Gene expression of enzymes regulating ketogenesis and fatty acid metabolism in regenerating rat liver

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Levels of mRNA for mitochondrial 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase, carnitine palmitoyltransferase I (CPT I) and carnitine palmitoyltransferase II (CPT II), fatty acid synthase (FAS) and actin were analysed during liver regeneration. mRNA levels for mitochondrial HMG-CoA synthase decreased rapidly, reaching a minimum 12 h after partial hepatectomy and returning to normal at 24–36 h. In contrast, CPT I, CPT II and FAS mRNAs increased throughout the period examined. Expression of actin increased significantly during regeneration. Levels of mRNA for mitochondrial HMG-CoA synthase also

decreased as a result of surgical stress, although the effect of hepatectomy was much greater. We determined the levels of mitochondrial HMG-CoA synthase using specific antibodies. The amount of protein rapidly decreased, although less markedly than the corresponding mRNA levels. These results show that the decrease described in ketogenesis in partially hepatectomized rats correlated with the decrease in the expression of mitochondrial HMG-CoA synthase, suggesting that this enzyme may also be a control point in ketogenesis in the regenerating liver, as it is in normal and diabetic rats.

INTRODUCTION

Partial hepatectomy (up to 70 %) induces a growth response that leads to a rapid resynthesis of the organ mass. The molecular signals that control this process are gradually being identified: among others, serum factors, growth factors and different nutrients are involved [1,2]. Several important metabolic changes are also produced: the metabolism steps up utilization of lipids and gluconeogenesis increases, glycolysis diminishes to maintain glucose homeostasis [3,4]. Recently, changes have been reported in fructose 2,6-bisphosphate, in 6-phosphofructo-2-kinase (PFK-2) activity and in the gene expression of the regulatory enzymes of the glycolytic/gluconeogenic pathways [5–7]. In addition, ketone-body synthesis is decreased in liver regeneration [8].

Hepatic ketogenesis is mainly controlled by two systems: (1) entry of fatty acids into mitochondria, catalysed by carnitine palmitoyltransferases (CPT I and II); and (2) enzymic activity of mitochondrial 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase. Schofield et al. [8] showed that, when fasted rats were subjected to hepatectomy, ketone-body concentrations decreased to lower values than in animals with acute surgical stress or trauma. The decrease was shown to occur not by accelerated extrahepatic utilization of ketone bodies, but by a decrease in their synthesis. These authors also pointed out that a locus distal from CPT I was strongly involved in the control of ketogenesis. This was an indication that mitochondrial HMG-CoA synthase could be a major regulatory site for the synthesis of ketone bodies in rat liver.

HMG-CoA synthase activity has been found to control ketogenesis in two ways: on the one hand, by a post-translational modification of the protein by succinylation/desuccinylation [9,10]; on the other hand, by control of gene expression. mRNA levels for mitochondrial HMG-CoA synthase change in a variety of physiological situations, such as fasting, refeeding, fat feeding and diabetes [11]. We also observed that, when HMG-CoA synthase mRNA levels were increased or decreased by these

factors, the levels of the protein changed in the same sense, depending on the experimental conditions, showing a co-ordinate mechanism between transcription and translation [12].

The aim of the present study was to determine whether mRNA and protein levels of the mitochondrial HMG-CoA synthase were modulated during rat liver regeneration, and, if so, to investigate whether this enzyme could also control ketogenesis after hepatectomy stress. Furthermore, other changes in the mRNA levels of lipid-regulatory enzymes related to the ketogenic process were also studied.

MATERIALS AND METHODS

Chemicals

[α -³²P]dATP (3000 Ci/mmol) was from Amersham. The random-primed DNA-labelling kit was from Boehringer Mannheim. The cDNA first-strand synthesis kit was from Stratagene. Other biochemical reagents were from Boehringer Mannheim, Sigma or Stratagene. All chemicals were of analytical grade.

Animals

Male Sprague–Dawley rats (180–220 g) fed *ad libitum* on chow and water were subjected to a 12 h-light/12 h-dark cycle (light periods starting at 08:00 h). To minimize the diurnal variation in liver DNA synthesis [13], partial hepatectomy or sham operation was performed between 08:00 and 10:00 h under diethyl ether anaesthesia [14], after which the animals were returned to the same conditions. Control rats (0 h) were not subjected to either anaesthesia or surgery. The animals were killed by decapitation at the time indicated and the livers were removed and immediately frozen in liquid nitrogen.

