Inhibition of proteolysis by cell swelling in the liver requires intact microtubular structures

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In the perfused rat liver, proteolysis is inhibited by cell swelling in response to hypo-osmotic media, glutamine and insulin. Colchicine, an inhibitor of microtubules, did not affect cell swelling in response to these agonists. However, the anti-proteolytic action of these effectors was largely blunted in the presence of colchicine or the microtubule inhibitors colcemid and taxol. On the other hand, inhibition of proteolysis by phenylalanine, asparagine or NH_4Cl , i.e. compounds which exert their antiproteolytic effects by mechanisms distinct from cell swelling, was not sensitive to colchicine. Swelling-induced inhibition of proteolysis was not affected by cytochalasin B. The anti-proteolytic effect of hypo-osmotic cell swelling and insulin was largely abolished in freshly isolated rat hepatocytes; however, it reappeared upon cultivation of the hepatocytes for 6–10 h. The

restoration of the sensitivity of proteolysis to cell volume changes was accompanied by a progressive reorganization of microtubule structures, as shown by immunohistochemical staining for tubulin. It is concluded that intact microtubules are required for the control of proteolysis by cell volume, but not for the control of proteolysis by phenylalanine, asparagine or NH_4Cl . These findings may explain why others [Meijer, Gustafson, Luiken, Blommaert, Caro, Van Woerkom, Spronk and Boon (1993) *Eur. J. Biochem.* **215**, 449–454] failed to detect an antiproteolytic effect of hypo-osmotic exposure of freshly isolated hepatocytes. This effect, however, which is consistently found in the intact perfused rat liver, also reappeared in isolated hepatocytes when they were allowed to reorganize their microtubular structures in culture.

INTRODUCTION

Alterations of hepatocellular hydration, which reflect changes in liver cell volume on a short-term scale, occur within minutes under the influence of hormones, cumulative substrate uptake or oxidative stress. These volume changes are the result of an altered activity of a variety of ion- and substrate-transporting systems in the plasma membrane (for reviews see [1,2]) and were recently recognized as another second messenger of hormone and amino acid action, which modulate a variety of metabolic liver functions. For example, cell swelling inhibits, whereas cell shrinkage stimulates, proteolysis in perfused rat liver. Regulation of proteolysis by insulin, glucagon or amino acids such as glutamine or glycine was explained by hormone- and amino acid-induced changes of cell volume [3–5]. The intracellular signal transduction mechanisms which couple alterations of cellular hydration to proteolytic activity in the liver are obscure. Former studies indicated that cytoskeletal elements are involved in autophagic proteolysis [6,7]. On the other hand, cell swelling affects cytoskeletal structures. Cell swelling increases actin polymerization [8] and increases the mRNA levels for β -actin and tubulin [8–10]. Some cell-volume-sensitive processes such as bile acid excretion [11] and the acidification of vesicular intracellular structures [12] were shown to be colchicine-sensitive.

Preliminary experiments indicated a colchicine-sensitivity of the antiproteolytic effect of hypo-osmotic cell swelling [10]. Nevertheless, it remained unclear whether colchicine affects specifically the cell-volume-dependent regulation of proteolysis or whether it inhibits regulation of proteolysis in general, i.e. also by compounds known to affect proteolysis independent of cell volume, such as NH_4Cl [13,14], phenylalanine [4,15] or as-

paragine [16,17]. Accordingly, we studied the effects of colchicine and other microtubule inhibitors on the regulation of proteolysis in the perfused rat liver by hypo-osmotic exposure, glutamine and insulin on the one hand and NH_4Cl , phenylalanine or asparagine on the other. The data suggest that regulation of proteolysis by the cellular hydration state requires intact microtubular structures, whereas regulation by compounds which act by mechanisms distinct from cell swelling are apparently not influenced by colchicine.

Whereas hypo-osmotic cell swelling inhibits proteolysis in the intact perfused rat liver [3–5,18], hypo-osmotic exposure had no effect on proteolysis in freshly isolated rat hepatocytes [19]. It was concluded in the same study that “although a decrease in medium osmolarity *per se* does not appreciably affect proteolysis, it does increase the sensitivity of proteolysis to inhibition by amino acids”. We therefore addressed the question of whether the disruption of microtubules, which accompanies the cell isolation procedure, might be responsible for the lack of effect of cell swelling on proteolysis in freshly isolated hepatocytes. The antiproteolytic action of hypo-osmotic cell swelling reappeared in isolated hepatocytes when they were allowed to reorganize their microtubule network in a short-term culture.

