Overexpression of Mitochondrial 3-Hydroxy-3-methylglutaryl-CoA Synthase in Transgenic Mice Causes Hepatic Hyperketogenesis*

Communication

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Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (HMG-CoA synthase) is a key enzyme in the ketone body pathway. To determine its role in the regulation of liver ketogenesis, transgenic mice expressing a P-enolpyruvate carboxykinase/HMG-CoA synthase chimeric gene have been obtained. An increase in the concentration of mitochondrial HMG-CoA synthase mRNA was detected in these mice, which was associated with a 3-fold increase in HMG-CoA synthase activity in liver mitochondrial extracts. Transgenic mice were normoglycemic and had normal levels of plasma triglycerides and lower free fatty acids. However, the plasma concentration of ketone bodies was about three times higher in transgenic mice than in control animals. Hepatocytes in primary culture from transgenic mice expressed the chimeric gene in a regulated manner and showed a 3-fold increase in β-hydroxybutyrylrate and acetoacete concentrations in the medium. This animal model thus shows that the overexpression of mitochondrial HMG-CoA synthase causes ketone body overproduction, suggesting that this enzyme may be a regulatory step in liver ketogenesis.

The main factor controlling hepatic ketone body production is the rate of acetyl-CoA generation from fatty acids. The latter, in turn, is governed by both the plasma free fatty acids level and the degree of suppression of carnitine palmitoyltransferase I by malonyl-CoA (1, 2). During starvation or diabetes, an increase in the plasma glucagon/insulin ratio suppresses malonyl-CoA synthesis, thus reducing the inhibition of carnitine palmitoyltransferase I and enhancing the capacity of the liver to oxidize fatty acids to acetyl-CoA (1, 2). Mitochondrial 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase catalyzes the condensation of acetoacetyl-CoA with acetyl-CoA to yield HMG-CoA and is believed to be a regulatory enzyme in the ketone body pathway. The activity of liver mitochondrial HMG-CoA synthase increases during starvation, fat feeding, and diabetes (3–5). For any given rate of acetyl-CoA generation, alterations in the level of HMG-CoA synthase could modulate the rate of ketogenesis. The increase in the enzyme activity (6) has been related to an increase in the concentration of the mitochondrial HMG-CoA synthase mRNA (7, 8). In addition, liver HMG-CoA synthase mRNA levels increase after birth (9, 10) because of the increase in plasma glucagon levels (11) and remain high throughout the suckling period (9, 10).

To study the role of mitochondrial HMG-CoA synthase in liver ketogenesis we have produced transgenic mice that overexpress mitochondrial HMG-CoA synthase under the control of the P-enolpyruvate carboxykinase (PEPCK) promoter. Transcription of the PEPCK gene is highly sensitive to glucagon and insulin. Glucagon activates, while insulin inhibits, PEPCK gene expression (12, 13). Thus, like mitochondrial HMG-CoA synthase gene, PEPCK gene expression is markedly increased by starvation and diabetes (14, 15). PEPCK gene transcription is inactive during fetal development but is greatly enhanced at birth (16, 17). The sequences necessary for many of the regulatory properties of the PEPCK promoter are contained within 500 base pairs of the start site of the gene transcription. Two sequences located at −90−−82 and −250−−234 are involved in the responsiveness of the PEPCK promoter to CAMP (18). An insulin-responsive element (−415−−400) and a glucocorticoid-responsive region (−450−−400) have also been described in the PEPCK promoter (18–20). This fragment of the promoter directs the expression of the PEPCK/growth hormone gene mainly to the liver in transgenic mice (21, 22). Thus, the PEPCK promoter may direct the expression of the HMG-CoA synthase in a physiologically regulated manner to the same tissues as the endogenous gene. Transgenic mice expressing the PEPCK/HMG-CoA synthase chimeric gene were normoglycemic and showed an increase in mitochondrial HMG-CoA synthase activity and in ketone body production.

