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Plasma membrane-bound carbonic anhydrase activity in the regenerating rat liver

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Part of the carbonic anhydrase activity of hepatocytes has been reported to be located in the plasma membrane. This strategic location suggests a physiological role other than that located within the cell and probably related to the specific secretory function of these cells. Furthermore, after two-thirds hepatectomy an enzymatic retrodifferentiation has been reported. We reasoned that liver regeneration probably affects the carbonic anhydrase activity in different ways depending upon its location and hence presumably physiological role. We measured, therefore, carbonic anhydrase activity in a soluble fraction or in a plasma membrane-enriched fraction obtained from liver homogenate from rats undergoing hepatectomy (two-thirds) one, three or seven days before liver resection and homogenation. No changes in carbonic anhydrase activity were found as far as soluble fraction was concerned. However, the carbonic anhydrase activity in plasma membrane was reduced (by 55%) soon after hepatectomy, there after it increased, returning to near control value at seven days. Lactate dehydrogenase activities in soluble and plasma membrane fractions were not modified by the regenerative process. Neither was 5′-nucleotidase activity determined in plasma membrane affected by liver regeneration. In summary, these results indicate a higher sensitivity of plasma membrane carbonic anhydrase activity to the regenerative process than soluble carbonic anhydrase activity. This suggests a different control of the turnover of these isoenzymes during rat liver regeneration. The phenomenon is consistent with a different physiological role for these activities; i.e., one (plasma membrane-bound carbonic anhydrase activity) may be involved in specific functions of differentiated hepatocytes, and another (soluble carbonic anhydrase activity) may be involved in general functions shared by both differentiated and undifferentiated cells.

Introduction

In a previous study [1] we have offered evidence for the presence of carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1) in the plasma membrane of rat hepatocytes. This isoenzyme (tentatively termed CA IV) accounts for only a low fraction of total hepatic carbonic anhydrase activity (probably less than 15%) in contrast to the high activity due to the soluble isoenzymes (CA II and CA III). However, the strategic location of CA IV suggests that this isoenzyme may play an important role, hitherto unknown, in cellular functions specific to mature hepatocytes such as the secretory function, similarly to what has been described for other bicarbonate transporting epithelia [2].

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It has been suggested that during regeneration in the rat liver following two-thirds hepatectomy, a step-wise retrodifferentiation occurs. The enzymic changes are later reverted because the regenerating liver retains the capacity to undergo retrodifferentiation towards a normal adult biochemical pattern. We reasoned that if soluble and membrane-bound carbonic anhydrase isoenzymes are involved in different cellular functions they probably undergo dissimilar post-hepatectomy retrodifferentiation. The aim of the present study was to test this hypothesis. Carbonic anhydrase activities were determined in soluble and plasma membrane-enriched fractions obtained from homogenate liver, together with a soluble enzyme, namely lactate dehydrogenase (EC 1.1.1.27) and a membrane-bound enzyme, namely 5′-nucleotidase (EC 3.1.5.1). These activities were monitored at the begining, at the midtime and at the end of the regeneration period in the two-thirds hepatectomized rat.

Materials and Methods

Animals

Non-fasting male Wistar CF rats (approx. 250 g) (Faculty of Pharmacy, Salamanca, Spain) were used. Two-thirds hepatectomy was carried out at 9.00 a.m. under ether anaesthesia as previously reported [3]. At different times after hepatectomy (1, 3 or 7 days) the rats were anaesthetised by i.p. administration (5 mg per 100 g body weight) of sodium pentobarbital (Claudio Barcia, Madrid, Spain). After an injection of 0.5 ml 0.15 M NaCl containing heparin (250 I.U.) into the penis vein, the portal vein was cannulated. The liver was perfused with cold (4°C) 0.15 M NaCl solution until the lobes appeared free of blood and the perfusate was colorless. The liver was then removed, thoroughly washed in cold NaCl solution, weighed (data given as liver wet weight) and used immediately. The total liver wet weight before undergoing hepatectomy was calculated from the weight of resected liver tissue, which was considered as 63% of total liver wet weight [3].

