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Regulation of the expression of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene

Its role in the control of ketogenesis

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We have explored the role of mitochondrial 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase in regulating ketogenesis. We had previously cloned the cDNA for mitochondrial HMG-CoA synthase and have now studied the regulation *in vivo* of the expression of this gene in rat liver. The amount of processed mitochondrial HMG-CoA synthase mRNA is rapidly changed in response to cyclic AMP, insulin, dexamethasone and refeeding, and is greatly increased by starvation, fat feeding and diabetes. We conclude that one point of ketogenic control is exercised at the level of genetic expression of mitochondrial HMG-CoA synthase.

INTRODUCTION

Ketogenesis occurs when the rate of acetyl-CoA formation through fatty acid oxidation exceeds the capacity of hepatic mitochondria to form citrate. Acetyl-CoA is then channelled through a series of reactions via 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthesis, which (i) release CoA needed for further fatty acid activation and (ii) generate acetoacetate and hydroxybutyrate, which are released by the liver and utilized by extrahepatic tissues.

Ketogenesis is observed under physiological conditions (fasting, long exercise, high-fat diet, and the transition from the foetal to the suckling state) as well as in the pathological condition of diabetes, and constitutes a mechanism to spare glucose. In all these cases, the glucagon/insulin ratio is increased [1,2], which suggests that cyclic AMP might be playing a role to trigger ketogenesis, but the main site of regulation is unknown.

It has been postulated that carnitine palmitoyltransferase I (CPT I), the enzyme responsible for the regulation of the entry of acyl-CoA into mitochondria, might control the rate of ketogenesis during the onset of diabetic ketosis and starvation. However, the acute reversal of these situations does not decrease the activity of CPT I in the short term [3,4], and it is still high when the plasma ketone-body concentration returns to basal levels. This suggests that CPT I is likely to be an important locus in the control of ketogenesis only during the onset of these two conditions, but not during their reversal [3,4].

It has been reported [5,6] that total HMG-CoA synthase activity (90% of which is mitochondrial) increases with fasting, fat feeding, diabetes, glucagon administration and with the transition from the foetal to the suckling state. We have therefore studied the regulation of ketogenesis at the level of the expression of the gene for mitochondrial HMG-CoA synthase in several conditions, using the cDNA which had recently been cloned in our laboratory [7], with the purpose of exploring the potential role of mitochondrial HMG-CoA synthase [8] in regulating ketogenesis.

We have observed that changes in the rate of hepatic ketogenesis are accompanied by equivalent changes in the levels of mRNA for mitochondrial HMG-CoA synthase under all the conditions tested.

MATERIALS AND METHODS

Animals

Sprague–Dawley rats weighing 150–250 g were used throughout this work. They were fed *ad libitum* with standard diet, except for the group fed with a fatty-acid-rich diet (43% lipid content versus 3%) and had free access to water. When using starved animals, food was withdrawn either 24 or 48 h before the beginning of the experiment. Diabetes was induced by a single dose of streptozotozin (70 mg/kg) injected intravenously under ether anaesthesia. The establishment of diabetes was attested by the presence of high concentrations of glucose and ketone bodies in the urine. After each treatment, rats were decapitated and their livers were quickly removed and placed in liquid N_2. The livers were then powdered in a porcelain mortar, under liquid N_2, distributed in about 1 g batches and kept at −80 °C until the RNA was isolated. Human insulin (Velosulin) was from Abelló Laboratories, Barcelona, Spain.

mRNA analysis

Total RNA was isolated [9], and 10 μg samples (within input-signal linearity) were subjected to agarose-gel electrophoresis and to Northern transfer. The RNA was fixed to Nytran filters by using u.v. at 254 nm for 2 min at 12 cm. After 6 h pre-hybridization, hybridization was carried out with a 1.5 kb probe corresponding to the *Kpn*I–*Kpn*I fragment of the cDNA for rat mitochondrial HMG-CoA synthase [7]. The probe was prepared by the random-primed method [10]. Filters were washed in 0.1 × SSC/0.5% SDS at 50 °C and placed in contact with Kodak X-ray film. Densitometry was performed and values were corrected by using a constitutive probe (rat γ-actin).