EXPERIMENTAL PROCEDURES

Materials—DNA-modifying enzymes, the random-primer DNA labeling kit, nylon membranes, and Bt2cAMP were obtained from Boehringer Mannheim. [32P]dCTP (3000 Ci/mmol) was from Amersham Corp. All media and sera were obtained from either Life Technologies, Inc. or Boehringer Mannheim. The other reagents used were of analytical grade.

Construction of the PEPCK/HMG-CoA Synthase Chimeric Gene and Generation of Transgenic Mice—The BamHI-XhoI fragment of the rabbit β-globin gene (23) was introduced at the BamHI-XhoI sites of the BlueScript polyninker. This fragment of the β-globin gene includes the last two exons, the last intron, and the 3’ region linked to the SV40 enhancer. The 2-kb EcoRI fragment containing the entire rat liver HMG-CoA synthase cDNA (7), obtained by partial digestion, was introduced at the EcoRI site of the second exon of the β-globin gene. Finally, the XhoI-BglII (−460 bp to +73 bp) fragment of the PEPCK promoter (24) was introduced at the XhoI-BamHI sites of the BlueScript polyninker (Fig. 1). This plasmid was designated pCHMG-CoAS. The

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strategy of introducing β-globin sequences and SV40 enhancer was used to ensure expression of the HMG-CoA synthase cDNA in transgenic mice. A 4.7-kb NotI-XhoI fragment, containing the entire chimeric gene, was microinjected into fertilized eggs (Fig. 1). This chimeric gene has two polyadenylation signals: one at the end of the HMG-CoA synthase cDNA and the other at the end of the β-globin gene.

The general procedures for microinjection of the PEPC/K/HMG-CoA synthase chimeric gene were as described (25). Fertilized mouse eggs were flushed from the oviducts of superovulated C57BL/6J/SJL mice 6–8 h after ovulation. Male pronuclei of the fertilized eggs were injected with 2 pl of DNA solution (approximately 2 ng/μl) and viable embryos were reimplanted in the oviducts of pseudopregnant mice. At 3 weeks of age, the animals were tested for the presence of the transgene by Southern blot of DNA tail samples. Mice were maintained under a light-dark cycle of 12 h (lights on at 8:00 a.m.).

DNA and RNA Analysis—Transgenic mice were identified on Southern blots prepared with 10 μg of tail DNA digested with EcoRI. Blots were hybridized with a 3.2-kb fragment, obtained after partial digestion of the pPHMG-CoAS with EcoRI and radiolabeled with [α-32P]dCTP by random oligopriming. Total RNA was obtained from liver (samples were obtained between 10 and 11 a.m.) or from primary cultures of hepatocytes by the guanidine isothiocyanate method (26), and RNA samples (30 μg) were electophoresed on a 1% agarose gel containing 2.2 M formaldehyde. Northern blots were hybridized to 32P-labeled cDNAs. The HMG-CoA synthase probe corresponded to a 1.43-kb EcoRI–EcoRI fragment of the rat cDNA (7); the rabbit β-globin probe corresponded to a 1.3-kb EcoRI–EcoRI fragment of the CDNA. These probes were labeled using α-32PdCTP, following the method of random oligopriming as described by the manufacturer. Membranes were placed in contact with Kodak XAR-5 film. The β-actin signal was used to correct for loading inequalities.

Preparation and Incubation of Hepatocytes—Hepatocytes were isolated as described by LoCascio et al. (27). After removing nonparenchymal cells and debris, aliquots of 5 × 109 hepatocytes, resuspended in Dulbecco’s minimal essential medium (DMEM) without serum, were incubated at 37°C for up to 90 min with continuous shaking in a 5% CO2 atmosphere. At 30-min intervals, samples (200 μl) were obtained and ketone body production was determined in the incubation medium. The nuclei of hepatocytes were resuspended in DMEM containing 0.2% albumin and 10% fetal calf serum. Cells (5 × 106) were then plated in 10 ml of this medium on collagen-coated dishes. After 4 h the medium was removed and cells were washed three times in DMEM. Then, 10 ml of DMEM was added to the cells, which were maintained in this medium for up to 24 h. Aliquots of 200 μl of the incubation medium were removed at different times, and ketone body production was measured.

