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Urea synthesis in freshly isolated and in cultured periportal and perivenous hepatocytes

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Periportal hepatocytes isolated by digitonin/collagenase perfusion produced urea faster than did similarly prepared perivenous hepatocytes, in both the presence and the absence of amino acids and various urea precursors. There was no difference between the two cell types in rates of intracellular proteolysis. The initial difference in urea synthesis persisted for 5 days during primary culture, but then gradually disappeared. Our results demonstrate that the periportal dominance of urea formation is unrelated to the currently existing acinar microenvironment in the intact liver, but probably reflects differences in acinar key enzyme activities only slowly converging during culture.

INTRODUCTION

The predominantly periportal location of amino acid catabolism and ammonia production in the acinus, the microcirculatory unit of the liver, is indicated by the distribution of activities of alanine aminotransferase, tyrosine aminotransferase and glutaminase (Welsh, 1972; Jungermann & Katz, 1982; Väänänen *et al.*, 1983; Sies & Häussinger, 1984; Quistorff *et al.*, 1985). Nitrogen from amino acids and ammonia serves as substrate for urea synthesis in the periportal zone, which also contains higher activities of urea-cycle key enzymes, such as ornithine carbamoyltransferase and carbamoyl-phosphate synthase (Mizutani, 1968; Gaasbeek Janzen *et al.*, 1981). Ammonia not used in the periportal zone for urea synthesis is effectively utilized for glutamine synthesis in the perivenous zone (Häussinger, 1983). This concept is supported by immunohistochemical evidence for the location of glutamine synthetase within a narrow zone around the terminal hepatic venules (Gebhardt & Mecke, 1983).

The existing evidence for heterogeneous nitrogen metabolism is based on enzyme-distribution studies (Jungermann & Katz, 1982) and perfusion experiments (Häussinger, 1983; Gebhardt & Mecke, 1984). Techniques developed fairly recently for separation of intact periportal or perivenous hepatocytes (Bengtsson *et al.*, 1981; Väänänen *et al.*, 1983; Lindros & Penttilä, 1985; Quistorff, 1985) now enable direct measurement of metabolic rates. In the present study we have used the new high-yield digitonin/collagenase technique, in which either periportal or perivenous cells are first destroyed with digitonin and the intact cells of the opposite zone are then isolated with conventional collagenase perfusion. Marked separation of the cell populations is demonstrated by the differences in marker enzyme activities. We observed heterogeneity in the rates of urea and ammonia production between freshly isolated periportal and perivenous hepatocytes in suspension. The persistence of these initial differences was followed during the culture of the hepatocytes for up to 9 days.

MATERIALS AND METHODS

Animals

Liver donors were 6–10-week-old male rats of the Alko mixed strain, which were housed five or six in each cage and fed on food pellets (R3; Ewos, Södertälje, Sweden) and tap water *ad libitum*.

Isolation of hepatocytes

Periportal or perivenous hepatocytes were isolated by the new digitonin/collagenase perfusion technique (Lindros & Penttilä, 1985). In brief, rats were anaesthetized (pentobarbital, 60 mg/kg intraperitoneally) and the livers were pre-perfused at 37 °C *in situ* for about 8 min with Hepes buffer, pH 7.4, including 1.1 mM-Ca^{2+}, at a rate of 40 ml/min. Then 7 mM-digitonin (BDH Chemicals, Poole, Dorset, U.K.) in the same buffer was infused for 20–50 s through either the portal or the venous cannula at 10 ml/min to destroy the periportal or perivenous acinar zone, respectively. Digitonin was washed out by perfusing the liver via the opposite cannula for 10 min with Ca^{2+}-free buffer (Seglen, 1976). Finally, intact hepatocytes from the digitonin-unaffected zone were liberated after conventional collagenase perfusion (Berry & Friend, 1969), washed, and suspended in Krebs–Henseleit (1932) bicarbonate buffer containing 4% (w/v) bovine serum albumin (Boehringer, Mannheim, Germany) and 10 mM-glucose to give a final cell concentration of 60–70 mg/ml.