Experimental procedure

Three small pieces (0.1–0.2 g) of liver were obtained and dried for 2–3 weeks at 60°C until their weight remained constant; the average result of the percentage of water content was used to calculate the liver dry weight.

Two or three livers were combined to obtain liver homogenate as previously reported [1]. The homogenate was used to obtain the soluble and plasma membrane-enriched fractions. In brief, after the homogenate had been filtered through two layers of gauze, part of the filtered homogenate was then centrifuged at $65000 \times g$ for 60 min. The pellet was discarded and the supernatant was used as the soluble fraction (S). The rest of the homogenate was used for plasma membrane isolation. In order to obtain the plasma membrane preparation, filtered homogenate was centrifuged at $500 \times g$ for 30 min using a Beckman L8-H centrifuge (Beckman Instruments, Madrid, Spain). The pellet was discarded and the supernatant was spun at $27500 \times g$ for 30 min, resulting in a pellet with a fluffy outer layer, which was collected, and a dark inner core, which was discarded together with the supernatant. 20 ml of buffer (50 mM Hepes-KOH (pH 7.4)/0.25 M sucrose) were added to the fraction collected, before carrying out two updown strokes of the motor-driven Teflon glass homogenizer. (Hepes was purchased from Boehringer, Mannheim, F.R.G.; sucrose was obtained from Merck, Darsmstad, F.R.G.). After making up to 40 ml with additional buffer, 10 ml of Percoll (Pharmacia Iberica, Barcelona, Spain) stock solutions (Percoll/2.5 M sucrose, 9:1, v/v) were added. Before centrifugation at $30900 \times g$ for 30 min, pH was adjusted to 7.4 with 50 mM KOH and the mixture was stirred for 5 min on ice. The resulting pellet and supernatant were discarded and the easily visible band containing plasma membranes was carefully removed and passed through a 23-gauge hypodermic needle three times before making up to 5 ml with the previously described buffer. The resulting solution was mixed and vigorously stirred (as described above) with 12 ml of Percoll stock solution/buffer (75% v/v). The mixture was transferred to the bottom of a discontinuous (0, 10, 18, 25 and 30% v/v) Percoll stock solution/buffer density gradient, prepared as described by Epping and Bygrave [4]. Loaded gradients were centrifuged at $48200 \times g$ for 2 min, excluding acceleration time. Milk-colored cellular material appeared distributed within four bands (F_1, F_2, F_3 and F_4, from top to bottom). Plasma membranes were included in fraction F_1 at the buffer/10% Percoll interphase. This fraction was collected and diluted 1:5 with 1 mM $NaHCO_3$. The mixture was then centrifuged at $48200 \times g$ for 30 min. The fluffy membrane pellet was easily washed free of the hard, glassy Percoll pellet adhering to the tube. The membranes were passed through a 23-gauge hypodermic needle three times before making up to 15 ml with additional buffer. They were incubated for 15 min on ice and then spun at $10000 \times g$ for 10 min. The pellet was similarly washed once more using buffer. The resulting pellet was resuspended in 2 ml of buffer using a hypodermic needle as described above.

