Cell hydration controls autophagosome formation in rat liver in a microtubule-dependent way downstream from p38^{MAPK} activation

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Autophagic proteolysis in rat liver is under the control of the cellular hydration state. Because the morphological site of swelling-dependent proteolysis regulation has not yet been identified, the formation of autophagosomes was investigated with transmission electron microscopy in slices from perfused livers. In livers from fed rats, hypo-osmotic exposure (185 mosmol/l) led within 30 min to a decrease in fractional cytoplasmic autophagosome volume that was sensitive to colchicine and p38^{MAPK} inhibition. Similarly, the decrease in autophagosome volume, but not the increase in cell volume caused by insulin or glutamine/glycine, was strongly inhibited by colchicine and SB 203580, an inhibition of p38^{MAPK} activation. Immune complex assays from perfused liver showed that hypo-osmotic activation of p38^{MAPK} was not inhibited by colchicine. Further, experiments using confocal laser microscopy in cultivated hepatocytes incubated with mouse-derived anti-(α-tubulin) showed that microtubular structures were not influenced by the inhibition of p38^{MAPK} by SB 203580. It is concluded that the sequestration of autophagic vacuoles is a major site of proteolysis regulation by cell hydration. Swelling-induced activation of p38^{MAPK} is required for this process and occurs upstream of the putative microtubule regulation site.

Key words: cell volume, colchicine, mitogen-activated protein kinase, protein degradation, SB 203580.

INTRODUCTION

Autophagic vacuole formation is the initial step of bulk protein degradation in liver. Hepatic autophagosomes have a half-life of approx. 10 min [1]. The exact biochemical events responsible for the regulation of autophagosome formation are only poorly understood. In rat liver, autophagic proteolysis is under the control of insulin [1,2], glucagon [3], intracellular ATP [4], amino acids [5–7] and nutritional state [8]. Changes in cellular hydration have been shown to mediate to a large extent the effects of glycine, glutamine, insulin and glucagon on proteolysis [6,9], and the anti-proteolytic effect of ethanol resides in its capacity to swell hepatocytes by a bumetanide-sensitive mechanism [10]. Intact microtubules are required in the link between cell hydration and proteolysis regulation [9], but little is known about the intracellular location of the putative proteolysis regulation site. Recently, activation of p38^{MAPK} has been shown to occur upon hypo-osmotic swelling in liver; the anti-proteolytic effect of swelling agonists such as insulin and glutamine/glycine was abolished by SB 203580, an inhibitor of p38^{MAPK} [11]. Insulin and certain amino acids inhibit autophagy at the sequestration step [12–14], but the exact mechanism of regulation is still obscure.

Therefore the purpose of the present study was to investigate the influence of cell swelling and shrinkage on the sequestration step. The results show that hypo-osmotic, insulin-induced and amino-acid-dependent swelling leads to a colchicine-sensitive decrease in the number of autophagic vacuoles, whereas shrinkage induces an increase in the number of autophagosomes. Hypo-osmotic activation of p38^{MAPK} is colchicine-insensitive and occurs upstream of the putative microtubule-dependent regulatory site.

MATERIALS AND METHODS

Liver perfusion

Livers, from male Wistar rats (160–230 g) fed on a standard chow, were perfused in an open non-recirculating manner as described previously [15]. The perfusion medium was bicarbonate-buffered Krebs–Henseleit saline plus lactate (2.1 mM) and pyruvate (0.3 mM), gassed with O_2/CO_2 (19:1). The NaCl concentration was varied to change the osmolarity. Additions were made either by use of micropumps (20 μl/min) or by dissolution in Krebs–Henseleit buffer. Colchicine was dissolved in DMSO. When indicated in the text, the influent perfusate contained multiples of a mixture of amino acids at a concentration found physiologically in the portal vein, i.e. 0.4 mM alanine, 0.1 mM proline, 0.2 mM serine, 0.3 mM glycine, 0.03 mM aspartate, 0.07 mM asparagine, 0.18 mM valine, 0.1 mM leucine, 0.07 mM isoleucine, 0.03 mM methionine, 0.04 mM tyrosine, 0.05 mM phenylalanine, 0.05 mM histidine, 0.05 mM arginine, 0.13 mM threonine, 0.25 mM lysine, 0.18 mM tryptophan, 0.06 mM cysteine, 0.1 mM glutamate and 0.5 mM glutamine plus 0.1 mM NH_4Cl. In these experiments, livers from rats starved for 24 h were used. Viability of the livers was assessed by monitoring effluent oxygen concentration and measuring lactate dehydrogenase leakage from livers, which did not exceed 20 m-units/min per g of liver.