RNA analyses and DNA-hybridization probes

Total RNA was extracted from frozen rat tissue by the LiCl/urea method [15]. Northern-blot analyses were performed by standard

Abbreviations used: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; CPT, carnitine palmitoyltransferase; PFK-2, 6-phosphofructo-2-kinase; FAS, fatty acid synthase.

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procedures [16]. The following fragments were used as probes: a 1.5 kb *KpnI* fragment derived from our cDNA for rat liver mitochondrial HMG-CoA synthase [17]; a 1.4 kb (*XhoI*–*BamHI*) fragment from the cDNA clone pBKS-CPT II.4 for rat CPT II [18]; a 1.7 kb *PvuII* fragment corresponding to a cDNA clone (pGFAS3) for goose fatty acid synthase (FAS) [19]; a 0.8 kb fragment from cDNA encoding rat actin [20]; and a 1.1 kb *PstI* fragment from the cDNA (pRSA13) clone for rat serum albumin [21].

A partial fragment of rat CPT I cDNA, covering the region corresponding to positions 1272–2020 in the rat CPT I mRNA [22], was produced by a reverse-transcriptase/polymerase chain reaction procedure using two specific primers, 5'-GCAGGGA-TACAGAGAGGAGG-3' (CPTa) and 5'-GGAAAGGTGAGT-CGACTGC-3' (CPTb). First-strand cDNA was synthesized from 100 ng of rat hepatic poly(A)⁺ RNA by using 300 ng of random hexamers and 20 units of Moloney murine leukaemia virus reverse transcriptase in accordance with the manufacturer's recommendations (Stratagene). Amplification of cDNA was performed by adding a 2 μ l sample of first-strand cDNA reaction mixture to 98 μ l containing 20 mM Tris/HCl (pH 8.3), 50 mM KCl, 1.25 mM MgCl₂, 0.2 mM dNTP, 0.1 μ M each of the two primers CPTa and CPTb, and 1.25 units of *Taq* polymerase. After an initial denaturation step at 94 °C for 5 min, the following amplification programme was used: denaturation at 94 °C for 1 min; annealing at 55 °C for 2 min; and extension at 72 °C for 3 min; 30 cycles were used for amplification. Upon completion of the last cycle, the mixture was incubated at 72 °C for 10 min. The amplified fragment was cloned in a *BamHI*–*SalI* site in pBlue-script SK⁺ vector (Stratagene) and sequenced by the dideoxy chain-termination method [23] to confirm the identity of the CPT I cDNA. This *BamHI*–*SalI* 715 bp fragment was used as a probe for the detection of CPT I mRNA.

All DNA probes were generated by labelling with [α -³²P]dATP to a specific radioactivity of 1.5×10^9 c.p.m./ μ g of DNA by random priming with Klenow polymerase. The levels of mRNAs were measured by densitometric scanning of the autoradiograms with an LKB Ultrosan XL laser densitometer and GelScan (2.1) software, and corrected for the amount of albumin mRNA that was used as control [7]. Statistical analysis was carried out by Student's *t* test, with significance level chosen as $P < 0.05$.

Western-blot analysis

Immunoblotting of mitochondrial HMG-CoA synthase was carried out as described by Serra et al. [12]. Specific antibody to mitochondrial HMG-CoA synthase (AS-79) was used, and this antibody did not cross-react with cytosolic HMG-CoA synthase [24].

RESULTS

To determine whether the changes in ketogenesis reported in regenerating liver [8] could be related to modifications in gene expression of mitochondrial HMG-CoA synthase, mRNA levels were determined by Northern-blot analyses. Values were corrected for the amount of albumin mRNA, which, due to the minimal changes observed, was taken as control [7]. As shown in Figure 1, the levels of mitochondrial HMG-CoA synthase mRNA decreased by up to 88% with respect to control at 12 h after partial hepatectomy. Afterwards, a continuous recovery was observed and values similar to control were reached after 48 h. In contrast, CPT I and CPT II showed increases of about 2–3-fold in the same period, decreasing slowly thereafter and returning to

Figure 1 Gene expression of mitochondrial HMG-CoA synthase, CPT I and CPT II, FAS and actin during liver regeneration