Analytical techniques

The plasma membrane marker enzyme 5'-nucleotidase was measured by the method of Bertrand and Buret [5]. The cytosolic marker enzyme lactate dehydrogenase was determined by the method of Vassault [6]. The results of enzyme activity for the above-cited enzymes are given as units/mg protein. One unit is defined as the activity able to convert 1 μmol substrate per min at 30°C. Carbonic anhydrase activity was assayed by a modification of the electrometric method of Wilbur and Anderson [7], in which the time required (in seconds) for a saturated CO_2 solution to lower the pH of 0.02 M Tris-HCl buffer from 8.3 to 6.3 is determined. Enzyme units for carbonic anhydrase activity are defined as 2 × (uncatalyzed time-catalyzed time)/catalyzed time, at 4°C. The method was qualitatively validated by inhibition with acetazolamide (Edemox, Wasserman, Barcelona, Spain) and quantitatively using dialyzed and lyophilized carbonic anhydrase from bovine erythrocytes (Sigma, St Louis, MO). Carbonic anhydrase activity was determined on the day of liver fractionation. All other enzymatic assays were carried out after overnight storage at 4°C. Protein concentrations were measured by the method of Lowry as modified by Markwell et al. [8] using bovine serum albumin (Sigma) as standard. After extraction of lipid from the plasma membrane fraction by the method of Folch et al. [9], phospholipids and cholesterol were

determined. Phospholipids were measured by the method of Yee [10] and cholesterol was determined according to three coupled enzymatic reactions catalysed by cholesterol esterase, cholesterol oxidase and peroxidase, respectively [11].

Results are expressed as means ± S.E. A paired *t*-test was used for calculating the statistical significance between liver fractions. The Bonferroni method of multiple range testing was used to compare experimental groups.

Results and Discussion

After two-thirds hepatectomy in the rat the remaining liver tissue undergoes a regenerative process. Fig. 1 summarizes the time course of recovery of liver weight in our experiments. Three days after liver resection the liver weight increased from the remaining 27% to 44%. At seven days post-hepatectomy liver weight was already close (93%) to the prehepatectomy weight. These data are in agreement with those obtained previously by us [3,12] and others [13]. This growth is known to be due to the compensatory hyperplasia undergone by the liver (for review, see Alison (1986) [14]).

Total protein content changes as liver weight does (Table I). However, during this period no significant changes in the content of protein were observed when expressed per gram of dry tissue (Table I). Table I shows that the amount of protein per ml of soluble or plasma membrane-enriched fraction was not significantly modified during the regenerative process. Although the liver content in proteins was quantitatively very similar, profound qualitative modifications were expected to occur during the regenerating process. These alterations in the normal adult protein pattern are presumably responsible for changes in the hepatic function following partial hepatectomy in the rat.

During hepatic regeneration some of the specific liver functions are modified. Namely, bile formation has been reported to be profoundly altered [3,12]. Shortly after hepatectomy the ability of ursodeoxycholate infusion to induce a bicarbonate-rich hypercholeresis is

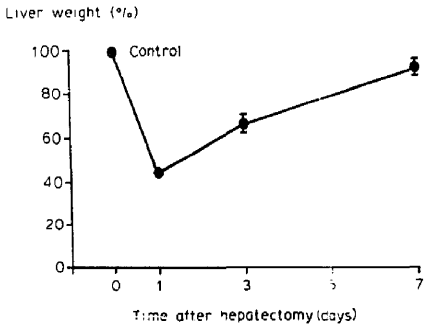

Fig. 1. Time-course of liver wet weight recovery after two-thirds hepatectomy. Values are expressed as percent of total liver weight before hepatectomy, which was calculated from the weight of the resected liver tissue (63% of total liver). Data are means ± S.E. The number of experiments for control and hepatectomized rats 1, 3 or 7 days were 7, 7, 6 and 7, respectively.

dramatically reduced in the rat [3]. By contrast, the choleretic ability of other bile acids, which do not strongly stimulate bicarbonate secretion into bile is not affected by partial hepatectomy. The change in ursodeoxycholate-induced bicarbonate output, and hence hypercholeresis [15], might be due in part to modifications in the conjugation pattern of this bile acid, although other possibilities cannot be ruled out. Among them, changes in carbonic anhydrase activity, which has been reported to play a role in ursodeoxycholate-induced bicarbonate secretion [16] must be considered. In this sense, although the physiological role of plasma membrane-bound carbonic anhydrase is not yet understood, it could be speculated that if such an activity is involved in the secretory function of the hepatocyte, e.g., by increasing the rate of bicarbonate transfer across this epithelium, a retrodifferentiation of these cells taking place during the liver regeneration might account at least partly for the loss of the ability of ursodeoxycholate to stimulate bicarbonate secretion into bile.