Determinations

Effluent perfusate pH was monitored continuously with a pH-sensitive electrode; effluent perfusate oxygen concentration was monitored with a Clark-type oxygen electrode (Biolytik, Bochum, Germany). The perfusion pressure was detected by a pressure transducer (Hugo Sachs Electronics, Hugstetten, Germany).

Abbreviation used: ERK, extracellular signal-regulated protein kinase.

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Basal portal pressure was 3–5 cmH_2O (400–670 Pa) and was not affected by the compounds used in this study. The intracellular water space was calculated from the difference between the washout profiles of simultaneously infused [^{14}C]urea and [^{3}H]inulin as described previously [16]. In fed animals, the cell water under control conditions was 591 ± 23 μl/g ($n = 16$). Proteolysis was determined in separate perfusion experiments as ^{3}H label release from rats that had been injected intraperitoneally with 150 μCi of L-[4,5-^{3}H]leucine 16 h before the perfusion experiment, as described previously [17]. Owing to different labelling of hepatic proteins after intraperitoneal injection the rate of proteolysis was set at 100 % under normotonic control conditions and the extent of inhibition of proteolysis was determined 30 min after institution of the relevant condition, a time point at which a new steady state had been reached.

Tissue processing for immune complex kinase assays, electron microscopy and morphometry

Rat livers were perfused for 130 min with iso-osmotic perfusion medium and thereafter with hypo-osmotic perfusion medium, insulin or amino acids. The desired osmolarities were achieved by the addition or omission of the relevant quantities of NaCl. When present, inhibitors were present for 30 min before institution of the different perfusion conditions. For determinations of immune complex kinase, liver lobes from perfused liver were excised at the relevant time points, homogenized with an Ultra-Turrax (Janke & Kunkel, Staufen, Germany) at 0 °C in lysis buffer containing 20 mM Tris/HCl (pH 7.4), 140 mM NaCl, 10 mM NaF, 10 mM sodium pyrophosphate, 1 % (v/v) Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 20 mM β-glycerophosphate and protease inhibitor cocktail (Boehringer, Mannheim, Germany).

For electron microscopic morphometry, fixation of the liver lobes was performed by the perfusion of glutaraldehyde (3 %, v/v) in Krebs–Henseleit medium for 30 s. From the fixed livers small cubes of approx. 1 mm^3 were cut, post-fixed for 2 h with 2 % OsO_4, 2 % (w/v) uranyl acetate and 1.5 % (w/v) lead citrate in PBS, dehydrated in a graded series of ethanol and embedded in Epon 812. Thin sections for electron microscopy were placed on copper grids, stained with uranyl acetate and lead citrate, and examined with a Philips CM10 electron microscope (Philips, Eindhoven, The Netherlands).

Immune complex kinase assays

The lysed samples from the perfused liver were centrifuged at 4 °C and aliquots of the supernatant were incubated with 1.5 μg of an antibody against extracellular signal-regulated protein kinase (ERK)-2 or p38^{MAPK} for 2 h at 4 °C. Immune complexes were collected by using Protein A–Sepharose 4 B (Pharmacia, Freiburg, Germany), washed three times with lysis buffer and four times with kinase buffer [10 mM Tris/HCl (pH 7.4)/150 mM NaCl/10 mM $MgCl_2$/0.5 mM dithiothreitol], and incubated with 1 mg/ml myelin basic protein, 10 μM ATP (only for the ERK assay), and 10 μCi of [γ-^{32}P]ATP for 30 min at 37 °C. The reactions were stopped by adding 2 × gel loading buffer, and activities of ERK-2 and p38^{MAPK} were monitored via autoradiography after SDS/PAGE [12.5 % (w/v) gel]. Densitometry was performed with the E.A.S.Y. RH system (Herolab, Wiesloch, Germany).