(a) Samples of total RNA (20 μ g/lane) extracted from normal and regenerating rat livers at the indicated times were transferred to nylon membranes after electrophoresis in 1.5% agarose and hybridized with mitochondrial HMG-CoA synthase (●), CPT I (▽), CPT II (▼), FAS (□), actin (○) and albumin (control) cDNAs as described in the Materials and methods section. The levels of these mRNAs were measured by densitometric scanning of the autoradiograms and corrected for the amount of albumin mRNA. The values represent means $\pm$ S.E.M. of three or four different animals: *significantly different from control rats ($P < 0.05$). Representative Northern blots are shown in Figures 1(b) and 2. (b) Northern blots of mitochondrial HMG-CoA synthase (mHCoA-S), CPT I and CPT II, FAS and actin during liver regeneration. Samples of total RNA (20 μ g/lane) extracted from normal (0 h) and regenerating rat livers at the indicated times were transferred to nylon membranes after electrophoresis in 1.5% agarose and hybridized with mHCoA-S, CPT II, FAS, actin and albumin cDNAs as described in the Materials and methods section. In the lower part of (b), the mRNA level of CPT I is shown together with that of the corresponding albumin. The mRNA size (kb) is indicated.

Figure 2 Northern blots of mitochondrial HMG-CoA synthase, CPT I and CPT II, FAS and actin after partial hepatectomy (a) or sham operation (b)

Samples of total RNA (20 μ g/lane) extracted from normal (0 h), sham-operated and regenerating rat livers at the indicated times were transferred to nylon membranes after electrophoresis in 1.5% agarose and hybridized with mitochondrial HMG-CoA synthase (mHCoA-S), CPT II, FAS, actin and albumin cDNAs as described in the Materials and methods section. CPT I is shown with its corresponding albumin in the lower part of the Figure. The mRNA size (kb) is indicated.

the basal values at 60 h. FAS mRNA levels remained unchanged for 8 h, then increased progressively up to 2-fold at 36 h, and decreased slowly thereafter. To check that all processes had been carried out correctly, we also studied the evolution of mRNAs for actin, which had been described to increase in regenerating liver. Values observed in Figure 1 clearly show that this gene appreciably increases its expression, since at 12 h values are 14-fold higher than control.

From the results described above, it seems clear that the most pronounced changes in the mRNA levels occur during the first 24 h after hepatectomy. To distinguish between the events which are specific to liver regeneration and those which are related to surgical stress, mRNAs were determined in sham-operated rats (animals subjected to a mid-ventral laparotomy without direct manipulation of the liver). Only mitochondrial HMG-CoA synthase showed decreased mRNA levels in these rats (Figure 2). However, quantitative differences were found in partially hepatectomized rats, compared with sham-operated rats. HMG-CoA synthase at 12 h showed a decrease in expression of about 50%, clearly less than the decrease observed after partial liver resection. CPT I showed an increase in expression of about 2-fold, similar to that observed after hepatectomy at the same time. CPT II and FAS, on the other hand, showed no change in expression in sham-operated rats at any time. Actin expression was not changed appreciably for the period of time studied, at variance to what happens in regenerating liver.

To ascertain whether changes in mitochondrial HMG-CoA synthase mRNA levels determined changes in levels of the protein, we measured the protein levels by Western blotting in

Figure 3 Western blot of mitochondrial HMG-CoA synthase after partial hepatectomy

Samples (50 μ g of protein) extracted from normal and regenerating rat livers at the indicated times were analysed by Western blotting using a specific antibody against mitochondrial HMG-CoA synthase (mHCoA-S).

hepatectomized livers at the same times as those shown in Figure 1. At 12 h the level of HMG-CoA synthase protein was decreased to 60% of control, and the levels returned quickly to normal values at 36 h (Figure 3). No change was observed in protein levels in sham-operated rats.

DISCUSSION

Studies on regenerating liver after partial hepatectomy have been performed in the past as a model to compare the mechanism of cell proliferation in normal as opposed to cancerous tissue. Some efforts have been directed to the fate of ketone-body metabolism during liver regeneration. Starved rats subjected to partial hepatectomy have much lower ketone-body concentrations in

plasma than do unoperated or sham-operated rats [25]. The possibility that the decrease in ketone bodies in blood was due to increased utilization by the peripheral tissues was ruled out by Schofield et al. [8]. These authors showed that in both hepatectomized and sham-operated rats ketone-body utilization was a linear function of the concentration in blood, ruling out the possibility that these manipulations have any real effect on the peripheral clearance of ketones. The conclusion was that ketone-body synthesis decreased in partially hepatectomized rats with respect to sham-operated rats. These authors also pointed out that a site of action distal to CPT was strongly involved in ketogenic control, since the administration of medium-chain triacylglycerols (precursors of the fatty acid entry into mitochondria without the mediation of CPT I and CPT II) did not promote ketone-body synthesis in hepatectomized or sham-operated rats. This regulatory site could be mitochondrial HMG-CoA synthase.