RESULTS

Throughout this study we used a probe expanding 1.5 kb of the cDNA from mitochondrial HMG-CoA synthase in the Northern-blot experiments described below. This probe detected only transcripts of 2.0 kb, corresponding to the mitochondrial enzyme, and not transcripts of 3.4 kb, corresponding to the cytosolic enzyme [7,11]. The use of this probe thus conferred the specificity needed to determine the expression of the mitochondrial HMG-CoA synthase.

Abbreviations used: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; CPT I, carnitine palmitoyltransferase I; IRS, insulin-responsive sequences.

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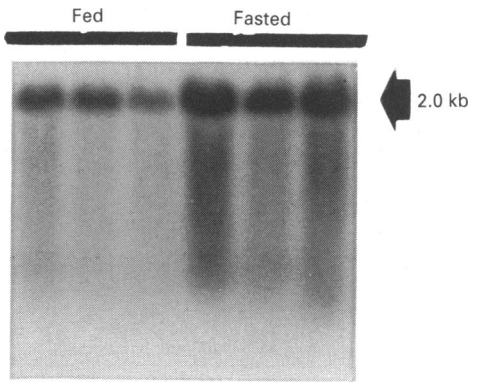

Fig. 1. Blot hybridization of mitochondrial HMG-CoA synthase mRNA of livers from fed and fasted rats

Three rats were used for each condition. The arrow shows the molecular size of the transcript.

We determined the levels of mitochondrial HMG-CoA synthase mRNA in those situations leading to changes in plasma ketone-body concentrations. Starvation (24 h) as shown in Figs. 1 and 2(*a*), increased mRNA levels 4-fold. The second day of starvation increased the expression by a further 10%. Given that the glucagon/insulin ratio increases in starvation, we studied the effect of cyclic AMP on the expression of the mitochondrial HMG-CoA synthase gene. Rats fed *ad libitum* were injected intraperitoneally with dibutyryl cyclic AMP. Figs. 2(*b*) and 4(*a*) show that the specific mRNA was increased by this cyclic nucleotide in a time-dependent manner such that 60 min after injection the mRNA level had doubled.

Upon refeeding of 24 h-starved rats, the levels of mitochondrial HMG-CoA synthase mRNA decreased very rapidly with time (Fig. 2*a*), so that mRNA values returned to the basal level of fed animals in 2.5 h.

A fat diet also correlated with high ketone-body concentrations [6,12] and, as shown in Fig. 4(*b*), this diet given for 3 weeks increased the level of mitochondrial HMG-CoA synthase mRNA 3.2-fold.

Diabetes is another state in which ketone-body concentrations rise sharply. Accordingly, we explored the expression of the mitochondrial HMG-CoA synthase gene in diabetic animals. Streptozotocin-treated animals started to develop high ketone-body and glucose concentrations in the urine after the second day of streptozotocin administration. As shown in Fig. 3(*a*), there was a large time-dependent increase in the mitochondrial HMG-CoA synthase mRNA levels compared with the basal values found in normal rats. Insulin, which is able partially to restore the effects of diabetes on ketone-body concentrations (urine levels of 0.5 mM) also decreased the expression of the mitochondrial HMG-CoA synthase gene by 50% when injected over 1 day (three times/day) in diabetic rats (Figs. 3*a* and 4*c*). We then wanted to test whether insulin could also control the

