Endocrine and amino acid regulation of liver macroautophagy and proteolytic function

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Bergamini, E., A. Del Roso, Z. Gori, P. Masiello, M. Masini, and M. Pollera. Endocrine and amino acid regulation of liver macroautophagy and proteolytic function. *Am. J. Physiol.* 266 (*Gastrointest. Liver Physiol.* 29): G118–G122, 1994.—Regulation of liver macroautophagy and protein degradation by hormones and direct regulatory amino acids were studied in male 2-mo-old Sprague-Dawley albino rats with the use of the antilipolytic agent 3,5′-dimethylpyrazole (DMP; 12 mg/kg body wt ip) as a stimulatory agent. Injection of DMP decreased glutamine plasma levels and glutamine release from the perfused liver. Autophagic vacuoles were observed in the pericanalicular area of liver cells after 30 min. Levels and release of other regulatory amino acids did not exhibit any significant decrease but subsequently increased. Intraperitoneal administration of glutamine inhibited the proteolytic response. In conclusion, these studies demonstrate that in vivo induction and control of liver macroautophagy and protein degradation by the physiological mechanism (i.e., by shortage of nutrients) involve unbalanced and asynchronous changes in the levels of selected direct regulatory amino acids (i.e., a decrease in glutamine and a subsequent increase in leucine and tyrosine levels).

rat liver; lysosomes; glucagon; insulin; glutamine; leucine

IT IS WELL KNOWN that intracellular protein is a major source of amino acids for metabolic needs when intestinal absorption slows or ceases (22, 23). Liver, kidney, and intestines are the main donors of amino acids in the first 48 h of starvation in laboratory rodents (1). The liver is particularly responsive to nutrition: in the rat, the weight of this organ varies considerably, decreasing during fasting and increasing after feeding (1, 20). The liver may lose as much as 20% of its protein and RNA content (1, 19, 20) during 24 h of starvation without any significant decrease in DNA content (20) or in the number of nuclei (15); it may promptly reintegrate these deficits during refeeding, by absorbing and storing as protein the entire gut output of amino acids in the absorptive period (9).

The lysosomal vacuolar system has been shown to play a major role in these losses of liver cell cytoplasm (3, 9, 22, 23, 27, 31); furthermore, the process responsible for this phenomenon, macroautophagy, is subject to acute regulation by amino acids, insulin, and glucagon (3, 10, 14, 22, 23, 25, 30). Regulation has been studied extensively in vitro, with the perfused liver and isolated liver cells. On the whole, results have led to the conclusion that amino acids are the primary regulators and that this primary control of macroautophagy is accomplished by a small group of direct regulators [Leu, Tyr/Phe, Gln, Pro, Met, His, and Trp (29)] and a specific coregulatory action of alanine (26). It appears that hormones may modulate amino acid regulation. Glucagon can induce macroautophagy and protein degradation in the perfused liver at normal amino acid concentrations, but its effects are abolished at amino acid concentrations greater than twice normal plasma levels; in contrast, insulin, calf serum, and other growth-promoting factors are uniformly inhibitory (4, 24).

The actual regulation of proteolysis in vivo is still obscure (22, 23). It has been suggested that combined effects of both amino acids and hormonal agents should be involved, but little experimental information has been obtained so far about the respective roles of amino acid and hormone regulation or about the nature of their interactions (22, 23). The present study was designed to explore the role of the regulatory response to amino acids and hormones by the use of a new convenient method of induction of autophagy in vivo by a physiological mechanism (5). With this method, an antilipolytic drug is injected into overnight-fasted rats to evoke an increase in glucagon and a decrease in insulin plasma levels (5, 7, 17).

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (Nossan, Milano) were used as liver donors. Unless otherwise stated, they weighed between 180 and 200 g at the time of the experiment and were maintained under natural lighting with water continuously available. Animals were fed standard laboratory chow ad libitum and all were starved for 18 h before perfusion. On the morning of the experiment, rats to be perfused were divided into groups at random and were given either the antilipolytic agent 3,5′-dimethylpyrazole (DMP) freshly dissolved in saline (12 mg/rat, in 0.2 ml saline) or saline only intraperitoneally.