MATERIALS AND METHODS

Liver perfusion

Livers from male Wistar rats (160–230 g), who were fed on a standard chow, were perfused in an open non-recirculating manner as described previously [20]. The perfusion medium was

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bicarbonate-buffered Krebs–Henseleit saline plus lactic acid (2.1 mmol/l) and pyruvate (0.3 mmol/l) gassed with O_2/CO_2 (19:1). In order to change the osmolarity the NaCl concentration was varied. Additions were made either by use of micropumps (20 μ l/min) or by dissolution in Krebs–Henseleit buffer or, in case of taxol, γ -luminolchicine and cytochalasin B, in DMSO. DMSO was added via micropumps at a rate of 20 μ l/min and did not significantly alter bile flow, taurocholate excretion, the cellular hydration state or the proteolysis rate.

Determinations

Effluent perfusate pH was registered continuously with a pH-sensitive electrode, and the K^+ concentration in the effluent perfusate was registered with an ion-selective electrode (Radiometer, Munich, Germany). Calculations of transmembrane K^+ fluxes were made by planimetry; a baseline drift, when present, was taken into account. The perfusion pressure was detected by a pressure transducer (Hugo Sachs Electronics, Hugstetten, Germany).

Liver mass was recorded continuously by placing the organ on a specially constructed balance pan with digital output (Sartorius, Göttingen, Germany), as described elsewhere [3]. Liver weights are given as wet weights.

Bile was collected at 2 min intervals. Bile flow was determined by gravimetry, assuming a specific mass of 1 g/ml. Taurocholate excretion into bile was assessed by liquid scintillation spectrometry of the radioactivity present in bile, based on the specific radioactivity of [3H]taurocholate in the influent perfusate.

The concentrations of urea and ammonia in the effluent perfusate were determined enzymically according to Bergmeyer [21].

Measurement of proteolysis in the perfused rat liver

To determine the rate of proteolysis in the perfused rat liver, the release of 3H label into the effluent perfusate was measured 16–20 h after having prelabelled the animals with an intraperitoneal injection of 150 μ Ci of L-[4,5- 3H]leucine [3]. After a pre-perfusion period of 80 min the release of 3H label had reached a steady state (about 500 c.p.m./min per g of liver), and more than 95% was associated with effluent leucine, as revealed by chromatographic analysis [3]. The release of 3H label remained almost constant for at least another 80–100 min and was seen to be derived from intracellular protein breakdown, since leucine is neither synthesized nor catabolized in the liver. Due to different labelling of hepatic proteins after intraperitoneal injection the rate of proteolysis was set to 100% under normotonic control conditions and the extent of inhibition of proteolysis was determined 30 min after institution of the respective conditions, a time point when a new steady state had been reached (e.g. see Figure 1). In these experiments, 100 μ mol/l unlabelled leucine was present in order to prevent further re-utilization of labelled leucine or protein synthesis.

Determination of the intracellular water space

The intracellular water space was calculated from the difference of washout profiles of simultaneously infused [^{14}C]urea and [3H]inulin (approx. 500 Bq/ml perfusate), as described previously [22]. For this, both isotopes were infused for 5 min, a time period sufficient to allow equilibration of both tracers within the intra- and extra-cellular compartments. Then tracer infusion was stopped and the effluent perfusate was collected for another 5 min, a time period that allowed complete washout of both isotopes. The effluent was monitored for the total sum of released

^{14}C and 3H labels by dual spectrum liquid scintillation spectrometry. Taking the $^{14}C/^3H$ ratio under steady-state conditions, a putative ^{14}C -, but not 3H -accessible space was calculated. This space, expressed as intracellular water space (μ l/g of liver), is seen to reflect the cellular hydration state. The cellular hydration state was measured under control conditions and again 30 min after institution of the respective conditions. Spontaneous decreases of the intracellular water space of 2.6% and 2.4% over 30 min in fed and 24-h-starved animals respectively were taken into account.

Isolation and subsequent cultivation of rat hepatocytes and assessment of proteolysis in isolated hepatocytes

Isolated rat hepatocytes were prepared by collagenase perfusion according to Hansen et al. [23] from livers of male Wistar rats that had been starved for 20 h and given an intraperitoneal injection of 150 μ Ci of L-[4,5- 3H]leucine 16 h prior to the hepatocyte isolation procedure in order to prelabel liver proteins *in vivo* [3]. After washing, rat hepatocytes were cultured for 2–10 h on collagen-coated culture dishes in Krebs–Henseleit medium which contained 100 μ mol/l L-leucine and 20 mmol/l D-glucose. After 2, 4 or 6 h of cultivation in the above-mentioned normo-osmotic medium (325 mosmol/l), the cells were washed twice with this medium supplemented with cycloheximide (5 μ mol/l) and subsequently incubated in normo-osmotic medium containing insulin (100 nmol/l) or in hypo-osmotic medium (205 mosmol/l) for another 2 h. The 3H radioactivity accumulating in the supernatant during this 2 h period was taken as a measure of the proteolytic activity of the cells. Total radioactivity at the end of the incubations (supernatant plus cell fraction) was taken as a measure of total label contained in liver protein. Proteolysis was also measured by the accumulation of valine plus isoleucine in the incubations (automatic amino acid analyser LKB 6151 Alpha Plus). Within an individual cell preparation, the rate of 3H label release or the rate of release of valine plus isoleucine in normo-osmotic incubations was set to 100% and the effect of hypo-osmotic exposure or insulin on proteolysis was expressed as a percentage of this. In order to guarantee sufficient inhibition of protein synthesis by cycloheximide, this inhibitor was not only present throughout the test period, but was also added 20 min prior to measurement. As shown previously, the concentration of cycloheximide (5 μ mol/l) was sufficiently high to inhibit protein synthesis by more than 95% [24]. Incubations were performed at 37 °C and were continuously gassed with water-vapour-saturated O_2/CO_2 (19:1). The incubations contained about 0.5 mg of hepatocyte protein per ml. The incubation volume was 7 ml. The protein content was determined according to Bradford [25]. Data on proteolysis are given as means $\pm$ S.E.M. (n = number of cell preparations).

