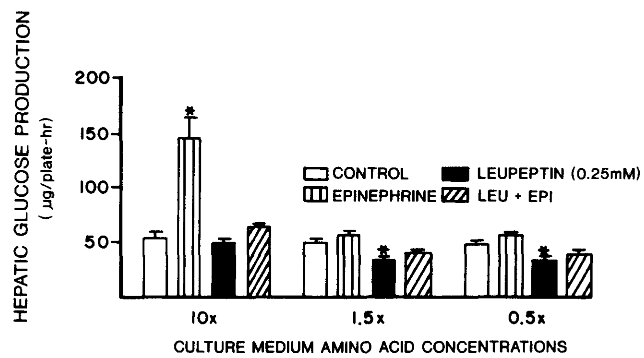

Figure 4. Hepatocyte glucose production is shown under the same conditions noted in Figure 2. Each value is the mean of three cultures. *$P < 0.05$ vs control (no additives).

tion. Epinephrine stimulated glucose output by 168% at nonlimiting (10 times) amino acid concentrations, and by 15% at both lower amino acid concentrations. Thus, a minimal concentration of amino acids (greater than 1.5 times) will dramatically stimulate active gluconeogenesis despite the presence of relatively plentiful substrate (lactate or alanine). Leupeptin suppressed the marked epinephrine effect at 10 times amino acids as well as the unstimulated glucose production (by 22–30%) at physiologic levels of medium amino acids (1.5 times and 0.5 times, respectively). These observations indicate a key role for endogenous amino acids in the control of gluconeogenesis.

At 10 times amino acids, hepatocyte urea production (Table II) remained essentially unchanged under all conditions. At the lowest medium amino acid level studied (0.5 times), leupeptin inhibited urea synthesis, but this could be restored to near control levels by the equimolar replacement of lactate with a nitrogen-containing substrate (alanine). Collectively, these data suggest that leupeptin affects both glucose and urea synthesis through its influence upon amino acid supply rather than a generalized toxic cellular effect.

Discussion

Insulin is undoubtedly the strongest short-acting stimulus of non-acute phase protein synthesis. This has been addressed in previous reports (4) and is also evident from the decline of albumin synthesis when insulin is withdrawn from hepatocyte cultures in the current study (compare Fig. 1 and Table I). The cellular proteolytic and gluconeogenic responses that generally accompany acute stress states are two additional factors that have been identified as regulators of hepatic protein synthesis. These processes are largely controlled by catabolic endocrine conditions and nutrient availability. For example, the glucogenic hormone glucagon is known to stimulate hepatic protein degradation and inhibit protein synthesis (11). Poso *et al.* (12) and Schworer and Mortimore (5) have reported that this hormonal stimulation of intracellular protein degradation in the liver is a manifestation of lysosomal mediated autophagy which results from a decrease in certain intracellular amino acids, particularly glucogenic amino acids. This intracellular "nutrient deficiency state" presumably arises from the increased consumption of these amino acids due to the glucogenic stimulus. Flaim *et al.* (2) have reported that rat livers perfused with amino-acid-deficient medium exhibit a depression of protein synthesis that may be restored to normal by enhanced proteolysis. When glutamine, a potent inhibitor of proteolysis, is introduced into the perfusate, this recovery of protein synthesis is prevented. These authors concluded that accelerated proteolysis or an increased extracellular amino acid concentration can serve as a substrate source to specific cellular compartments where one or more amino acids function in a regulatory capacity with respect to protein synthesis.

In the present study, the apparent independence of albumin production with amino acid concentration (Table I) probably derives from enhanced cellular protein degradation (Fig. 2), which functions to maintain the availability of amino acids for processes such as gluconeogenesis and protein synthesis. When amino acids are nonlimiting (10 times amino acids), leupeptin does not significantly influence secretory protein production or cellular protein degradation. However, at 1.5 times and 0.5 times amino acids, concentrations to which periportal and perivenous hepatocytes are normally exposed, addition of a proteolytic inhibitor reduces albumin secretion by 17% and 41% percent, respectively. Thus, the integrity of the proteolytic response mechanism is important in sustaining protein production at physiologic amino acid concentrations.