Quantitative evaluation of intracellular organelles

The autophagic vacuoles were defined as portions of cytoplasm sequestered from the remaining cytoplasm by one or two membranes. The morphology of autophagic vacuoles has been described in detail elsewhere [1]. The square fields defined by the copper grids (127 μm × 127 μm) were used as test fields and systematically searched for autophagic vacuoles at a magnification of × 10 500. The area of cytoplasm examined was in the range 7500–11 500 μm^2 ($n = 6$). The area of the autophagic vacuole was measured at magnification × 21 000. Low-power electron micrographs of the test fields were mounted and the area of the hepatocytic cytoplasm was calculated by a count pointing method (144 test points). The fractional volume of autophagic vacuoles, defined as the volume of autophagic vacuoles per volume of liver cell cytoplasm (V_{av}/V_c), was calculated as described previously [18,19]. Similarly, the volumes of mitochondria (V_{mito}/V_c), glycogen (V_{glyc}/V_c), rough endoplasmic reticulum (V_{rer}/V_c) and residual intracellular organelles (V_{resid}/V_c) per volume of cytoplasm were determined and expressed as fractional cytoplasmic volumes [20]. Volume-weighted mean hepatocyte volumes were calculated with the I_0^3 method of Gundersen [21] and expressed in μm^3.

Hepatocyte isolation and cell culture

Isolated rat hepatocytes were prepared from livers of male Wistar rats (160–180 g, fed *ad libitum*) by modified collagenase perfusion [22] as described previously [23]. Cell viability was more than 90 % as assessed by Trypan Blue exclusion; cell purity 48 h after preparation was more than 96 %. Isolated rat hepatocytes were cultured for 48 h on collagen-coated coverslips in DMEM (Dulbecco's modified Eagle's medium) containing glucose (6 mM), gentamycin (100 μg/ml), fetal bovine serum (10 %, v/v), dexamethasone (100 nM), insulin (100 nM) and aprotinin (1 μg/ml). The cell cultures were maintained in an air/CO_2 (19:1) atmosphere at 37 °C. In the indicated experiments, osmolarity was changed by altering the NaCl concentration of the DMEM. The inhibitors colchicine and SB 203580 were present for 30 min each, as indicated in the text.

Immunocytochemistry

For immunofluorescence the cells were washed with PBS, fixed with ice-cold methanol for 10 min, permeabilized with Triton X-100 (0.1 %, v.v) for 5 min and blocked with 1 % (w/v) BSA for 1 h. The glass slides were incubated overnight with mouse anti-(α-tubulin) antibody (Clone DM 1A, dilution 1:600; Calbiochem, Bad Soden, Germany) at 4 °C, washed with PBS and stained with goat anti-mouse Alexa-488-conjugated antibodies (dilution 1:600; Molecular Probes, Eugene, OR, U.S.A.) at room temperature for 90 min. Coverslips were mounted in fluorescent mounting medium (Dako, Carpinteria, CA, U.S.A.).

Confocal laser scanning microscopy

Immunostained cell samples were analysed with a Leica TCS NT confocal laser scanning system with an argon–krypton laser on a Leica (Bensheim, Germany) DM IRB inverted microscope. Images were acquired from a channel at a wavelength of 488 nm.

Materials

L-Lactic acid was from Roth (Karlsruhe, Germany). Colchicine and insulin were from Sigma (Deisenhofen, Germany). Glutaraldehyde was from Serva (Heidelberg, Germany). SB 203580 was from Calbiochem. Radiochemicals were from Amersham (Braunschweig, Germany). DMEM, fetal bovine serum and gentamicin were purchased from Biochrom (Berlin, Germany). Enzymes were from Boehringer (Mannheim, Germany). All other chemicals were from Merck (Darmstadt, Germany).

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Statistics

Results from different perfusion experiments are given as means ± S.E.M. (n being the number of independent experiments). Conditions were compared with Student's t test. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

In the perfused rat liver, lowering the extracellular osmolarity from 305 to 185 mosmol/l for 30 min led to a significant decrease in fractional cytosolic volume (V_{av}/V_c) of autophagic vacuoles that was sensitive to inhibition by colchicine (Table 1). Insulin and glutamine/glycine, whose anti-proteolytic effect in liver depends to a major extent on their swelling capacity [24,25], also induced a colchicine-sensitive decrease in V_{av}/V_c. In the presence of insulin (35 nM), increasing extracellular osmolarity from 305 to 385 mosmol/l for 30 min led to a statistically significant increase in V_{av}/V_c, from $(12.9 \pm 0.8) \times 10^{-4}$ to $(29.0 \pm 3.5) \times 10^{-4}$ ($n = 3$, $P < 0.05$), which was paralleled by an increase in proteolysis in the perfused rat liver [26]. Decreasing extracellular osmolarity to 185 mosmol/l led to an increase in the mean hepatocyte volume, whereas the fractional volumes of cytoplasmic intracellular organelles, e.g. mitochondria, rough endoplasmic reticulum, glycogen and residual organelles, did not change significantly during hypo-osmotic perfusion (Table 2). The numerical density of autophagic vacuole profiles per 10000 μm^2 of hepatocytic cytoplasmic area, which reflects the absolute number of autophagic vacuoles, changed from 104 ± 8 ($n = 8$) to 50 ± 5 ($n = 5$) during hypo-osmolarity (185 m-osmol/l, $P < 0.001$) and the ratio of numerical density of autophagic vacuole profiles and V_{av}/V_c remained constant under these conditions (Table 1). The results show that the average cytoplasmic area of a single autophagic vacuole does not change during hypo-osmolarity and that the swelling-induced decrease in V_{av}/V_c is not a consequence of simple dilutional effects of hypo-osmolarity-dependent expansion of the cytoplasmic space but reflects a true decrease in the number of autophagic vacuoles. It can be concluded that sequestration of autophagic vacuoles is under the control of cell hydration.