Liver perfusion. Livers were perfused in situ as described by Mortimore and Mondon (21). Surgery was performed under Nembutal anesthesia (50 mg/kg body wt ip). At given times (i.e., 15, 30, 60 and 150 min after injection of DMP or saline), blood samples were taken from the inferior vena cava and from the vena porta to assay plasma levels of free fatty acids (FFA), glucose, amino acids, and hormones, and perfusion was performed in the single-pass mode with a medium free from amino acids. After a 7-min washout, two adjoining 1.5-min fractions of the outflow of the liver (~12 ml each) were taken for the measurement of amino acid release.

Perfusion medium. The perfusion medium was composed of the following: Krebs-Ringer bicarbonate buffer and bovine serum albumin (fraction V, Sigma Chemical, St Louis, MO), 10 mM glucose, 18 μM cycloheximide, and freshly washed, no longer usable human erythrocytes (27% vol/vol). Before its addition to the medium, a concentrated aqueous solution of albumin was dialyzed overnight at 5°C against 4 liters of glass-distilled water and was then passed through 3-μm and 0.2-μm Millipore filters.

Determination of protein degradation and analytical procedure. Total values of liver protein degradation were determined by analyzing another aliquot of the fraction of perfusate

for the valine content with the use of high-performance liquid chromatography (11).

Electron microscopy observations. Very small fragments of liver tissue were taken ~5 min after the beginning of perfusion and were fixed in glutaraldehyde in 0.1 M phosphate buffer for 2 h. Then samples were washed in phosphate buffer for a few hours, freed from gross connective tissue, reduced to smaller pieces, postfixed in OsO_4 in 0.1 M phosphate buffer, dehydrated, and embedded with Polybed 812 by standard procedures. Thin sections were stained with uranyl acetate and lead citrate and were observed with a Siemens Elmiskop 1 A electron microscope.

RESULTS

Effects of administration of DMP on lysosomal vacuolar system of liver cells. Figure 1 depicts the changes in the lysosomal vacuolar system of liver cells at different times after the intraperitoneal injection of the antilipolytic agent DMP. In the control (injected with saline) fasted rats, lysosomal structures of varying appearances were encountered frequently (Fig. 1*A*) and were found to be a prominent feature in animals killed 15 min after DMP treatment (Fig. 1*B*). However, macroautophagic vacuoles were not generally observed in the liver cells of these two experimental groups. By contrast, 30 min after treatment with DMP, numerous macroautophagic vacuoles were seen in the pericanalicular area of most liver cells, often containing recognizable material (Fig. 1*C*). It may be added that the number of macroautophagic vacuoles, like that of the remaining secondary lysosomes, was found to increase at longer times after treatment (e.g., 60 and 150 min).

Effects of administration of DMP on release of valine and other amino acids from the liver. Table 1 shows the effects of the administration of DMP on the release of valine and direct regulatory amino acids. Valine release from the liver into the perfusate during short-term single-pass liver perfusion is a widely accepted index of the rate of proteolysis in vivo (21). In view of the results of electron microscopic observations, as well as the fact that stimulation of proteolysis should exhibit longer latencies, in this experiment the 15-min time point was omitted and an additional time point was included (60 min). Valine release increased slightly after 30 min and was significantly higher at the 60-min time point. In regard to regulatory amino acids, different kinds of behavior were observed. At 30 min, only glutamine release changed, decreasing significantly; no other regulatory amino acids underwent significant changes, and mean values were either slightly below (5 of 8) or above (3 of 8) control values. At 60 min, a different picture was observed: glutamine values were still significantly lower than controls; leucine exhibited a highly significant increase (+80%); none of the other regulatory amino acids significantly changed, but in most cases (5 of 7 amino acids) mean values were above controls.