The phenomenon of enzymatic retrodifferenciation has been described for various enzymatic systems [17,18,19,20,21]. Little more is known about the role of

TABLE I

Protein content of homogenate, and soluble fraction and plasma membrane preparations, and lipid composition of plasma membrane preparations during the regeneration of two-thirds hepatectomized rat liver

Values are means ± S.E. ($n \geq 4$). * $P < 0.05$, ** $P < 0.01$ as compared with controls by the Bonferroni method of multiple range testing.

Days after hepatectomy	Homogenate		Soluble fraction	Membrane		
	protein (g)	protein/dry liver wt. (mg/g)	protein (mg/ml)	protein (mg/ml)	cholesterol (μmol/mg protein)	phospholipids (μmol/mg protein)
Control	1.23 ± 0.17	381 ± 27	5.45 ± 0.36	1.95 ± 0.26	0.07 ± 0.02	0.27 ± 0.03
1	0.47 ± 0.05 **	400 ± 54	5.58 ± 0.19	2.33 ± 0.16	0.11 ± 0.01 *	0.25 ± 0.03
3	0.94 ± 0.04 *	443 ± 22	5.78 ± 0.19	2.67 ± 0.23	0.06 ± 0.01	0.26 ± 0.02
7	1.05 ± 0.02	485 ± 70	5.51 ± 0.49	1.92 ± 0.42	0.23 ± 0.04 **	0.29 ± 0.04

carbonic anhydrase activity found in the soluble fraction, but, in contrast to the plasma membrane-bound activity, cytosolic carbonic anhydrase activity is presumably involved in cellular functions less specific for hepatic tissue, such as the control of intracellular pH. Thus, during liver regeneration a retrodifferentiation of carbonic anhydrase activity would be expected to affect the plasma membrane-bound more than the cytosolic activity.

The results (Fig. 2) indicate that the specific activity of carbonic anhydrase per mg of protein found in the homogenate was markedly decreased (by 39% one day after hepatectomy). Part of this reduction was due to the lowered plasma membrane-bound carbonic anhydrase activity. However, a role of other non-soluble isoenzymes of carbonic anhydrase not measured in this study cannot be ruled out as possible contributors to the total reduction of carbonic anhydrase specific activity. When this activity was compared between the soluble and plasma membrane fractions, as shown in Fig. 2, the membrane-bound carbonic anhydrase activity was markedly decreased (by 55%) one day after hepatectomy, thereafter increasing until a total recovery was reached at the seventh day, while the activity of the