In line with previous results [11], hypo-osmotic liver perfusion led to a rapid but transient 2.1 ± 0.4-fold activation of $p38^{MAPK}$ ($n = 3$) with a maximum within the first 10 min (Figure 1). An early 1.8 ± 0.3-fold activation of the ERK-2 ($n = 3$) was also observed. The hypo-osmotic activation of both $p38^{MAPK}$ and ERK-2 was not inhibited by colchicine (5 μM). There was a 2.3 ± 0.5-fold maximum activation of $p38^{MAPK}$ ($n = 3$) in the presence of hypo-osmolarity and colchicine; the maximum hypo-osmolarity-induced activation of ERK-2 was 1.7 ± 0.3-fold ($n = 3$) in the presence of colchicine. Colchicine itself had no detectable effect on MAP (mitogen-activated protein) kinase activation under normo-osmotic conditions. SB 203580, a specific inhibitor of $p38^{MAPK}$ [27], largely prevented the hypo-osmotic [11], insulin-induced and glutamine/glycine-dependent decrease in autophagic vacuoles (Table 1).

As shown by confocal laser microscopy studies in 48 h-cultivated rat hepatocytes incubated with antibodies against α-tubulin, the meshwork-like appearance of microtubules (Figure 2A) remained unchanged in the presence of SB 203580 (250 nM) (Figure 2B) but was destroyed by colchicine (5 μM) (Figure 2C). These findings suggest that inhibition of $p38^{MAPK}$ does not affect the transmission of the swelling signal to the autophagosome regulation site by a partial destruction of microtubular structure or function.

As shown previously, effector-induced cell swelling was not inhibited by SB 203580 [11]. Neither aniso-osmotic nor agonist-dependent cell swelling was prevented by colchicine (Table 3). Hypo-osmolarity, insulin or glutamine/glycine led to a colchicine-sensitive decrease in proteolysis [9]. Although the inhibition of proteolysis by hypo-osmolarity (185 m-osmol/l) was $31.4 \pm 0.9\%$ ($n = 4$) in the perfused rat liver and was decreased to $2.3 \pm 4.0\%$ ($n = 4$) in the presence of colchicine, the inhibition of proteolysis induced by insulin and amino acids was not fully sensitive to colchicine [9]. Swelling-independent signalling mechanisms, such as the well-known activation of ribosomal protein S 6 by insulin [28], which inhibits hepatic autophagy [29], might be involved in insulin-induced protein retention; the residual anti-

Table 1 Effect of hypo-osmotic perfusion conditions, insulin and amino acids on fractional volume (V_{av}/V_c) of autophagic vacuoles in perfused rat liver in the presence of the microtubule inhibitor colchicine and the $p38^{MAPK}$ inhibitor SB 203580

Livers from fed male Wistar rats were perfused in an open non-recirculating manner for a total duration of 160 min. Hypotonic perfusion medium (185 mosmol/l), insulin (35 nM) or glutamine/glycine (2 mM) were infused from 130 min to 160 min. At 160 min the livers were perfused with 3% (v/v) glutaraldehyde for 30 s and tissues were further processed for electron microscopy. When present, an inhibitor [i.e. colchicine (5 μM) or the $p38^{MAPK}$ inhibitor SB 203580 (250 nM)] was infused from 100 min to 160 min. Results are expressed as fractional autophagic vacuole volume, which is defined as the volume of autophagic vacuoles per cytoplasmic volume (V_{av}/V_c), and are shown as means ± S.E.M. with the number of experiments in parentheses. Significance was calculated by means of Student's t test. $^{***}P < 0.001$ compared with the respective control; n.s., not significant.