Effects of administration of DMP on plasma levels of valine and direct regulatory amino acids. Table 2 shows that administration of DMP has no significant effect on the plasma levels of direct regulatory amino acids either at 15 or 30 min after the injection. At 15 min, most

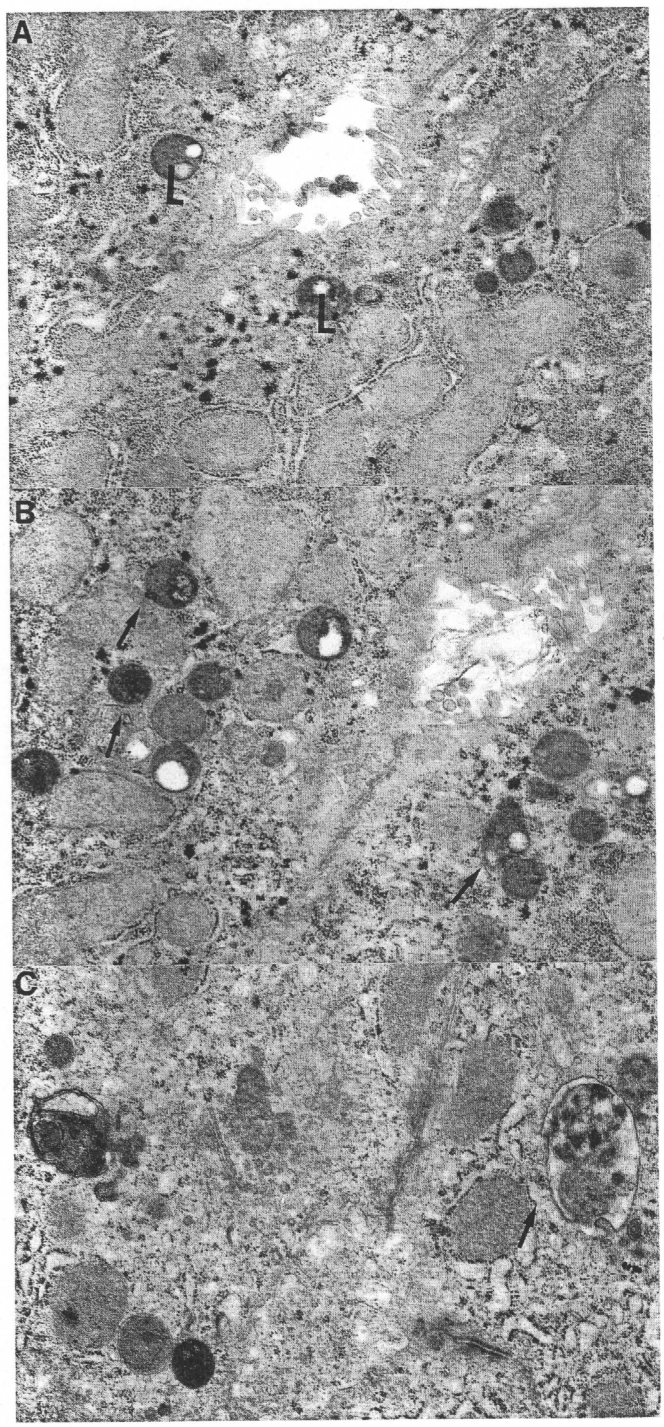

Fig. 1. Effects of administration of 3,5′-dimethylpyrazole (DMP) on liver autophagy in 2-mo-old rats. Representative pictures are given of the pericanalicular area of liver cells from control rats (*A*) and rats treated with DMP for 15 (*B*) and 30 min (*C*). Magnification, ×14,000. *A* (untreated control): few lysosomes (L) and no autophagic vacuoles are seen. *B* (15 min after DMP injection): lysosomal structures are seen with a varying appearance (e.g., elements containing single or multiple membranes; bound vesicles that may contain cytoplasm (arrows). Autophagic vacuoles are encountered very rarely, as in the case of controls. *C* (30 min after DMP injection): 2 autophagic vacuoles are seen containing unidentifiable material and partially degraded organelles (arrow).