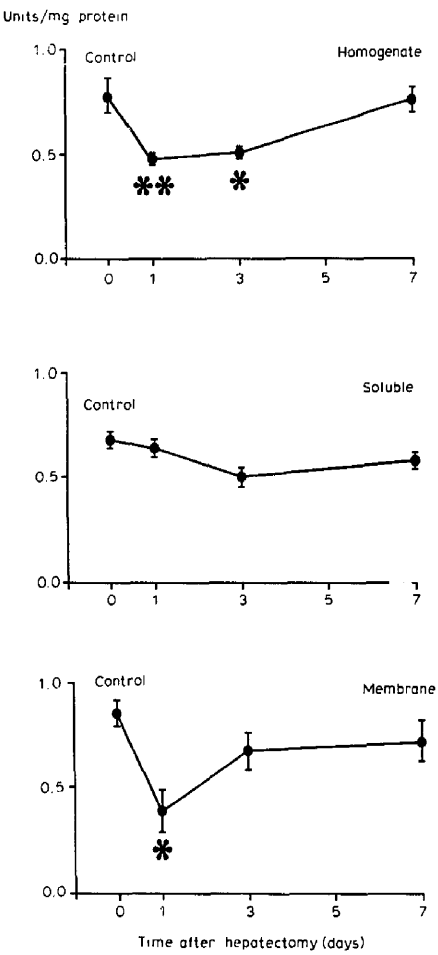

Fig. 2. Time-course of specific carbonic anhydrase activity after two-thirds hepatectomy, measured in homogenate, and soluble and plasma membrane-enriched fractions. Values are means ± S.E. The number of experiments for control and hepatectomized rats 1, 3 or 7 days before the enzyme activity determination were, 6, 7, 5 and 7, respectively. *, $P < 0.05$, ** $P < 0.01$, as compared to controls by the Bonferroni method of multiple range testing.

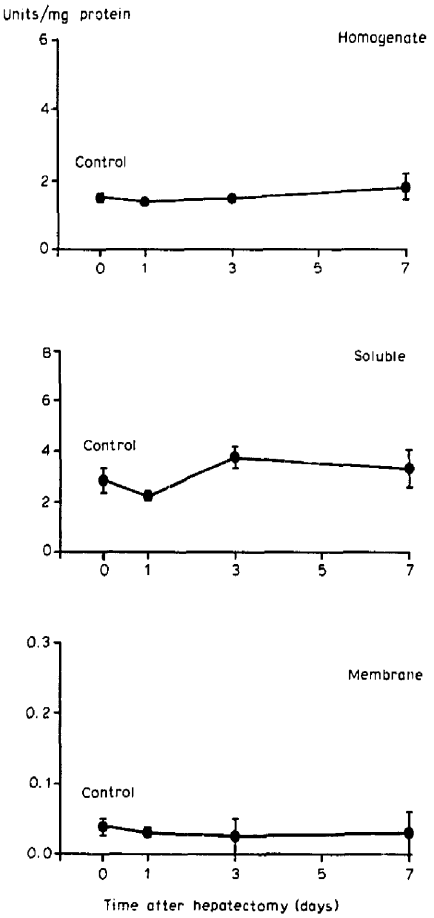

Fig. 3. Time-course of specific lactate dehydrogenase activity after two-thirds hepatectomy, measured in homogenate, and soluble and plasma membrane-enriched fractions. Values are means ± S.E. The number of experiments for control and hepatectomized rats 1, 3 or 7 days before the enzyme activity determination were 7, 7, 5 and 7, respectively. No significant difference as compared to controls by the Bonferroni method of multiple range testing was found.

soluble fraction was not significantly modified during the regeneration period.

In order to test the possibility of a lower degree of contamination by cytosolic protein during the purification of the plasma membrane fraction from regenerating livers, we measured the activity of a soluble marker enzyme, i.e., lactate dehydrogenase, in homogenate and the soluble and plasma membrane-enriched fractions. The results are shown in Fig. 3. As compared to the controls no significant changes were observed in the homogenate or the soluble fraction during the regenerative period. The activity of this enzyme was very low in the plasma membrane fraction (less than 3% of the specific activity found in the homogenate). This suggested that the carbonic anhydrase activity found in this fraction was not due to contamination by adsorption of cytoplasmic enzyme onto the plasma membrane or by enclosure of the cytoplasm into membrane vesicles. Moreover, this activity was not modified by partial hepatectomy. The posibility that the reduction in carbonic anhydrase activity detected in plasma membrane fraction during regeneration could be due to changes in cytoplasmic contamination can therefore reasonably be ruled out.

We also wondered whether the reduction in carbonic anhydrase specific activity in the plasma membrane fraction might be due to a lower enrichment in the plasma membrane obtained during the regenerating period. To test this hypothesis, 5′-nucleotidase activity was assayed in homogenate and the soluble and plasma membrane fractions. The results of this study are shown in Fig. 4. This activity is located mainly in the plasma membrane although a certain amount is found within the cell [22,23]. Our results show that the 5′-nucleotidase activity found in the fraction enriched in plasma membrane is not significantly modified during the liver regeneration period. However, a transient reduction in cytoplasmic 5′-nucleotidase activity was found at 1 and 3 days after hepatectomy.