For primary culture, hepatocytes were isolated aseptically and suspended in phosphate-free Hepes suspension buffer, pH 7.6 (Seglen, 1976). The buffer was supplemented with 2 mM-pyruvate, 50 μg of amphotericin B (Fungizone; Gibco, Paisley, Scotland, U.K.)/ml and 10 μg of gentamycin/ml.

Incubation of hepatocytes

Hepatocytes (total volume 0.4 ml) were incubated under O_2/CO_2 (19:1) for 120 min at 37 °C in a shaking water bath in 20 ml rubber-stoppered glass vials. When

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indicated, a stock solution of 20 amino acids was added to give final concentrations of 10 times normal plasma values (Schworer *et al.*, 1981). The capacity for urea synthesis was measured in incubations where saturating concentrations of ammonia, lactate and ornithine were used (Meijer *et al.*, 1985). Incubations were terminated with 0.4 ml of ice-cold 0.6 M-$HClO_4$, and the neutralized supernatant was used for determination of ammonia and urea.

Intracellular protein degradation

Hepatic proteins were prelabelled with L-[U-^{14}C]valine (sp. radioactivity 280 μCi/mmol; Amersham International, Amersham, Bucks., U.K.) as described by Woodside & Mortimore (1972). Two injections were given, 18 and 4 h before the cells were isolated; the total dose of [^{14}C]valine was 50 μCi/rat (Grinde & Seglen, 1981). The rate of protein degradation was measured as described by Seglen (1978) and Grinde & Seglen (1981) in Krebs–Henseleit bicarbonate buffer. The rates of proteolysis were measured from 60 to 120 min to avoid interference by short-lived protein degradation (Woodside & Mortimore, 1972). The samples were treated and the radioactivity was measured as described by Seglen (1978).

Hepatocyte primary culture

Cells were inoculated on collagen-gel-coated culture dishes (8.5 cm diameter; Nunc) at a density of 0.6×10^6 cells/ml (6.5 ml per dish). The medium was a 1:1 mixture of Waymouth MB 752/1 and Ham F-12 supplemented with 5% (v/v) fetal-calf serum, 5% (v/v) newborn-calf serum (Gibco), 20 mM-Hepes, 5 mM-$NaHCO_3$, 10 mM-Tricine, 10 units of insulin/l, 1 μM-dexamethasone, 50 μg of glucagon/ml and 10 μg of gentamycin (Sigma, St. Louis, MO, U.S.A.)/ml. The medium was changed at 4 h and then every 24 h. Urea was analysed in the medium, and DNA in the cells.

Analytical methods

Activities of periportal and perivenous marker enzymes were assayed as cited previously (Lindros & Penttilä, 1985), and DNA was measured as described by Richards (1974). Urea and ammonia were assayed by standard enzymic methods (Gutmann & Bergmeyer, 1974; Kun & Kearney, 1974), with correction for protein-bound ammonia (Wanders *et al.*, 1980).

RESULTS

Separation of periportal and perivenous hepatocytes

The validity of the digitonin/collagenase method has been discussed in detail elsewhere (Lindros & Penttilä, 1985; Quistorff, 1985). In the present study the mean periportal/perivenous ratios for the periportal markers alanine aminotransferase and γ-glutamyltransferase were 1.71 and 3.46 respectively, and 0.77 and 0.79 respectively, for the perivenous markers glutamate dehydrogenase and pyruvate kinase. These ratios are similar to those previously observed (Lindros & Penttilä, 1985) and demonstrate reasonably good separation.

Protein degradation in periportal and perivenous hepatocytes

The physiological sources of nitrogen for urea synthesis are ammonia from portal blood and amino acids derived either from portal blood or via intracellular proteolysis. In non-saturating conditions heterogeneity in the zonal distribution of intracellular proteolysis would influence the microenvironment and thus the rate of urea formation. Therefore we measured the rate of intracellular protein degradation in freshly isolated periportal and perivenous hepatocytes. In the absence of amino acids both periportal and perivenous cells degraded proteins at high but similar rates (Table 1). Excess amino acids, which are the primary physiological inhibitors of hepatic lysosomal proteolysis, reduced the release of valine by 45 and 46% in periportal and perivenous hepatocytes respectively. Our observation that the rate of proteolysis was only slightly higher in periportal than in perivenous hepatocytes is consistent with a previous study (Smith-Kielland *et al.*, 1982) and with data indicating even acinar distribution of lysosomal components (Schworer *et al.*, 1981).