Immunofluorescence microscopy

For indirect immunofluorescence microscopy, rat hepatocytes were precultured as described above for 2–8 h on collagen-coated culture dishes. The procedure for cell fixation and immunological labelling of microtubules was performed as described previously [26], with some modifications outlined previously [10,27]. All specimens were analysed in a Reichert-Jung Polyvar microscope (Leitz, Wetzlar, Germany) equipped with epifluorescence illumination. Micrographs were obtained on Kodak 400 ektachrome slide films.

Materials

Colchicine, colcemid, taxol, γ -lumicolchicine, cytochalasin B, cycloheximide and taurocholate were from Sigma (Munich, Germany). L-4,5- ^3H Leucine was from Amersham Buchler (Braunschweig, Germany). All enzymes, pyruvate, 2-oxoglutarate, NAD^+ and NADH were from Boehringer (Mannheim, Germany). Lactic acid was from Roth (Karlsruhe, Germany). Scintillation cocktail (Quickszint 301) was from Zinsser Analytic (Frankfurt, Germany). Collagenase was obtained from Biochrom (Berlin, Germany), protein reagent was from Bio-Rad (Munich, Germany). All other reagents were of analytical grade and were obtained from Merck (Darmstadt, Germany).

Statistics

Statistical significance was calculated by means of Student's *t* test for unpaired and paired samples. Data from different perfusion experiments are given as means $\pm$ S.E.M. (number of experiments).

RESULTS

Effect of microtubule inhibitors on proteolysis

Livers were exposed to colchicine, colcemid or taxol ($5\text{ }\mu\text{mol/l}$) for 30 min before institution of the respective antiproteolytic conditions. A time period of 20 min of colchicine infusion has been shown to result in steady-state conditions of colchicine uptake by the perfused rat liver [11] and to a destruction of hepatic microtubules [10]. Colchicine decreased proteolysis by $7.6 \pm 0.4\%$ ($n = 14$) and $12.0 \pm 1.0\%$ ($n = 14$) in fed and 24-h-starved animals respectively. Within 30 min, colcemid ($5\text{ }\mu\text{mol/l}$) led to a decrease of the proteolytic rate by $7.7 \pm 0.7\%$ ($n = 4$) in livers from fed rats; in the presence of taxol ($5\text{ }\mu\text{mol/l}$), proteolysis decreased by $5.1 \pm 1.9\%$ ($n = 3$) within 30 min.

Microtubule inhibition selectively blocks swelling-induced inhibition of proteolysis, but has no effect on cell swelling

The antiproteolytic effect of hypotonic exposure was inhibited by 75–90% in the presence of colcemid (Figure 1) and colchicine (Table 1); a smaller extent of inhibition was seen in the presence of taxol at $5\text{ }\mu\text{M}$. Colchicine, colcemid and taxol had no effect on the aniso-osmotic liver mass increase and the concomitant volume-regulatory K^+ efflux from the perfused rat liver. Under aniso-osmotic conditions, the steady-state liver mass reflects the cellular hydration state [22]. It can therefore be concluded that these microtubule inhibitors do not affect hypo-osmotic cell swelling or the volume-regulatory response. The functionally inert stereoisomer of colchicine, γ -lumicolchicine ($5\text{ }\mu\text{mol/l}$), did not affect the antiproteolytic effects of hypo-osmolarity, the liver mass increases or the transmembrane K^+ fluxes.

Colchicine also inhibited the antiproteolytic effects of glutamine plus glycine and insulin, but had no effect on liver mass changes or net K^+ fluxes induced by these effectors (Table 1). The residual antiproteolytic effect of glutamine/glycine in the presence of colchicine may be ascribed to ammonia formation from glutamine [4].