Table 1. *Effects of administration of DMP on release of amino acids from the liver into perfusate during single-pass liver perfusion*

Treatment	Controls	30 min	60 min
Valine	133 ± 12.0	157 ± 30.8	212 ± 27.5*
Leucine	100 ± 10.0	122 ± 19.2	181 ± 22.5*
Tyrosine	50 ± 3.3	40 ± 5.0	64 ± 6.7
Phenylalanine	45 ± 5.8	52 ± 7.5	67 ± 8.3
Glutamine	680 ± 50.8	494 ± 40.8*	462 ± 45.8*
Proline	227 ± 39.2	152 ± 29.2	194 ± 45.4
Methionine	20 ± 1.7	16 ± 5.0	21 ± 3.3
Histidine	82 ± 5.8	71 ± 11.7	79 ± 13.3
Tryptophan + ornithine	93 ± 6.7	87 ± 9.2	112 ± 15.8
Alanine	193 ± 10.8	206 ± 23.3	223 ± 20.0

Values (in nmol · min^{-1} · 100 g body wt^{-1}) are means ± SE of at least 5 cases. 3,5′-Dimethylpyrazole (DMP) was administered at 12 mg/kg body wt ip. For control, no significant difference was found between rats given saline 30 or 60 min before death, and results were pooled. * Statistically significant difference ($P < 0.05$; F test and Bonferroni's multiple t test) with respect to *time 0*.

values (7 of 9 amino acids) fell below control values, and at 30 min most values (7 of 9) were higher. At 60 min, levels of all direct regulatory amino acids with the only exception of glutamine were higher than control values, although to different extents (leucine: +49%; tyrosine: +54%; phenylalanine: +13%; proline: +2%; methionine: +28%; histidine: +29%; tryptophan: +17%; alanine: +20%). In general, the effects of DMP on the plasma levels of regulatory amino acids parallel changes in amino acid release from the perfused liver, methionine being a notable exception. In view of the significant changes observed in Table 1, it may be worthwhile to stress once more that glutamine plasma levels were always found to be below control values after DMP injection. Changes in glutamine plasma levels, although not statistically significant, may be essential for the stimulation of liver protein breakdown: Table 3 shows that the intraperitoneal injection of glutamine (250 mg/kg body wt, in 2 equal doses 20 and 40 min after injection of DMP) fully prevented the rise in the rate of

Table 2. *Effects of administration of DMP on levels of regulatory amino acids in plasma of blood samples taken from inferior vena cava of 18-fasted rats*

Treatment	Controls	15 min	30 min	60 min
Valine	147 ± 8.1	123 ± 10.0	168 ± 33.2	204 ± 19.8*
Leucine	100 ± 10.0	101 ± 8.5	105 ± 15.9	170 ± 16.5*
Tyrosine	69 ± 3.5	63 ± 4.0	88 ± 12.7	106 ± 7.2†
Phenylalanine	63 ± 5.5	51 ± 1.8	61 ± 5.8	71 ± 5.5
Glutamine	604 ± 39.2	516 ± 46.7	534 ± 33.9	504 ± 45.7
Proline	167 ± 9.0	159 ± 16.0	177 ± 20.0	172 ± 22.5
Methionine	43 ± 3.3	46 ± 3.7	54 ± 4.1	55 ± 2.0*
Histidine	75 ± 2.2	65 ± 3.3	79 ± 11.9	97 ± 10.0
Tryptophan	164 ± 13.6	140 ± 12.2	178 ± 27.8	192 ± 24.2
Alanine	597 ± 49.4	614 ± 62.8	725 ± 120.0	716 ± 101.3

Values given (in nmol/ml) are means ± SE of 6 cases. For control, no significant difference was found between rats given saline 30 or 60 min before deaths and results were pooled. 3,5′-Dimethylpyrazole (DMP) was administered at 12 mg/kg body wt ip. * and †, statistically significant difference ($P < 0.05$ and $P < 0.01$, respectively; F test and Bonferroni's multiple t test) compared with *time 0*.