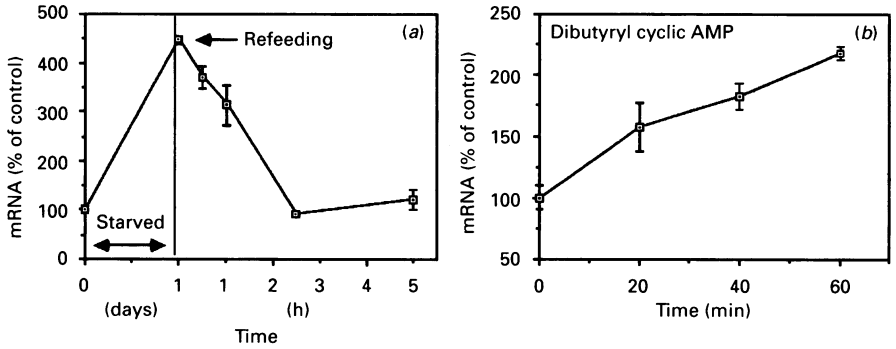

Fig. 2. Effects of refeeding and of cyclic AMP on mitochondrial HMG-CoA synthase mRNA levels

(*a*) Rats were starved for 24 h and refed from 20 min to 5 h. At each time rats were decapitated and their livers quickly removed and frozen in liquid N_2. RNA was isolated and subjected to electrophoresis and to Northern transfer. Data represent the means of three experiments. (*b*) Fed animals were injected with dibutyryl cyclic AMP (30 mg/kg) together with the same dose of caffeine.

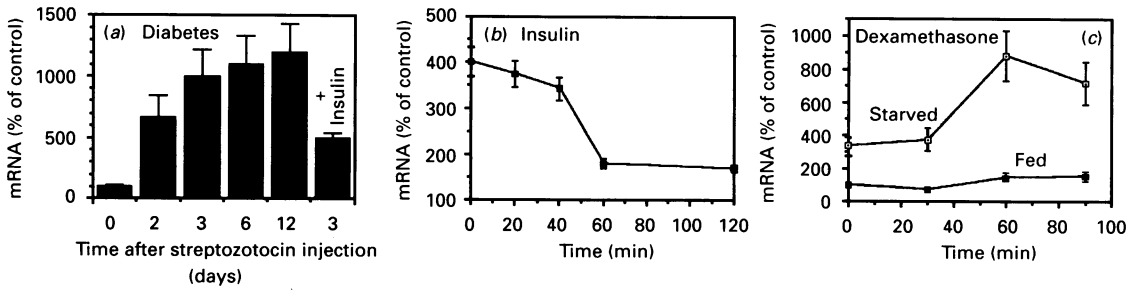

Fig. 3. Mitochondrial HMG-CoA synthase mRNA levels under different treatments

(*a*) Effect of experimentally induced diabetes. Streptozotocin was injected intravenously (70 mg/kg) and liver RNA isolated at different days. Reversion of diabetes by insulin was tested on day 3 after streptozotocin injection. In this case insulin was injected (6 units/kg) three times: 24 h, 12 h and 2 h before removing the liver. mRNA values are expressed as percentages of the levels found in normal fed animals. (*b*) Effect of insulin on mitochondrial HMG-CoA synthase gene expression. Starved rats (24 h) were injected intraperitoneally with insulin (40 units/kg) and the specific mRNA levels corresponding to different times of action of the hormone were determined. (*c*) Action of dexamethasone (5 mg/kg) on mRNA levels of mitochondrial HMG-CoA synthase in either fed rats or animals starved for 24 h.

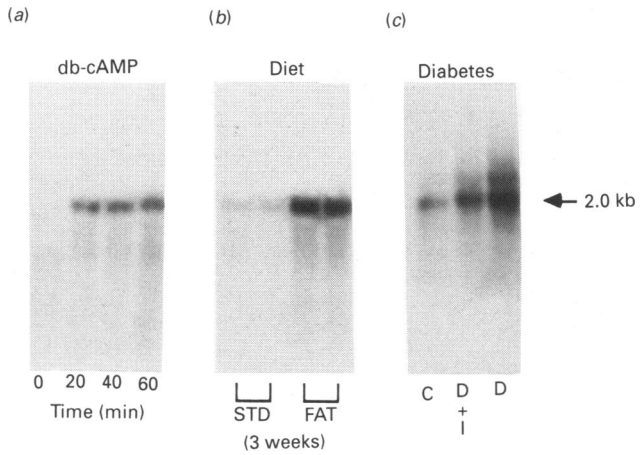

Fig. 4. Blot hybridization of mitochondrial HMG-CoA synthase mRNA in rat liver (*a*) after injection of dibutyryl cyclic AMP (db-cAMP), (*b*) after 3 weeks of fatty diet feeding, or (*c*) in diabetes