On the other hand, colchicine did not significantly impair the antiproteolytic action of phenylalanine (Figures 2a and 2b), NH_4Cl or asparagine, i.e. compounds which exert their antiproteolytic effect by mechanisms distinct from cell swelling (Table 1). Ammonia in the effluent increased from $6 \pm 1\text{ }\mu\text{mol/l}$ under basal conditions to $19 \pm 3\text{ }\mu\text{mol/l}$ ($n = 4$) in presence of phenylalanine; urea in the effluent was $31 \pm 2\text{ }\mu\text{mol/l}$ before

Figure 1 Effect of hypo-osmotic cell swelling (-80 mosmol/l) on ^3H leucine release from the perfused rat liver in the absence (○) and presence (●) of $5\text{ }\mu\text{mol/l}$ colcemid

Rats were preinjected intraperitoneally with ^3H leucine 16 h prior to the perfusion experiment. The release of ^3H label from the perfused rat liver is plotted versus time. Data are given as means $\pm$ S.E.M. and are from 3–4 different perfusion experiments with livers from fed rats. Colcemid abolished the inhibition of proteolysis caused by a decrease in the extracellular osmolarity.

and $32 \pm 1\text{ }\mu\text{mol/l}$ ($n = 4$) after a 30 min infusion of phenylalanine. With colchicine present, the ammonia concentration in the effluent was $8 \pm 2\text{ }\mu\text{mol/l}$ under basal conditions and $15 \pm 3\text{ }\mu\text{mol/l}$ ($n = 4$) during phenylalanine infusion, and that of urea was $33 \pm 3\text{ }\mu\text{mol/l}$ under basal conditions and $35 \pm 3\text{ }\mu\text{mol/l}$ ($n = 4$) in the presence of phenylalanine.

The antiproteolytic effect of NH_4Cl (5 mmol/l), which leads to alkalization of lysosomes in liver [13,14], was $34.0 \pm 2.4\%$ ($n = 4$) under control conditions (Table 1). In the presence of colchicine ($5\text{ }\mu\text{mol/l}$) the ammonia-induced inhibition of proteolysis was $38.2 \pm 3.3\%$ ($n = 3$).

Asparagine, which has been shown to cause cell swelling in hepatocytes [28] and to prevent the fusion between autophagosomes and lysosomes in liver [17], caused an inhibition of proteolysis at 2 mmol/l of $18.7 \pm 2.3\%$ ($n = 5$) in livers from fed animals. There was also some slight swelling, as indicated by a liver mass increase of about $2.2 \pm 0.2\%$ ($n = 11$) and an increase in the cellular hydration state by $3.7 \pm 0.4\%$ ($n = 9$). Asparagine (2 mmol/l) caused an increase in ammonia in the effluent from $9 \pm 2\text{ }\mu\text{mol/l}$ to $35 \pm 3\text{ }\mu\text{mol/l}$ ($n = 11$). Urea in the effluent increased from $35 \pm 3\text{ }\mu\text{mol/l}$ to $124 \pm 11\text{ }\mu\text{mol/l}$ ($n = 11$). Colchicine influenced neither the liver mass increase nor the increase in the cellular hydration state upon addition of asparagine (Table 1). With colchicine present, asparagine infusion led to an increase in the effluent ammonia concentration from $10 \pm 2\text{ }\mu\text{mol/l}$ under basal conditions to $44 \pm 9\text{ }\mu\text{mol/l}$ ($n = 8$) by 30 min after beginning the infusion of asparagine (2 mmol/l).

Table 1 Effect of microtubule inhibitors on proteolysis control by hypo-osmotic exposure, various amino acids, insulin and NH_4Cl and on liver mass, intracellular water space and K^+ fluxes in the perfused rat liver

For experimental details see the Materials and methods section. The microtubule inhibitors colcemid, taxol and colchicine or its functionally inert stereoisomer γ -lumicolchicine were added 30 min prior to addition of the agonists (see Figure 1). The decrease in proteolysis under the respective conditions in the presence of the different inhibitors was calculated with iso-osmotic medium plus inhibitor as the control. The different parameters were determined 30 min after institution of the respective conditions, a time point when K^+ fluxes had been completed and liver mass and proteolysis had reached a new steady state. Positive net K^+ flux refers to net uptake of K^+ , whereas negative net K^+ flux means net release of K^+ from the perfused rat liver. Colchicine and γ -lumicolchicine had no effect on liver mass and portal pressure and did not induce significant K^+ fluxes. In control experiments, the decrease in proteolysis 30 min after addition of colchicine was $7.6 \pm 0.4\%$ ($n = 14$) and $12.0 \pm 1.0\%$ ($n = 14$) in fed and starved animals, respectively. Under control conditions the intracellular water space was $552 \pm 17 \mu\text{l/g}$ ($n = 106$). The change in intracellular water space in the presence of colchicine was $-2.4 \pm 1.6\%$ ($n = 16$) and $-2.6 \pm 2.4\%$ ($n = 13$) in livers from fed and starved animals respectively. In the case of addition of glutamine and glycine, livers were from 24-h-starved animals; in all other experiments fed animals were used. The numbers in parentheses indicate the number of perfusion experiments. Data are given as means $\pm$ S.E.M. Significance was calculated by means of Student's *t* test: *significant versus control, $P < 0.01$. n.d., not determined.