When hepatocyte glucose production was augmented using epinephrine (in the presence of glucagon and dexamethasone), protein synthesis was mildly stimulated and the effects of leupeptin were antagonized (Fig. 2). This response was also observed with norepinephrine and at all amino acid concentrations, although the overall magnitude of this increase was small. This is paradoxical, since Chin *et al.* (23) have reported previously that α-adrenergic agonists inhibit liver pro-

Table II. Hepatocyte Urea Production

Condition	Urea production[a] (μmol/plate-hr)			
	Control	Epinephrine	Leupeptin	Leupeptin + epinephrine
Culture amino acids 10 × normal				
Lactate (5 m*M*)	0.476 ± 0.036	0.540 ± 0.057	0.559 ± 0.026	0.582 ± 0.012[b]
Culture amino acids 0.5 × normal				
Lactate (5 m*M*)	0.320 ± 0.016	0.366 ± 0.010	0.252 ± 0.003[b]	0.274 ± 0.014[b]
Alanine (5 m*M*)	0.559 ± 0.026	0.582 ± 0.012	0.458 ± 0.025[b]	0.539 ± 0.007

[a] Hepatocyte urea production was determined at different media amino acid concentrations (control) and after the addition of epinephrine (2 μM) and/or a lysosomal proteolytic inhibitor, leupeptin (0.25 m*M*).
[b] Statistically different from control ($P < 0.05$).

tein synthesis. Additionally, based upon reports by Ayuso *et al.* (9) and Requero *et al.* (24), accelerated gluconeogenesis is expected to inhibit protein production. In view of the previously discussed interrelationship between proteolysis, protein synthesis, and glucogenic amino acids, it might be expected that metabolic competition for the intracellular amino acid pool would affect protein synthesis negatively. However, an inhibitory influence was not observed in this culture system. Therefore, a modulatory role for gluconeogenesis upon protein synthesis cannot be demonstrated. It should be noted that previous reports demonstrating an interrelationship between these processes have primarily utilized *in vivo* liver studies in which small amounts of insulin may have inhibited the compensatory proteolytic response. Also increased catecholamine concentrations may stimulate protein synthesis directly by increasing cellular amino acid uptake (25), thus buffering the consumption of key amino acids.

Hepatocyte glucose production depends upon amino acid availability to a greater extent than protein synthesis. Although basal glucose synthesis rates are insensitive to medium amino acid concentration, catecholamine-stimulated increases are markedly dependent upon the extracellular amino acid levels and the integrity of the proteolytic response.

Under nonlimiting (10 times) amino acid concentrations, leupeptin completely inhibited the prominent catecholamine-induced glucose production (Fig. 4). Similar responses were noted at lower amino acid concentrations, but they were of a smaller magnitude. Studies using 5 m*M* lactate or alanine (not shown) were qualitatively similar, which indicates that insufficient glucogenic substrate concentration or type can not account for this phenomenon. It appears more likely that proteolysis exerts a regulatory role on hepatic gluconeogenesis similar to that experienced by protein synthesis, since these processes are linked by common factors. Thus, the markedly increased glucose output noted at 10 times amino acids and epinephrine (Fig. 4) probably requires a critical intracellular concentration of certain amino acids that are provided by proteolysis. At lower medium amino acid concentrations, the collective contribution of proteolysis and cellular uptake of amino acids is more limited, resulting in an attenuated glucose response. In fact, the magnitude of this smaller glucogenic response is comparable to that observed when hepatocytes are suspended in amino-acid-free physiologic solutions (26, 27).

The observed dependence of glucose production upon amino acid concentration may provide insight into the heterogeneous metabolic nature of the hepatic acinus. Previous studies have indicated that glucose synthesis predominates in the periportal acinar zones of the liver (28). Although the mechanism of this metabolic pattern has not been established, based upon the studies reported here, increased amino acid availability in the upstream regions of the liver lobule may play a role in this phenomenon.

The process of hepatic proteolysis plays a significant role in regulating hepatic secretory protein synthesis and gluconeogenesis. Since these latter metabolic processes appear important for host survival under stress, the stimulatory influence which protein degradation offers must be considered to have at least a short-term benefit to the acutely ill host and warrants continued study.

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