	$10^4 \times V_{av}/V_c$			
Condition	Control	Hypotonic	Insulin	Glutamine/glycine
No inhibitor	51.8 ± 1.9 (7)	28.2 ± 3.5*** (5)	12.9 ± 0.8*** (3)	16.6 ± 1.4*** (3)
Colchicine	43.5 ± 4.2 (3)	38.8 ± 4.7$^{n.s.}$ (3)	32.5 ± 4.1$^{n.s.}$ (3)	33.2 ± 3.4$^{n.s.}$ (3)
SB 203580	52.7 ± 5.9 (3)	53.0 ± 6.9$^{n.s.}$ (3)	39.5 ± 6.3$^{n.s.}$ (3)	41.7 ± 3.3$^{n.s.}$ (3)

Table 2 Mean hepatocyte volumes and fractional cytoplasmic volumes of mitochondria, glycogen, rough endoplasmic reticulum and residual cytoplasmic organelles in slices from perfused rat liver under iso-osmotic and hypo-osmotic conditions

Livers from fed male Wistar rats were perfused for 160 min. If present, hypo-osmotic perfusion conditions (305 → 185 m-osmol/l) had been installed from 130 min to 160 min. At that time point, livers were fixed with 3% (v/v) glutaraldehyde and further processed for transmission electron microscopy. Fractional cytoplasmic volumes of mitochondria (V_{mito}/V_c), glycogen (V_{glyc}/V_c), rough endoplasmic reticulum (V_{rer}/V_c) and residual cytoplasmic organelles (V_{resid}/V_c) and volume-weighted mean hepatocyte volumes were determined as described in the Materials and methods section. Each of the sets of fractional cytoplasmic organelle volumes (first four results columns) sums to 100%; the numbers of perfusion experiments are given in parentheses. $^{**}P < 0.01$ compared with control.

Condition	V_{mito}/V_c (%)	V_{glyc}/V_c (%)	V_{rer}/V_c (%)	V_{resid}/V_c (%)	Mean hepatocyte volume (μm^3)
Normo-osmotic	23.8 ± 1.0 (9)	18.3 ± 5.0 (9)	31.8 ± 2.0 (9)	26.1 ± 3.4 (9)	2700 ± 200 (9)
Hypo-osmotic (185 mosmol/L)	24.3 ± 3.0 (5)	28.0 ± 5.0 (5)	25.5 ± 1.2 (5)	22.2 ± 3.5 (5)	3719 ± 136 (5)**

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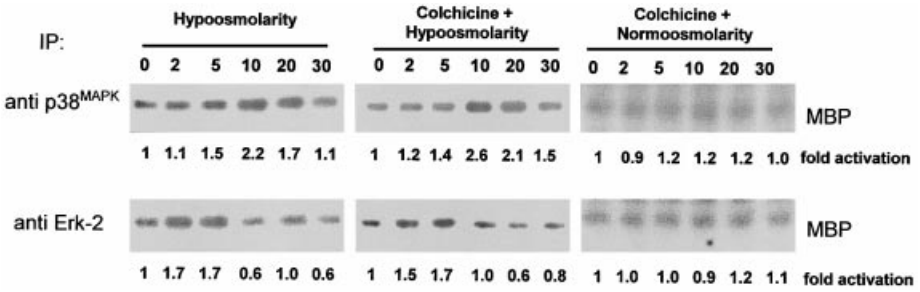

Figure 1 Effect of colchicine on hypo-osmolarity-induced MAP kinase activation in perfused rat liver

Representative results from three independent experiments are shown. After a 130 min normo-osmotic preperfusion period (305 m-osmol/l), the perfusion medium was switched to hypo-osmolarity (185 m-osmol/l) and liver samples were taken at the indicated time points. MAP kinase activity was measured with immune complex assays (IP) with antibodies against p38^{MAPK} and ERK-2. Myelin basic protein (MBP) was used as a substrate for the immunoprecipitated ERK-2 and p38^{MAPK}. In experiments with colchicine, the inhibitor was present after 100 min of perfusion. Quantification for three independent experiments was performed densitometrically.