Conditions	Decrease in proteolysis (%)	Change in liver mass (%)	Change in intracellular water space (%)	Net K^+ flux ($\mu\text{mol/g}$)
Hypo-osmotic (-80 mosmol/l)				
Control	22.6 ± 1.5 (17)	$+10.9 \pm 0.6$ (16)	n.d.	-11.6 ± 0.3 (27)
Lumicolchicine ($5 \mu\text{mol/l}$)	22.1 ± 2.2 (4)	$+9.8 \pm 0.3$ (4)	n.d.	-8.6 ± 0.5 (3)
Colchicine ($5 \mu\text{mol/l}$)	5.8 ± 1.4 (4)*	$+10.4 \pm 0.5$ (4)	n.d.	-11.9 ± 0.8 (4)
Colcemid ($5 \mu\text{mol/l}$)	3.7 ± 0.4 (4)*	$+10.7 \pm 1.4$ (4)	n.d.	-11.5 ± 0.7 (3)
Taxol ($5 \mu\text{mol/l}$)	12.4 ± 2.0 (3)*	$+9.3 \pm 0.2$ (3)	n.d.	-11.2 ± 1.1 (4)
Hypo-osmotic (-120 mosmol/l)				
Control	28.6 ± 1.4 (8)	$+15.7 \pm 0.7$ (11)	n.d.	-18.6 ± 0.8 (11)
DMSO ($20 \mu\text{l/min}$) present	23.5 ± 1.5 (3)	$+13.9 \pm 0.5$ (3)	n.d.	-17.5 ± 0.7 (3)
Lumicolchicine ($5 \mu\text{mol/l}$)	28.1 ± 0.8 (3)	$+16.4 \pm 2.4$ (4)	n.d.	-15.7 ± 0.8 (4)
Colchicine ($5 \mu\text{mol/l}$)	2.3 ± 4.0 (5)*	$+16.3 \pm 0.9$ (5)	n.d.	-15.8 ± 0.6 (5)
Glutamine-glycine (2 mmol/l)				
Control	46.1 ± 2.9 (5)	$+9.9 \pm 2.5$ (3)	$+24.4 \pm 1.1$ (4)	-1.8 ± 0.2 (3)
Colchicine ($5 \mu\text{mol/l}$)	18.6 ± 2.2 (4)*	$+11.5 \pm 1.0$ (8)	$+25.7 \pm 5.5$ (4)	-2.3 ± 0.2 (4)
Insulin (35 nmol/l)				
Control	22.9 ± 1.0 (23)	< 0.5	$+12.2 \pm 1.4$ (8)	$+4.5 \pm 0.2$ (15)
Colchicine ($5 \mu\text{mol/l}$)	11.7 ± 1.2 (6)*	< 0.5	$+12.6 \pm 0.8$ (4)	$+5.2 \pm 0.3$ (9)
Phenylalanine (2 mmol/l)				
Control	21.2 ± 2.1 (4)	$+0.8 \pm 0.1$ (5)	-0.3 ± 1.3 (4)	-1.0 ± 0.2 (7)
Colchicine ($5 \mu\text{mol/l}$)	21.8 ± 1.6 (3)	$+0.9 \pm 0.1$ (7)	-0.9 ± 0.2 (4)	-0.9 ± 0.1 (8)
NH_4Cl (5 mmol/l)				
Control	34.0 ± 2.4 (4)	$+3.7 \pm 0.4$ (9)	n.d.	-6.3 ± 0.9 (4)
Colchicine ($5 \mu\text{mol/l}$)	38.2 ± 3.3 (3)	$+3.3 \pm 0.2$ (3)	n.d.	-4.2 ± 0.4 (3)
Asparagine (2 mmol/l)				
Control	18.7 ± 2.3 (5)	$+2.2 \pm 0.2$ (11)	$+3.7 \pm 1.5$ (6)	< 0.5
Colchicine ($5 \mu\text{mol/l}$)	14.6 ± 2.1 (3)	$+2.6 \pm 0.2$ (10)	$+3.0 \pm 1.5$ (4)	< 0.5

Urea in the effluent increased from a basal $34 \pm 4 \mu\text{mol/l}$ to $124 \pm 9 \mu\text{mol/l}$ ($n = 8$) in the presence of asparagine (2 mmol/l). The antiproteolytic effect of asparagine in the presence of colchicine was $14.6 \pm 2.1\%$ ($n = 3$).