The decreased mRNA and protein levels for mitochondrial HMG-CoA synthase presented herein are consistent with reported data, showing that ketone-body synthesis decreases during liver regeneration. We had previously shown that an increase in mitochondrial HMG-CoA synthase gene expression was followed by an increase in protein [12], and also that this enzyme catalysed a regulatory step in the control of ketone bodies in normal, fed, starved and diabetic rats [11]. It appears that in a similar way decreases in mRNA levels are qualitatively coordinated with decreases in protein level in hepatectomized animals. Experimental results in sham-operated rats, which were, however, clearly different from a quantitative point of view, show that the levels of protein do not change appreciably.

The molecular mechanism of decreased transcription of mitochondrial HMG-CoA synthase is difficult to establish. It has been shown that in regenerating liver there is an increase in serum glucagon levels [26,27], an increase in cyclic AMP levels [5,28], a decrease in insulin concentrations [3,29], and an increase in glucocorticoid concentrations [3]. All these changes may mediate the transcription of phosphoenolpyruvate carboxykinase and PFK-2/fructose-2,6-bisphosphatase genes [6,7] in hepatectomized rats. However, we have previously shown that all these parameters produced increased expression of mitochondrial HMG-CoA synthase gene. It is concluded that an unknown and possibly stronger repression mechanism may abolish the expected positive effect on expression caused by these factors. The delayed production of glucocorticoids may speed new ketone-body synthesis during the increase in liver mass.

Other genes tested show a lower response in hepatectomized rats. CPT I and CPT II, which mediate the entry of fatty acids to mitochondria, present a low response after hepatectomy. However, a 2–3-fold increase in these mRNA levels is consistently produced. This is in agreement with the increase in mitochondrial CPT activity [26] with the increase in mitochondrial respiration seen when palmitoylcarnitine was used as a substrate, and with the increase in the ATP synthesis throughout 72 h after hepatectomy. These effects show the necessity to increase the transport of fatty acids into mitochondria to promote a higher β -oxidation process and facilitate the tricarboxylic-acid-cycle flux in response to greater energy demand, as observed by Katagiri [26]. In addition, Nakatani et al. [30,31] have shown that fatty acid oxidation is essential to DNA synthesis and mitosis.

The results obtained in mRNA levels for CPT I, which change in the opposite sense to those of mitochondrial HMG-CoA synthase in hepatectomized animals, reinforce the hypothesis expressed by Grantham and Zammit [32,33] that ketogenic mitochondrial control is exerted in other mitochondrial sites than CPT I. These authors reported that acute depression of

ketogenic capacity of the liver, either on refeeding of starved rats or on insulin treatment of diabetic rats, was not accompanied by any reversal of changes in CPT I in the first hours of these treatments. Other authors confirmed that this ketogenic control is exerted at the level of mitochondrial HMG-CoA synthase [9,11,34].

We have also observed that amounts of mRNA for FAS are approximately double after day 2 of liver regeneration. This result correlates with those obtained by Gove and Hems [35] and Schofield et al. [36,37], who observed similar changes in fatty acid synthesis within the first 3 or 4 days.

Although not related to the ketogenic pathway, results on actin expression show the importance of this protein in regenerating liver, where it may be used as the cytoskeleton of the newly formed cells. This response, also reported by Sobczak et al. [38], may validate the results obtained in mitochondrial HMG-CoA synthase.