proteolytic activity of glutamine/glycine in livers from fed animals in the presence of colchicine is due to the significant formation of lysosomotropic ammonia, which is insensitive to colchicine [9]. Colchicine (5 μM) was without effect on proteolysis inhibition when livers from rats fasted for 24 h were perfused with a mixture of amino acids representing 5-fold the concentration usually found in the portal vein (Figure 3). Under these artificial conditions, proteolysis was inhibited by $74 \pm 4\,\%$ ($n = 3$) and the results show that in the presence of non-physiologically high amino acid concentrations, swelling-independent anti-proteolytic mechanisms, known for phenylalanine and asparagine [11] and the formation of lysosomotropic ammonia from amino acids might overcome swelling-dependent mechanisms of proteolysis regulation. The results show further that colchicine does not lead to a stimulation of proteolysis from non-lysosomal sources. Nevertheless, the fractional volume of autophagic vacuoles does not necessarily reflect the actual proteolytic activity in liver and it is not exactly known how the half-lives of autophagic vacuoles are modulated by cell hydration, hormones and amino acids. However, as shown in Table 3, an increase in cell volume was accompanied by a qualitatively parallel decrease in V_{av}/V_c.

The results in this paper show that the modulation of autophagosome formation is under the control of cell hydration and involves the activation of p38^{MAPK} upstream of the putative microtubule-dependent signal transduction element. This hypothesis is demonstrated by (1) the rapid modulation of V_{av}/V_c by aniso-osmolarity, insulin and amino acids, (2) the sensitivity to colchicine of both the swelling-dependent regulation of autophagic sequestration and the anti-proteolytic action of cell swelling by aniso-osmotic conditions, hormones or amino acids, as shown previously [9,30], (3) the sensitivity of autophagic vacuole formation to inhibition of p38^{MAPK} activation by SB 203580, (4) the insensitivity of cell swelling and the consequent swelling-induced p38^{MAPK} activation to inhibition by colchicine and (5) the lack of toxic effects of p38^{MAPK} inhibition on microtubular structures. The results suggest that the swelling signal in liver is transmitted to the sequestration site via the activation of p38^{MAPK} and that the colchicine-sensitive regulation element is probably located between p38^{MAPK} and the autophagosomes (Scheme 1).

The control values found in the absence of amino acids and insulin agree with the results found by Schworer et al. [31], who described a fractional autophagosome volume in rat liver of 58.4×10^{-4}. During amino acid deprivation, macroautophagy accounts for a total of 60–75 % of total protein degradation in perfused rat liver, as shown in Figure 3 and described by others [3,32]. In the absence of insulin and amino acids, autophagosome formation is already maximally stimulated [31] and autophagy-derived proteolysis cannot be increased further by exposure to glucagon or hypertonic conditions [26]. However, a hyperosmotic increase of autophagosome formation is observed when proteolysis is preinhibited by insulin.

Colchicine led to an approx. 15 % decrease in basal fractional autophagic vacuole volume in liver (Table 1) and a decrease in proteolysis by approx. 10 % (Table 3) [33]. This is in accord with the results of Seglen et al. [34], who described little effect of vinblastine on autophagic sequestration in rat liver. The concentration of colchicine was sufficient to destroy microtubules in liver completely [35] and was orders of magnitude lower than the concentrations needed to exert a strong anti-proteolytic effect in isolated hepatocytes [36]. In the perfused rat liver, proteolysis was inhibited by cell swelling in response to hypo-osmolarity and the anti-proteolytic action of cell swelling was largely blunted by the destruction of microtubules with colchicine, vinblastin or taxol [30,33] but not by the analogue γ-lumicolchicine, which has no effect on microtubules. It can therefore be concluded that the microtubule-dependent inhibition of autophagosome formation ('sequestration step') is involved in the swelling-dependent inhibition of proteolysis in liver.

The signalling cascades involved in autophagosome formation are poorly understood. Protein phosphorylation has an important role in hepatic autophagy [11,37]; an interesting observation was that the inhibitory effect of okadaic acid on autophagy in isolated hepatocytes could be ascribed to modulation of the cytokeratin cytoskeleton [38]. Work by Blommaart et al. [29,39] showed an inhibitory role of S 6 phosphorylation and a requirement for phosphoinositide 3-kinase for autophagy in liver. Nevertheless, the inhibitory action of hypo-osmotic conditions on proteolysis in the perfused rat liver was sensitive neither to inhibition by LY 294002 or wortmannin, i.e. inhibitors of phosphoinositide 3-kinase, nor to inhibition by rapamycin [11], an inhibitor of p70 S 6 kinase, indicating that other pathways are involved in the hydration-dependent control of proteolysis. Recent findings from perfused rat liver demonstrated a sensitivity of the anti-proteolytic action of cell swelling, insulin and glutamine/glycine to inhibitors of p38^{MAPK} activation [11]. The activation of p38^{MAPK} upon hypo-osmotic cell swelling is insensitive to colchicine (Figure 1), as is the increase in cell hydration under aniso-osmotic conditions with insulin or glut-