Effects of cytochalasin on swelling-induced inhibition of proteolysis, bile flow and taurocholate excretion

Under control conditions, the steady-state inhibition of proteolysis following hypo-osmotic exposure (-120 mosmol/l) was $23.5 \pm 1.5\%$ ($n = 3$) (Figure 3). In the presence of cytochalasin B, the extent of inhibition was $22.5 \pm 1.7\%$ ($n = 4$). Cytochalasin B itself induced a slight inhibition of proteolysis of $4.0 \pm 0.3\%$ ($n = 4$); this effect was also found upon infusion of DMSO, the vehicle used for dissolution of cytochalasin (Figure 3). The increase in liver mass upon hypo-osmotic exposure (-120 mosmol/l) was $16.2 \pm 1.1\%$ ($n = 4$) in the presence of cytochalasin B versus $13.9 \pm 0.5\%$ ($n = 3$) under control conditions (see also Table 1). The volume-regulatory K^+ efflux was $17.5 \pm 0.7 \mu\text{mol/g}$ of liver ($n = 3$) under control conditions versus $14.9 \pm 1.5 \mu\text{mol/g}$ of liver ($n = 4$) with cytochalasin B present.

In the presence of $100 \mu\text{mol/l}$ taurocholate, infusion of cytochalasin B ($2 \mu\text{mol/l}$) decreased bile flow from 2.3 ± 0.2 to $0.5 \pm 0.1 \mu\text{l/min}$ per g of liver ($n = 4$) (Figure 4). Excretion of taurocholate decreased from 129 ± 5 to $28 \pm 6 \text{ nmol/min}$ per g of liver ($n = 4$). After cessation of cytochalasin B infusion there was a partial restoration of bile flow and taurocholate excretion. This is in line with previous reports on the cholestatic effect of cytochalasins in rat liver [29,30].

Effect of cycloheximide on proteolysis inhibition in the perfused rat liver under hypo-osmotic conditions

In order to resolve the discrepancy between the finding of an antiproteolytic effect of cell swelling in the perfused rat liver [3,18] and the experiments in hepatocytes published by Meijer et al. [19], in which there was no antiproteolytic effect of cell swelling *per se*, we transferred their experimental conditions to the perfused liver by using livers from starved rats and adding cycloheximide ($25 \mu\text{mol/l}$) to the perfusion medium, with leucine, lactate and pyruvate absent. Glucose was present at a con-

Figure 2 Effect of phenylalanine (2 mmol/l) on ^3H label release from the perfused rat liver in the absence (a) and presence (b) of 5 $\mu\text{mol/l}$ colchicine

Phenylalanine was infused for 30 min (●). Control conditions (○) refer to no further addition (a) or addition of colchicine alone (b). Data are given as means $\pm$ S.E.M. and are from 3–4 different perfusion experiments with livers from fed rats. Colchicine did not affect the inhibition of proteolysis by phenylalanine. This amino acid did not cause substantial liver cell swelling (see also Table 1).

Figure 3 Effect of hypo-osmotic exposure (–120 mosmol/l) on ^3H label release from the perfused rat liver in the absence (●) and presence (○) of cytochalasin B (2 $\mu\text{mol/l}$)

Perfusate osmolality was lowered by omission of 60 mmol/l NaCl from the Krebs–Henseleit buffer. The inhibition of proteolysis by hypo-osmotic cell swelling was unaffected by 2 $\mu\text{mol/l}$ cytochalasin B. Control experiments were run in the presence of DMSO as dissolution vehicle for cytochalasin B. Data are given as means $\pm$ S.E.M. and are from 3–4 different perfusion experiments with livers from fed rats.

Figure 4 Effect of cytochalasin B (2 $\mu\text{mol/l}$) on bile flow (○) and taurocholate excretion (●) in the perfused rat liver

In these experiments, 100 $\mu\text{mol/l}$ taurocholate was present (1 $\mu\text{Ci/l}$). There was a substantial cholestatic effect of cytochalasin B at 2 $\mu\text{mol/l}$, which was partially reversible. The data reflect a series of four different experiments and are given as means $\pm$ S.E.M.

Figure 5 Effect of hypo-osmotic exposure (-80 mosmol/l) on proteolysis in the perfused rat liver in the presence of cycloheximide ($25 \mu\text{mol/l}$)

Livers were from 24-h-starved rats. The perfusion medium contained 5 mmol/l glucose and was devoid of lactate plus pyruvate. In the perfused rat liver, the antiproteolytic effect of exposure to a hypo-osmotic perfusion medium (225 mosmol/l) was not affected by the protein synthesis inhibitor. Data are given as means $\pm$ S.E.M. and are from four different experiments.

centration of 5 mmol/l (20 mmol/l in the experiments published by Meijer et al. [19]). As shown in Figure 5, upon infusion of cycloheximide ($25 \mu\text{mol/l}$) there was a marked transient increase of ^3H label release within the first 5 min of infusion and a subsequent steady-state decrease of $22.2 \pm 3.1\%$ ($n = 4$).

It should be emphasized that the experiments were carried out under conditions favouring reincorporation of proteolysis-derived ^3H label into newly synthesized protein, i.e. in the absence of unlabelled leucine. Thus the findings in Figure 4 suggest that cycloheximide inhibition of protein synthesis precedes a strong inhibitory effect on proteolysis. The inhibition of proteolysis by cycloheximide in the perfused liver is different from the situation in hepatocytes, which show no inhibition [19,31].