Table 3. *Effects of administration of DMP and of glutamine on release of amino acids from the liver into perfusate during single-pass liver perfusion*

Treatment	Controls	Glutamine	DMP	DMP + Glutamine
Valine	153 ± 16.0	145 ± 18.3	217 ± 17.2	162 ± 13.3*
Leucine	109 ± 10.3	102 ± 17.2	163 ± 22.5	133 ± 9.2*
Tyrosine	36 ± 4.0	35 ± 5.8	47 ± 5.8	37 ± 2.7
Phenylalanine	52 ± 5.2	48 ± 7.1	71 ± 6.7	60 ± 4.7*
Glutamine	732 ± 96.8	566 ± 89.0	417 ± 66.7	450 ± 48.0*
Proline	156 ± 21.9	138 ± 14.4	171 ± 18.9	129 ± 9.6
Methionine	40 ± 3.3	31 ± 2.9	29 ± 3.2	32 ± 2.1*
Histidine	75 ± 8.3	68 ± 7.8	77 ± 7.5	76 ± 3.8
Tryptophan + ornithine	45 ± 6.6	45 ± 8.1	72 ± 8.1	45 ± 9.9
Alanine	160 ± 28.8	142 ± 11.3	146 ± 20.0	128 ± 6.6

Values (in nmol · min^{-1} · 100 g body wt^{-1}) are means ± SE of 4 cases. For details of 3,5′-dimethylpyrazole (DMP; 12 mg/kg body wt ip) and glutamine (250 mg/kg body wt ip) administration, see text. *Statistically significant difference (F test). Valine: effects of both DMP and glutamine are significant ($P < 0.05$, in both cases). Leucine: effect of DMP is significant ($P < 0.025$). Phenylalanine: effect of DMP is significant ($P < 0.01$). Glutamine: effect of DMP is significant ($P < 0.01$). Methionine: interaction between DMP and glutamine is significant ($P < 0.025$). A significant difference between controls and DMP- and glutamine-treated rats is observed with the amino acid glutamine.

valine release from the perfused liver into perfusate at 60 min. Changes in glucagon and insulin plasma levels had already taken place and were not affected by the injection of glutamine.

DISCUSSION

Treatment of fasted rats with DMP causes dramatic metabolic and endocrine alterations and affects levels of regulators of liver autophagy and protein breakdown (7, 17). The temporal pattern of the alterations is reproducible (7). After the injection of DMP in the young adult animal, the levels of plasma FFA fall very rapidly, and hypoglycemia and conspicuous endocrine changes are observed shortly thereafter. In other words, treatment with the antilipolytic agent DMP appears to affect regulatory mechanisms that control the production of endogenous sugar and oxidizable substrate. It is conceivable that to make up for the sudden shortage of FFA fuel, the animal concentrates and enlarges, on a very short time scale, the metabolic and endocrine changes that normally occur more slowly during the smooth, "physiological" transition from the fed to the fasted state. Thus new evidence in this article demonstrates that treatment with DMP causes a rapid alteration in plasma levels of other nutrients in addition to FFA and glucose, including amino acids.

From the point of view of the control of liver autophagy and protein breakdown, all of the endocrine and metabolic regulators are seen to be recruited by the administration of DMP. This evidence clearly substantiates the hypothesis that the regulation of proteolysis in vivo must involve combined effects of amino acids with hormonal agents (22). From the quantitative point of view, however, the changes in amino acid concentrations appear to remain within comparatively narrow limits, whereas changes in insulin and glucagon levels

are much larger. Hence, it may be speculated that the ability of amino acids to exert wide-ranging control may be less important in vivo than in vitro and that changes in insulin and glucagon levels may make the biggest contribution to the dramatic increase in the number of autophagic vacuoles in liver cells after DMP administration (6) and the subsequent significant increase in the rate of liver protein breakdown (Table 1; see also Ref. 5).