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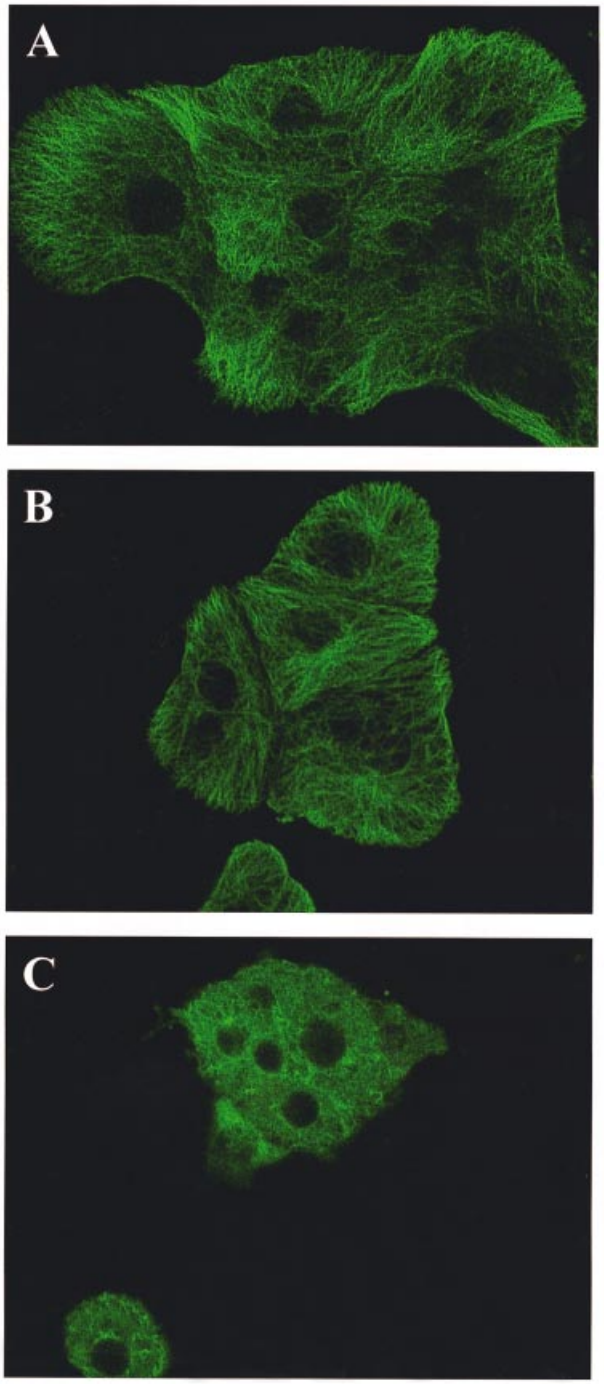

Figure 2 p38^{MAPK}-independent microtubular structures in cultivated rat hepatocytes as revealed by confocal laser microscopy

Morphological analysis of the distribution of α-tubulin in 48-h-cultivated rat hepatocytes was performed by confocal laser microscopy of cultivated hepatocytes incubated with mouse-derived anti-(α-tubulin). (**A**) Untreated control cells (305 m-osmol/l) showed a radial distribution pattern of α-tubulin. (**B**) When hepatocytes were incubated for 30 min with the p38^{MAPK} inhibitor SB 203580 (250 nM), the cells showed the same meshwork-like distribution pattern of α-tubulin as in control experiments without the inhibitor. (**C**) After incubation with colchicine (5 μM) for 30 min, a pronounced disassembly of α-tubulin was observed. Representative results for a series of four experiments for each condition are shown.

amine/glycine (Table 3). The phosphorylation of p38^{MAPK} therefore seems to have a key role in the signalling cascade induced by swelling conditions in the liver.