Lanes in (*b*): STD, standard diet; FAT, Fatty diet. Lanes in (*c*) C, Control; D, Diabetic; D+I, diabetic rat treated with insulin over 1 day.

expression of mitochondrial HMG-CoA synthase gene in normal rats. Given that the level of expression of this mRNA is low in fed animals, we performed this series of experiments with starved rats. Human rapid-acting insulin was injected into 24 h-starved rats, and the levels of specific mRNA were measured at the times indicated. As shown in Fig. 3(*b*), insulin caused a time-dependent decrease in mitochondrial HMG-CoA synthase mRNA. This effect could already be observed 20 min after the injection of insulin, and after 1 h the mRNA values had returned to the level of animals fed *ad libitum*.

Dexamethasone is a glucocorticoid that stimulates gluconeogenesis, which in many instances proceeds in a parallel fashion to ketogenesis. In view of these actions, we tested whether dexamethasone was able to alter mitochondrial HMG-CoA synthase gene expression. We found (Fig. 3*c*) that the levels of specific mRNA increased within 60 min of dexamethasone injection. However, although the increase was rather small (1.5-fold) in fed rats, it was much more dramatic (9-fold) in rats fasted for 24 h.

DISCUSSION

For the last 15 years a great effort has been made to elucidate the main locus of control of ketogenesis. This effort has been focused on the study of the regulatory role of CPT I. A key question to answer, however, if CPT I were the main regulator, would be how the activity of this enzyme could simultaneously control ketogenesis and oxidation of acetyl-CoA in the citric acid cycle. Indeed, regulation of CPT I activity by malonyl-CoA plays a role in control of ketogenic flux in livers of normal fed rats [13] and has been widely researched [14]. However, Grantham & Zammit [4] have studied the role of CPT I in the regulation of hepatic ketogenesis during the onset and reversal of chronic diabetes in adult rats. They concluded that CPT I is an important locus for the control of hepatic fatty acid oxidation and hence ketogenesis during the onset of diabetic ketosis. In contrast, it does not appear to play such a role during the acute reversal of ketosis. These conclusions are similar to those drawn from previous studies on the acute effects of refeeding of starved rats [3]. Acute depression of the ketogenic capacity of the liver on refeeding is not accompanied by any reversal of changes in CPT I (sensitivity to inhibition by malonyl-CoA and fatty acyl-CoA) induced by starvation during the first hours of refeeding, and there must therefore be other sites that would regulate ketogenesis during certain transition periods (starved-to-fed and diabetic-to-insulin). Schofield *et al.* [15], studying ketonemia after partial hepatectomy, reached the same conclusion: that ketogenesis is not only controlled at the level of CPT I. CPT II has recently been cloned [16], but it is known that this enzyme has no regulatory effect on ketogenesis [17]. In fact, results reported by Williamson *et al.* [18], Dashti & Ontko [19] and Quant *et al.* [20,21], suggest this ketogenic control may be exerted at the level of mitochondrial HMG-CoA synthase.

We now present data that clearly demonstrate that, in adult rats, regulation at the level of genetic expression of mitochondrial HMG-CoA synthase gene constitutes a major site of control of ketogenesis. We have found that this control is exerted by cyclic AMP, insulin, fasting and refeeding conditions, diabetes, a fat diet and dexamethasone.

Quant *et al.* [20] have shown that injection of glucagon in adult rats activates both ketogenesis and the enzyme HMG-CoA synthase by decreasing intramitochondrial succinyl-CoA levels and hence succinylation of the HMG-CoA synthase. This mechanism operates in the same direction as the increased expression of HMG-CoA synthase gene under conditions in which hepatic cyclic AMP levels are high (e.g. the fasting condition and direct dibutyryl cyclic AMP injection). The opposite effect also holds true, namely that refeeding (or insulin injection) decreases HMG-CoA synthase gene expression in starved rats and increases succinylation of HMG-CoA synthase (with a concomitant inactivation of the enzyme). The latter effects result in a parallel fall in ketone-body concentrations in blood [4].