The inhibitory effect of hypo-osmotic exposure (-80 mosmol/l) on proteolysis was $25.2 \pm 3.5\%$ ($n = 4$) in the presence of cycloheximide. This value is similar to the inhibition of proteolysis of $27.3 \pm 3.3\%$ ($n = 7$) upon hypo-osmotic cell swelling in the absence of cycloheximide. These data indicate that the antiproteolytic effect of cell swelling in the perfused rat liver is maintained even in the presence of cycloheximide, although this inhibitor may interfere with proteolysis.

Microtubule reorganization and restoration of volume-sensitive proteolysis control in isolated hepatocytes

Cytoskeletal derangements occurring during the preparation of isolated hepatocytes could explain why proteolysis control by

Figure 6 Reorganization of microtubular structures of hepatocytes during short-term cultivation as visualized by immunofluorescence

After 2 h (a) and 4 h (b) of cultivation, cells were processed for immunofluorescence with fluorescein isothiocyanate-labelled anti-tubulin antibodies. After 4 h there was a significant improvement in the visualization of the microtubular network. Two photographs representative of four pictures at each time point in two different cell preparations are shown.

cell volume is present in the intact hepatocyte *in situ* but not in freshly isolated rat hepatocytes. In immunofluorescence studies, a gradual reorganization of hepatocyte microtubule structure was seen (Figures 6a and 6b) when the freshly isolated hepatocytes were kept in culture for 4 h. A further gradual improvement of microtubule visualization was also seen after 6 and 8 h of cultivation (results not shown). This process was accompanied by the restoration of proteolysis control by hypo-osmotic and insulin-induced cell swelling (Tables 2 and 3). After 4, 6, 8 and 10 h of cultivation there was a significant increase in the anti-proteolytic effect in isolated hepatocytes which had been exposed either to hypo-osmotic incubation media (205 mosmol/l ; Table 2) or to insulin (100 nmol/l ; Table 3).

In the same series of experiments the inhibition of proteolysis, after previous prelabelling of the rats by an intraperitoneal injection of $150 \mu\text{Ci}$ of $\text{L-[4,5-}^3\text{H]leucine}$ 16 h prior to the experiment, was determined as ^3H radioactivity in the supernatant of the incubation medium, as described in the Materials and methods section. Upon hypo-osmotic exposure (-120 mosmol/l) the corresponding inhibition of proteolysis was $11 \pm 1\%$ after 4 h of cultivation; this increased to $19 \pm 4\%$ after 10 h of cultivation ($n = 9$). In the presence of insulin (100 nmol/l) the inhibition of proteolysis increased from $3 \pm 2\%$ after 4 h of cultivation to $13 \pm 3\%$ after 8 h of cultivation ($n = 8$).

Table 2 Effect of the duration of cultivation on the sensitivity of hepatocytes to swelling-induced inhibition of proteolysis upon hypo-osmolar exposure (–120 mosmol/l)

Hepatocytes were isolated from livers of 20-h-starved rats (see the Materials and methods section). After isolation, the cells were cultured for 4, 6, 8 or 10 h, with washes performed every 2 h. At 140 min prior to the termination of cultivation the cells were exposed to cycloheximide (5 μ mol/l) and 20 min later they were incubated for 120 min in normo-osmotic (325 mosmol/l) or hypo-osmotic (205 mosmol/l medium). The incubation was ended by addition of perchloric acid (2 mol/l) and, after neutralization, the supernatant was assayed for valine plus isoleucine and the cells for protein concentration. The proteolysis rate was determined during the last 2 h of cultivation. Data are given as means $\pm$ S.E.M. (with numbers of cell preparations in parentheses). Significance was calculated by Student's *t* test for paired samples: **P* < 0.05; ***P* < 0.01.

Cultivation time (h)	Rate of proteolysis (nmol of Val + Ile/ h per mg of cell protein)		
	Control	Hypo-osmotic (–120 mosmol/l)	Inhibition (%)
4	28.7 $\pm$ 3.3	24.8 $\pm$ 2.5	12 $\pm$ 8 (4)
6	26.2 $\pm$ 1.7	21.3 $\pm$ 1.7**	19 $\pm$ 4 (5)**
8	20.8 $\pm$ 0.9	16.6 $\pm$ 1.1**	21 $\pm$ 2 (5)**
10	17.5 $\pm$ 2.1	12.4 $\pm$ 0.8*	27 $\pm$ 9 (5)*

Table 3 Reconstitution of the antiproteolytic effect of insulin upon cultivation of isolated hepatocytes

Conditions were identical to those described in Table 2. Cells were exposed either to normo-osmotic perfusion medium (325 mosmol/l) or to normo-osmotic perfusion medium containing insulin (100 nmol/l). Significance was calculated by Student's *t* test for paired samples: ** significant versus control, *P* < 0.01.