Additional information about the respective roles of the different regulators of liver autophagy and proteolysis can be obtained by looking at the temporal pattern of the effects of the drug in more detail, screening liver cells for the presence of autophagic vacuoles, and making a parallel assessement of the plasma levels of regulators of autophagy, at different time points after administration of DMP. Under the electron microscope, it was found that an increase in the sequestration of volumes of cytoplasm within the lysosomal particles and in the formation of autophagic vacuoles was observed consistently 30 min after injection of the antilipolytic agent; at this time point, the increase in the release of valine from the perfused liver into the perfusate was very small and was not yet significant. In other words, evidence supports the hypothesis that at the 30-min time point the process of vacuole formation has only just taken place. In this perspective, it may be concluded that the endocrine changes, together with the decrease in glutamine levels, take place before the onset of autophagy, whereas the changes in the levels of most of the relevant amino acids (i.e., those included in the group of the primary regulators or modulators of the process of autophagy in vitro with the only exception of glutamine) follow the appearance of the autophagic vacuoles. In conclusion, the former changes can be considered as the possible cause of the process of autophagy, whereas the latter cannot and may rather be a consequence of the increase in protein degradation, in view of the rise in the concentration of many plasma amino acids.

The roles of direct regulatory amino acids deserve comment, in particular their respective individual roles in the in vivo control of liver autophagy. In in vitro experiments, the effects of balanced alterations in the levels of the regulatory amino acids (as multiples of the physiological concentration) were explored. The changes in plasma amino acid concentrations observed during induction of autophagy in our in vivo experiment are different; data show that many glucogenic amino acid levels are reduced by the administration of DMP, whereas valine, isoleucine, and leucine increase in plasma and are released in increased amounts from the perfused liver with a longer time lag. These alterations closely resemble the changes caused by the administration of glucagon, diabetes, or fasting (18, 30). Hence, the induction of autophagy and proteolysis caused by the administration of DMP may be mediated by the depletion of glycine, glutamate,and glutamine caused by the alteration in the secretion of pancreatic hormone(s) (30). Glutamine may be the most effective amino acid and the ultimate inducer of autophagy: in the present work the catabolic responses to DMP were blocked by relatively small amounts of the amino acid (that caused a 10% increase in plasma glutamine levels at death) given at the appropriate time. With regard to the changes in leucine, which is the most effective direct regulator of autophagy in vitro (e.g., see Refs. 22, 29) longer time lags are required (from 30 to 60 min), and plasma levels and outputs move toward higher nonstimulatory values. In addition to leucine, most regulatory amino acids tend toward higher levels at these later times (60 and 150 min). Hence, we may speculate that in vivo these amino acids do not contribute to the enhancement of autophagy but rather they may counteract stimulation and tune down the rate of liver protein breakdown to adjust it more finely to the needs of peripheral tissues (29, 32, 33).

In conclusion, evidence so far available suggests that the enhancement of autophagy induced by DMP administration is caused by concerted changes in insulin and glucagon plasma levels and is mediated by a depletion of intracellular amino acids (an effect attributable to primary actions of glucagon on gluconeogenesis, amino acid transport, and metabolic interconversion of glutamine, see Ref. 18), and that this physiological mechanism may operate in vivo in every case of shortage of nutrients. The role of glutamine in the in vivo regulation of macroautophagy and protein breakdown in the liver may not be a minor one, compared with leucine, perhaps because of its action as the most important mediator of the effects of glucagon. On the other hand, leucine may act in vivo as a direct regulator (suppressor) of proteolysis in the liver and in different tissues, including skeletal and cardiac muscles (8, 12). In addition, in vivo leucine may affect the rate of liver protein breakdown indirectly by participating in the regulation of glucagon, insulin, and somatostatin secretion (see, e.g., Ref. 16).

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