Taken together, these results indicated that plasma membranes from regenerating livers were not less pure than that from control rats. Thus, changes in carbonic anhydrase activity expressed as per mg of protein can be assumed to represent modifications in the activity of the plasma membrane-bound enzyme. This is consistent with the quantitative differences in cell surface proteins during liver regeneration [24]. However, other possibilities should be considered, among them, a modification in the activity of the membrane-bound enzyme due to changes in the physical-chemical properties of the membrane and related to changes in its lipid composition during the replicative cycle.

As has been reported [25] and confirmed by us (Table I), the phospholipid composition of the plasma membrane of hepatocytes does not appear to be modified during liver regeneration, at least as far as total

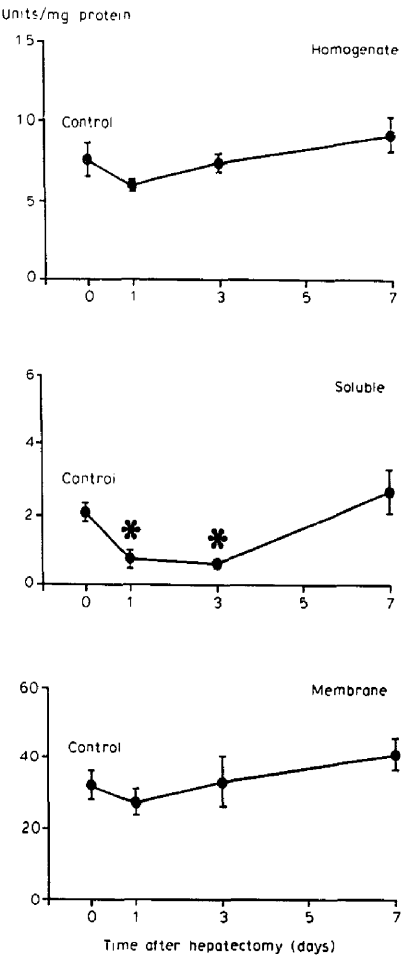

Fig. 4. Time-course of specific 5′-nucleotidase activity after two-thirds hepatectomy, measured in homogenate, and soluble and plasma membrane-enriched fractions. Values are means ± S.E. The number of experiments for control and hepatectomized rats 1, 3 or 7 days before the enzyme activity determination were 7, 7, 5 and 7, respectively. *, $P < 0.05$, as compared to controls by the Bonferroni method of multiple range testing.

phospholipid content is concerned. By contrast, the cholesterol/protein ratio is increased at 24 h after hepatectomy and at the end of the times considered (7 days). This is in agreement with an increase in hepatic cholesterol synthesis [26] required for cell growth during liver regeneration. Thus, as shown in table I, the cholesterol/phospholipid ratio is modified at 1 and 7 days after hepatectomy. This might modify membrane fluidity and hence the membrane-bound enzymes [25]. However, the changes in the cholesterol/phospholipid ratio correlate with a modification in plasma mem-

brane-bound carbonic anhydrase activity only at 1 day after hepatectomy when the value of the ratio is increased by 57% as compared with control rats, but not at 7 days when this ratio is increased even more (by 186%) and no significant change in carbonic anhydrase activity was observed. These results do not allow us to rule out a role of modification in plasma membrane fluidity as being the cause of the reduction in carbonic anhydrase activity shortly after hepatectomy, although they suggest that this is probably not the case.

In summary, the present findings indicate that a difference in the time-course of soluble and plasma membrane-bound carbonic anhydrase activities occurs during the regenerative process. This may be interpreted as the result of different control for the turnover of these isoenzymes, which is consistent with the hypothesis of a different physiological role for cytoplasmic and plasma membrane carbonic anhydrase in the hepatocyte.

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