Cultivation time (h)	Rate of proteolysis (nmol of Val + Ile/ h per mg of cell protein)		Inhibition (%)
	Control	+ Insulin (100 nmol/l)	
4	26.6 $\pm$ 1.2	26.8 $\pm$ 2.3	0 $\pm$ 4 (4)
8	22.5 $\pm$ 2.4	19.5 $\pm$ 2.4**	14 $\pm$ 3 (4)**

DISCUSSION

Requirement of intact microtubules for proteolysis regulation by the cellular hydration state

In the intact perfused rat liver, the antiproteolytic effect of hypo-osmotic cell swelling was almost completely abolished in the presence of colchicine, whereas proteolysis inhibition by NH₄Cl, phenylalanine and asparagine was not affected. These latter agonists do not appreciably alter cell volume and accordingly affect proteolysis by mechanisms distinct from cell volume changes. The antiproteolytic effects of insulin and glutamine/glycine, which were largely ascribed to cell swelling, were also found to be colchicine-sensitive, albeit to a somewhat lesser extent than those of hypo-osmotic cell swelling (Table 1). This may indicate that mechanisms other than cell swelling, such as ammonia formation, may contribute to their antiproteolytic action.

Taken together, these observations suggest that the regulation of proteolysis by cell volume, but not by other mechanisms, depends on intact microtubular structures. Cell swelling was

shown to induce an alkalinization of cellular vesicular compartments as assessed by Acridine Orange and fluorescein isothiocyanate/dextran fluorescence studies [12,32], and this effect was also shown to be colchicine-sensitive [12,33]. Thus one might speculate that a microtubule-dependent regulation of the acidification of vacuolar structures may contribute to the effect of cell volume on autophagic proteolysis, although further mechanisms cannot be ruled out. In this respect it is of interest to note that vesicular acidification occurs early in the pathway of autophagic proteolysis [34]. The link between cell volume, microtubules and proteolysis inhibition, however, is unclear, and it remains to be established to what extent colchicine-sensitive alkalinization in endocytotic compartments mediates the volume-dependent regulation of proteolysis and whether the sequestration process itself, which is subject to regulation by amino acids [31,35], is affected by cell volume changes.

Cell swelling and proteolysis in isolated hepatocytes

Following isolation of hepatocytes by means of collagenase treatment the microtubular network is grossly altered, as indicated by the clumpy appearance of microtubules when examined by immunofluorescence staining. Upon cultivation of freshly isolated hepatocytes, filamentous microtubular structures progressively reappear (Figure 6), suggesting that microtubular structures reorganize over the first 6 h of cultivation. In parallel with the morphological reappearance of a fine microtubule network, the sensitivity of proteolysis to hypo-osmotic cell swelling is restored (Table 2). Although the absolute rate of proteolysis declines in cultured hepatocytes, an effect which is probably due to exhaustion of the short-lived protein fraction, a significant inhibitory effect of hypo-osmotic cell swelling is observed upon cultivation of hepatocytes.

Considering the requirement of intact microtubular structures for the cell-volume-dependent control of proteolysis in the perfused liver, these data suggest that the lack of responsiveness to hypo-osmotic cell swelling of freshly isolated hepatocytes [19] may be ascribed to the microtubular disintegration accompanying the cell isolation procedure. Thus the claim that cell swelling *per se* does not affect proteolysis is correct for freshly isolated hepatocytes, but should not be extrapolated to the hepatocyte *in situ* with intact microtubular structures. The finding that intact microtubules are required for the volume-dependent regulation of proteolysis may also explain a variety of conflicting results in the literature. For example, insulin was shown to inhibit proteolysis in perfused liver [5,36–38] and in cultured hepatocytes [39], but was found to be ineffective in freshly isolated hepatocytes [40]. Recent work from Luiken et al. [41] showed an 8% inhibition of proteolysis upon addition of insulin to freshly isolated hepatocytes from starved rats, an effect which was enhanced by amino acids.

In our system the antiproteolytic effect of insulin reappears when hepatocytes are kept in culture for at least 8 h (Table 3). Although not proven, it is tempting to ascribe this phenomenon to the described reorganization of microtubules which occurs during the first hours of cultivation. Nevertheless, other effects of cultivation, such as those on insulin sensitivity, might also play a role. Likewise, glycine inhibits proteolysis in the perfused rat liver [4] but not in hepatocyte suspensions [42]. Since both insulin and glycine exert their antiproteolytic effects via an increase in cellular hydration [4,5], it is not surprising that under conditions of microtubule disruption, i.e. in freshly isolated hepatocytes, their effects on proteolysis are masked. The postulated permissive effect of amino acids to the inhibitory effect of insulin on proteolysis in hepatocytes from starved rats [41] could possibly

be ascribed to amino-acid-dependent stabilization of microtubules [10]. It might therefore be speculated that one answer to the search for the 'anticatabolic factor' that is observed upon cultivation of hepatocytes [40] is the reconstruction of cytoskeletal elements.

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