Table 1. Protein degradation in freshly isolated periportal and perivenous hepatocytes

Periportal and perivenous hepatocytes were isolated from livers prelabelled with [^{14}C]valine and incubated at 37 °C for 2 h in Krebs–Henseleit bicarbonate buffer supplemented with 4% bovine serum albumin, 10 mM-glucose and 15 mM-valine. Protein degradation was measured as the net release of radioactive valine and is expressed as a percentage of the initial protein-incorporated radioactivity. Results are means ± S.E.M. ($n = 7$).

Amino acid supplementation (10 × plasma concn.)	Protein degradation (%/h)		
	Periportal cells	Perivenous cells	Periportal/perivenous ratio
−	5.62 ± 0.18	5.35 ± 0.46	1.05
+	3.08 ± 0.34	2.89 ± 0.27	1.07

Urea synthesis in freshly isolated cells

In the absence of any added substrates, when the proteolysis-derived amino acids are the only source of nitrogen, the rate of urea synthesis, although low in both cell types, was 3 times as high in periportal as in perivenous cells (Table 2). The calculated average rate of urea synthesis, 18.3 μmol/h per g of cells, was similar to that reported for conventionally isolated heterogeneous hepatocytes (Seglen, 1977; Wojtczak *et al.*, 1978; Meijer *et al.*, 1985). In the presence of amino acids (10 × normal plasma concentrations) the rate of urea synthesis was stimulated 4.5-fold in periportal hepatocytes and 6-fold in perivenous cells, but the periportal − perivenous difference was maintained.

These results may reflect the higher capacity of the periportal hepatocytes not only to synthesize urea, but also to degrade amino acids. Indeed, periportal hepatocytes have been reported to contain higher activities of alanine aminotransferase, glutaminase and tyrosine aminotransferase (Welsh, 1972; Jungermann & Katz, 1982; Väänänen *et al.*, 1983; Sies & Häussinger, 1984; Quistorff *et al.*, 1985). Therefore, urea synthesis was measured also in the presence of various precursors (Table 3). Because the capacity of the urea cycle may be overestimated in the presence of arginine (Hensgens &

Table 2. Urea and ammonia accumulation by freshly isolated periportal and perivenous hepatocytes

Periportal and perivenous hepatocytes were incubated as described in the legend to Table 1, in the absence or presence of a physiological mixture of 20 amino acids at 10 times normal plasma concentration, except that valine was omitted. Results are means ± S.E.M. ($n = 6$): ***$P < 0.001$ and **$P < 0.01$ for the significance of the difference between periportal and perivenous cells.

	Amino acid supplementation (10 × plasma concn.)	Accumulation (μmol/h per g of cells)		
		Periportal cells	Perivenous cells	Periportal/perivenous ratio
Urea	−	27.2 ± 3.3	9.4 ± 1.1***	2.99
	+	121.1 ± 12.3	60.9 ± 4.4***	2.10
Ammonia	−	1.08 ± 0.15	< 0.2	—
	+	2.36 ± 0.23	1.46 ± 0.15**	1.66

Table 3. Effect of precursor supplementation on urea synthesis

Periportal and perivenous hepatocytes were incubated as in Table 2 in the presence of all 20 amino acids (10 × aa) except arginine; other supplementations are given in the Table. Results are means ± S.E.M. ($n = 3$): *$P < 0.05$ and **$P < 0.01$ for the significance of the difference between the periportal and perivenous cells.