The mechanism(s) for the decrease in the expression of this gene by insulin could be based on a counteraction of the effect of cyclic AMP on the gene and/or on the binding of a transcription factor to an insulin-responsive sequence (IRS). Thus insulin has been shown to decrease the sensitivity of hepatic cyclic-AMP-dependent protein kinase to this cyclic nucleotide [22], which could decrease the phosphorylation state of CREB (CRE binding protein) thus decreasing the transcription rate of the gene. It is also known that a sequence in the gene for phosphoenolpyruvate carboxykinase (which is another insulin-dependent protein) mediates the negative effect of insulin on the transcription of this gene [23]. In this regard, the mitochondrial HMG-CoA synthase gene promoter contains putative sequences similar to CRE (cyclic response element) and to the AP-2 binding site, which confer regulation to cyclic AMP [24] and to IRS (G. Gil-Gómez, J. Ayté & F. G. Hegardt, unpublished work). Specific degrees of binding to these sequences by transcription factors could mediate the control exerted by insulin on the expression of this gene.

Diabetes is another state in which the effect of the shortage of insulin produces elevated ketone-body synthesis. We have shown in the present study that ketone-body levels were dramatically increased in blood and urine in diabetes and that this state correlated with a great expression of mitochondrial HMG-CoA synthase, reaching values of 1200%, which strongly suggests a control of ketogenesis by HMG-CoA synthase gene expression. In our conditions, insulin administration to diabetic rats produced both a major decrease (but not full disappearance) of ketone bodies in urine and a 50% decrease in HMG-CoA synthase gene expression.

Fat feeding is a condition in which ketogenesis is increased, although its effects are not completely understood. Benito *et al.* [25], studying the effects of different fatty acids on ketogenesis in isolated hepatocytes of suckling or weaned rats, concluded that regulation of ketogenesis may involve distribution of long-chain fatty acyl-CoA between the esterification pathway and the β-oxidation pathway. Quant *et al.* [26], on the other hand,

supported the hypothesis that fatty acids (octanoate better than palmitate) added to isolated mitochondria resulted in desuccinylation and activation of mitochondrial HMG-CoA synthase, resulting in a stimulus to ketogenesis. Results presented here do not question these findings, which appear to occur as a short-term mechanism. What is really new is that a fatty diet increases HMG-CoA synthase gene expression by more than 300%, a process which occurs previous to a post-translational modification of the enzyme. The combination of the two processes results in a dual control of ketogenesis, by increasing not only the activity of the enzyme but also its amount, once its mRNA is translated.

The different sensitivity of mitochondrial HMG-CoA synthase gene expression to dexamethasone in fasted and fed rats could be explained if the effect of insulin were dominant over that of the glucocorticoid, as described for phosphoenolpyruvate carboxykinase [23]. In the fed state, when the glucagon/insulin ratio is low, insulin could trigger a preferential binding to IRS, thus preventing binding to the accessory factor 2 (AF-2), an element of the glucocorticoid response complex.

Several authors have suggested that mitochondrial HMG-CoA synthase is a major site of control of ketogenesis in different metabolic situations [3–5], and this conclusion has been confirmed by others in the light of modern metabolic control theory [20,21,26]. From our studies, a new picture of how ketogenesis is regulated emerges: an important locus of control lies in the expression of the mitochondrial HMG-CoA synthase gene. Modulation of mitochondrial HMG-CoA synthase activity occurs *in vivo* in different transition periods by a succinylation–desuccinylation process. However, expression of the gene accompanies this process and modulates the amount of enzyme which may be covalently modified. Triggered by different signals, genetic expression of mitochondrial HMG-CoA synthase into mRNA is accurately regulated, providing a strong basis for translational and post-translational processes that would lead to the final activity measured in the liver under ketogenic or non-ketogenic conditions.

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