Table 3 Fractional autophagic vacuole volumes and cell hydration in perfused rat liver under normotonic and aniso-osmotic conditions, with insulin or amino acids in the presence or absence of colchicine

Livers from fed male Wistar rats were perfused for 160 min. At that time point, livers were fixed with glutaraldehyde and further processed for transmission electron microscopy. Hypotonic and hypertonic perfusion conditions respectively were installed for 30 min. If present, colchicine (5 μM) was infused 30 min before the installation of hypotonic perfusion conditions or the infusion of insulin or glutamine/glycine. Fractional autophagic vacuole volumes (V_{av}/V_c), cell hydration and proteolysis were determined in separate perfusion experiments. In control experiments, V_{av}/V_c was $(51.8 \pm 1.9) \times 10^{-4}$ ($n = 7$). The intracellular water space under control conditions was 591 ± 23 μl/g of liver weight ($n = 16$). Colchicine led to a decrease in V_{av}/V_c of 16.0 ± 8.1% ($n = 3$) and an inhibition of proteolysis of 10.1 ± 2.4% ($n = 3$). No detectable changes in cell hydration were observed with colchicine alone. For experimental details see the Materials and methods section. In parentheses the number of different perfusion experiments is given. $^{***}P < 0.001$, $^{**}P < 0.01$ compared with control; n.s., not significant.

Condition	Colchicine	Change V_{av}/V_c (%)	Change cell hydration (%)
Hypotonic (−120 mosmol/l)	Absent	$-45.6 \pm 6.8^{***}$ (5)	$+21.4 \pm 1.2^{***}$ (5)
Hypotonic (−120 mosmol/l)	Present	-10.8 ± 9.7n.s. (3)	$+20.3 \pm 3.2^{***}$ (3)
Insulin (35 nM)	Absent	$-75.1 \pm 1.5^{***}$ (3)	$+12.2 \pm 1.4^{***}$ (13)
Insulin (35 nM)	Present	-25.2 ± 9.4n.s. (3)	$+12.6 \pm 0.8^{***}$ (6)
Glutamine/glycine (2 mM)	Absent	$-68.0 \pm 2.7^{***}$ (3)	$+10.1 \pm 1.4^{***}$ (6)
Glutamine/glycine (2 mM)	Present	-23.7 ± 7.8n.s. (3)	$+11.3 \pm 2.4^{**}$ (3)

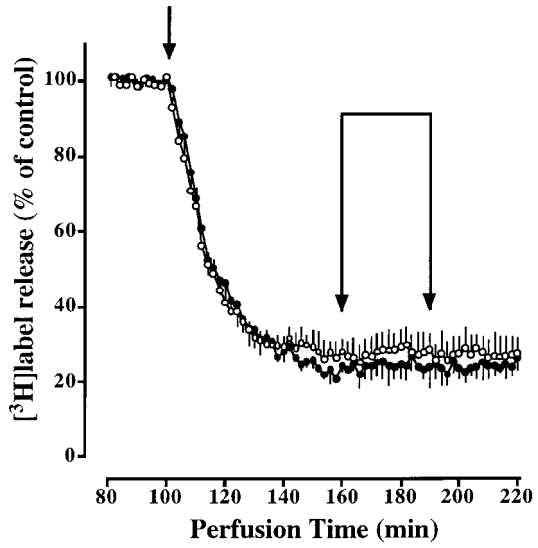

Figure 3 Lack of effect of colchicine on inhibition of autophagic proteolysis by an amino acid mixture at 5-fold physiological portal amino acid concentrations

Livers from 24-h-fasted rats were perfused with a 5-fold concentrated physiological amino acid mixture from 100 to 220 min of perfusion time (●; start of addition of amino acids indicated by the single arrow). From 160 to 190 min (paired arrows), 5 μM colchicine was present (○). Results are from three perfusion experiments and are shown as means ± S.E.M.

In summary, we show here that the modulation of autophagosomes by cell swelling is sensitive to SB 203580 and involves a microtubule-dependent signal element downstream of p38^{MAPK} activation. Potential microtubule-associated protein candidates could be tau [40,41] or protein kinases such as the MAP kinase signal integrating protein kinase MNK-1 [42].

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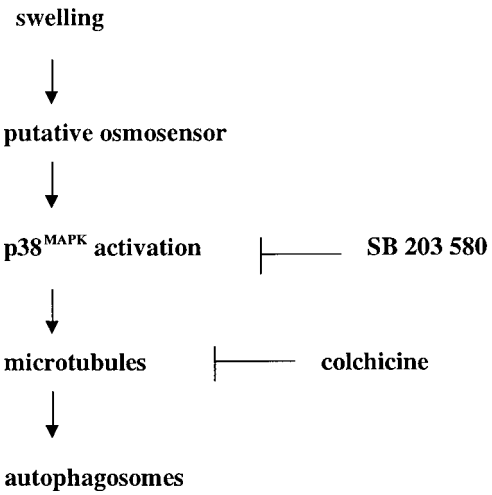

Scheme 1 Hypothetical regulation steps in the swelling-dependent modulation of autophagic proteolysis in perfused rat liver

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