Incubation conditions	Urea synthesis (μmol/h per g of cells)		
	Periportal cells	Perivenous cells	Periportal/perivenous ratio
10 × aa − arginine	43.6 ± 6.0	19.1 ± 1.6*	2.28
10 × aa − arginine + 3 mM-ornithine	54.6 ± 5.0	25.8 ± 2.0**	2.12
10 × aa − arginine + 2 mM-ornithine + 10 mM-lactate + 20 mM-NH_4Cl	53.8 ± 7.0	23.5 ± 1.3*	2.29
0 × aa + 2 mM-ornithine + 10 mM-lactate + 20 mM-NH_4Cl	88.7 ± 6.7	66.8 ± 4.9	1.32
0 × aa + 1.76 mM-arginine	121.8 ± 6.8	110.9 ± 11.2	1.10

Meijer, 1979), the effect of adding arginine alone was tested (Table 3). Both periportal and perivenous hepatocytes produced urea at high but similar rates, suggesting a rather homogeneous distribution of arginase. On the other hand, in the absence of arginine, or when it was replaced with ornithine, the difference in the rate of urea synthesis between the periportal and perivenous hepatocytes was maintained. Also, in the presence of saturating concentrations of ammonia (20 mM), lactate (10 mM) and ornithine (3 mM) periportal cells produced more urea (Table 3). Under these conditions removal of amino acids stimulated urea production. However, the absolute difference between the periportal and perivenous cells persisted, although the difference was not statistically quite significant ($P < 0.06$). These results demonstrate that the periportal cells have higher capacity to synthesize urea, although at non-saturating concentrations of ammonia faster catabolism of amino acids may contribute.

Ammonia metabolism

In the absence of exogenously added amino acids, no detectable amount of ammonia was found in the incubation medium of the perivenous hepatocytes (Table 1). In contrast, ammonia was slowly produced by periportal cells, which is in accordance with previous liver-perfusion studies (Häussinger, 1983), showing active glutaminase in the periportal area. To our knowledge the zonal distribution of other ammonia-producing enzymes in the liver is not known. In the presence of amino acids both cell types accumulated ammonia, the rate for the periportal cells still being twice that of the perivenous cells.

Primary culture of periportal and perivenous hepatocytes

During early culture both types of cells produced urea at high rates (Fig. 1), indicating high catabolic activity of freshly isolated hepatocytes (Seglen, 1977; Tanaka *et al.*, 1978). The initial rates of urea synthesis during culture were similar to those of freshly isolated cells in the presence of excess amino acids. This was expected, since the content of amino acids in the culture medium allows maximal rates of urea synthesis (see the Materials and methods section). The significantly higher rate of urea formation in periportal hepatocytes than in perivenous hepatocytes was maintained during culture. The dif-

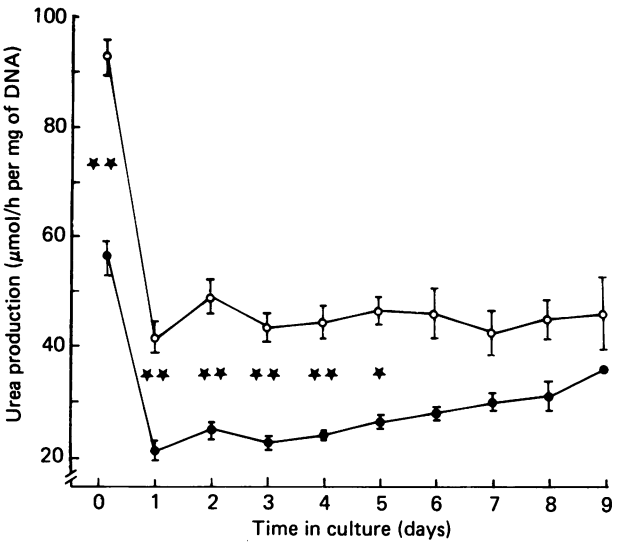

Fig. 1. Changes in the rate of urea production by cultured periportal and perivenous hepatocytes

Cells were cultured from 4 h to 9 days and the concentration of urea was measured in the culture medium, which was changed at 4 h and then every 24 h. The numbers of cell preparations were four and three for periportal and perivenous cells respectively. Results are means ± S.E.M.: $^{**}P < 0.01$ and $^{*}P < 0.05$ for the difference between periportal and perivenous cell populations.

ference persisted for 5 days, but gradually disappeared during prolonged culture. This disappearance was due to increased urea synthesis by perivenous cells, whereas that of periportal cells did not change. The linear correlation of urea synthesis rate with time during days 1–9 was $r = 0.127$ (not significant) for periportal cells and $r = 0.936$ ($P < 0.001$) for the perivenous hepatocytes.