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REFERENCES

- 1 Fausto, N. and Mead, J. E. (1989) *Lab. Invest.* **60**, 4–13
- 2 Michalopoulos, G. K. (1990) *FASEB J.* **4**, 176–187
- 3 Leffert, K. L., Koch, K. S., Moran, T. and Rubalcava, B. (1979) *Gastroenterology* **76**, 1470–1482
- 4 Katz, N. (1979) *Eur. J. Biochem.* **98**, 535–542
- 5 Rosa, J. L., Ventura, F., Carreras, J. and Bartrons, J. (1990) *Biochem. J.* **270**, 645–649
- 6 Rosa, J. L., Tauler, A., Lange, A. J., Pilakis, S. J. and Bartrons, R. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 3746–3750
- 7 Rosa, J. L., Bartrons, R. and Tauler, A. (1992) *Biochem. J.* **287**, 113–116
- 8 Schofield, P. S., French, T. J. and Sugden, M. C. (1987) *Biochem. J.* **241**, 475–481
- 9 Quant, P. A., Tubbs, P. K. and Brand, M. D. (1989) *Biochem. J.* **262**, 159–164
- 10 Quant, P. A., Tubbs, P. K. and Brand, M. D. (1990) *Eur. J. Biochem.* **187**, 169–174
- 11 Casals, N., Roca, N., Guerrero, M., Gil-Gómez, G., Ayté, J., Ciudad, C. J. and Hegardt, F. G. (1992) *Biochem. J.* **283**, 261–264
- 12 Serra, D., Casals, N., Asins, G., Royo, T., Ciudad, C. J. and Hegardt, F. G. (1993) *Arch. Biochem. Biophys.* **307**, 40–45
- 13 Barbiroli, B. and Potter, V. R. (1971) *Science* **172**, 738–741
- 14 Higgins, G. M. and Anderson, R. M. (1931) *Arch. Pathol.* **12**, 186–202
- 15 Auffray, C. and Rougeon, F. (1980) *Eur. J. Biochem.* **107**, 303–314
- 16 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor
- 17 Ayté, J., Gil-Gómez, G., Haro, D., Marrero, P. F. and Hegardt, F. G. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3874–3878
- 18 Woeltje, K. F., Esser, V., Weis, B. C., Sen, A., Cox, W. F., McPhaul, M. R., Slaughter, C. A., Foster, D. W. and McGarry, J. D. (1990) *J. Biol. Chem.* **265**, 10720–10725
- 19 Back, D. W., Goldman, M. J., Fisch, J. E., Ochs, R. S. and Goodridge, A. G. (1986) *J. Biol. Chem.* **261**, 410–417
- 20 Brown, C. W., McHugh, K. M. and Lessard, J. L. (1990) *Nucleic Acids Res.* **18**, 5312
- 21 Sargent, T. D., Wu, J. R., Sala-Trepat, J. M., Wallace, R. B., Reyes, A. A. and Bonner, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3256–3260
- 22 Esser, V., Britton, C. H., Weis, B. C., Foster, D. W. and McGarry, J. D. (1993) *J. Biol. Chem.* **268**, 5817–5822
- 23 Sanger, F., Niklen, S. and Maniatis, T. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463–5467
- 24 Royo, T., Pedragosa, M. J., Ayté, J., Gil-Gómez, G., Vilaró, D. and Hegardt, F. G. (1993) *J. Lipid Res.* **34**, 867–874
- 25 Schofield, P. S., McLees, D. J., Myles, D. D. and Sugden, M. C. (1985) *Biochem. J.* **231**, 225–228
- 26 Katagiri, S. (1988) *Nippon Geka Gakkai Zasshi* **89**, 694–702
- 27 Morley, C. G. D., Kuku, S., Rubenstein, A. H. and Boyer, J. L. (1975) *Biochem. Biophys. Res. Commun.* **67**, 653–661
- 28 Koide, Y., Shelton, H., Ong, S. H. and Steiner, A. L. (1978) *J. Biol. Chem.* **253**, 4439–4445
- 29 Bucher, N. L. and Weir, G. C. (1976) *Metab. Clin. Exp.* **25**, 1423–1425

-
- 30 Nakatani, T., Ozawa, K., Asano, M., Ukikusa, M., Kamiyama, Y. and Tobe, T. (1981) *J. Lab. Clin. Med.* **97**, 887–898
- 31 Nakatani, T., Yasuda, K., Ozawa, K., Kawashima, S. and Tobe, T. (1982) *Clin. Sci.* **62**, 295–297
- 32 Grantham, B. D. and Zammit, V. A. (1986) *Biochem. J.* **239**, 485–488
- 33 Grantham, B. D. and Zammit, V. A. (1988) *Biochem. J.* **249**, 409–414
- 34 Dashti, N. and Ontko, J. A. (1979) *Biochem. Med.* **22**, 365–374
- 35 Gove, C. D. and Hems, D. A. (1978) *Biochem. J.* **170**, 1–8
- 36 Schofield, P. S., Sugden, M. C., Corstorphine, C. G. and Zammit, V. A. (1987) *Biochem. J.* **241**, 469–474
- 37 Schofield, P. S., French, T. J., Goode, A. W. and Sugden, M. C. (1985) *FEBS Lett.* **184**, 214–220
- 38 Sobczak, J., Tournier, M. F., Lotti, A. M. and Duguët, M. (1989) *Eur. J. Biochem.* **180**, 49–53
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