DISCUSSION

In this study we demonstrate that in both the presence and the absence of exogeneous amino acids hepatocytes enriched in periportal cells are able to synthesize urea 2–3 times as fast as cells from the perivenous region. Since the rates of lysosomal proteolysis in periportal and perivenous hepatocytes are similar (Table 1), the difference seems to be unrelated to the source of amino acids. Furthermore, because the cell incubation conditions were identical, the periportal dominance of urea synthesis seen in liver-perfusion studies (Häussinger, 1983) cannot be due to substrate or oxygen gradients, as suggested by Kari & Thurman (1985). Rather, our results agree with the observation on the acinar distribution of carbamoyl-phosphate synthase (Gaasbeek Janzen *et al.*, 1981) and of enzymes involved in amino acid catabolism (Welsh, 1972; Jungermann & Katz, 1982; Väänänen *et al.*, 1983; Sies & Häussinger, 1984; Quistorff *et al.*, 1985).

Quistorff (1985) observed a periportal dominance of gluconeogenesis of the same magnitude as that of urea synthesis in our study, thus indicating a tight coupling between these two functions (Meijer *et al.*, 1985) also in subpopulations of hepatocytes. Although many reactions may contribute to the observed accumulation of ammonia into the incubation medium of hepatocytes (Povers & Meister, 1982), its periportal dominance can be explained by the hypothesis of the intercellular glutamine cycle (Häussinger, 1983). According to this model, ammonia is produced in the periportal zone by glutaminase, which is more active in this zone (Sies & Häussinger, 1984). Utilization of ammonia in the periportal zone is limited by the high K_m of carbamoyl-phosphate synthase (Lusty, 1978). Ammonia is, however, effectively removed by glutamine synthetase, which is located in the perivenous acinar zone (Gebhardt & Mecke, 1983; K. O. Lindros & K. E. Penttilä, unpublished work) and has a low K_m for ammonia (Deuel *et al.*, 1978). Thus both the absence of glutaminase and the presence of glutamine synthetase can explain the lower accumulation of ammonia in the perivenous hepatocytes.

The fast rate of urea production by both cell types during initial culture is probably determined by high rates of intracellular proteolysis and subsequent oxidation of amino acids, rather than by any initial changes in urea-cycle enzyme activities, which, according to Lin & Snodgrass (1975), only slowly decrease during the first 5 days of culture. These results are consistent with the notion that under physiological conditions, when amino acids provide nitrogen for urea synthesis, key enzymes exert only minor control over the urea synthesis (Meijer *et al.*, 1985).

The persistent capacity of the periportal cells to produce urea faster than the perivenous cells during culture demonstrates for the first time that periportal–perivenous difference in a metabolic pathway, appearing initially as a consequence of different circulatory microenvironments, can be maintained during culture of the cell populations under identical conditions. Thus our results suggest that the periportal–perivenous differences, at least for the urea-cycle pathway, are due to differences in enzyme activities rather than supply of substrates. Although the activities of the urea-cycle enzymes were not measured, the persistence of the periportal – perivenous difference in urea synthesis is probably explained by their relatively slow turnover (Gumucio & Miller, 1982). The slow disappearance of the difference also suggests regulation at the level of gene expression, the gradual increase in urea synthesis by the perivenous cells implying enhanced synthesis of the rate-limiting enzymes in these cells. Finally, our results indicate that the acinar distribution of urea synthesis *in vivo* is not directly governed by acute changes in acinar substrate or oxygen availability.

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