To determine the regulation of the expression of the chimeric gene, hepatocytes were cultured for 4 h with DMEM or DMEM with 0.5 mM β2cAMP plus 1 μM dexamethasone, followed by total RNA isolation from the cells.

Enzyme and Metabolite Analysis—Glucose was measured in serum by the hexokinase method (GlucoQuant System, Boehringer Mannheim, Germany). The β-hydroxybutyrate levels in perchloric acid extracts of both serum and incubation medium were determined by the β-hydroxybutyrate dehydrogenase technique (Boehringer Mannheim, Germany). The acetoacetate levels in perchloric extracts of serum and incubation medium were determined as described previously (28). Serum-free fatty acids were measured by the acyl-CoA synthetase and acyl-CoA oxidase method (Wako Chemicals, Germany). Serum triglycerides were determined enzymatically (GPO-PAP, Boehringer Mannheim, Germany).

HMG-CoA synthase activity was determined in mitochondrial extracts obtained from livers of fed control and transgenic mice, using a modification of the method described by Reed et al. (29). One gram of fresh isolated liver was homogenized with 2 ml of buffer in a loose fitting glass homogenizer with a Teflon pestle (clearance, 0.5 mm). The homogenization buffer contained 2 mM Hepes pH 7.2, 0.25 mM sucrose, and 0.1 mM EDTA. The homogenate was diluted with an equal volume of the homogenization buffer and centrifuged at 160 × g for 15 min. The supernatant was centrifuged at 9,000 × g for 15 min. Pellets were rehomogenized with 100 mM potassium phosphate buffer, pH 7.0, containing 0.1 mM EDTA and 0.1 mM diethiothreitol and then centrifuged at 12,000 × g, and pellets were resuspended in the same buffer. Mitochondria were disrupted using a motorized homogenizer (Omni International). HMG-CoA synthase activity was measured in the extracts by spectrophotometric assay as described by Flanagan and West (1976).

Statistical Analysis—All values are expressed as the means ± S.E. Significance of differences was established using the Wilcoxon signed-rank test.

RESULTS AND DISCUSSION

The PEPC/K/HMG-CoA synthase chimeric gene used to obtain transgenic mice is indicated in Fig. 1. Two founder mice were obtained after microinjection, as confirmed by Southern blot analysis. After hybridization of the membranes, three diagnostic bands of 0.6, 1.17, and 1.43 kb were detected in the transgenic mice that were not present in the controls (Fig. 2). These mice were seen to carry about 10 copies of the transgene when compared with standard DNA. In the experiments described below we used F1 and F2 generations (2–4 months old) from the PHMG-1 line, since they expressed higher levels of the transgene. Littermates were used as controls.

To determine the expression of the transgenic, RNA was obtained from the liver of fed and starved control and transgenic mice. Two transcripts, of 2.0 and 2.5 kb, were detected in the liver of transgenic mice when Northern blots were hybridized with HMG-CoA synthase cDNA (Fig. 3). The 2.0-kb transcript came from the expression of the endogenous gene and also that of the chimeric gene when the transcript was polyadenylated at the first signal. The 2.5-kb transcript was polyadenylated at the signal at the end of the β-globin gene. Indeed, this was the only transcript detected when the same Northern blot was probed with the last exon of the rabbit β-globin gene (data not shown). The levels of both mRNA transcripts (2.0 and 2.5 kb) were higher (about 3-fold) in the liver of transgenic mice than that of the endogenous transcript in control mice. Starvation resulted in an increase in the expression of both the endogenous gene and the transgene (Fig. 3).

The overexpression of liver mitochondrial HMG-CoA synthase mRNA in transgenic mice was related with an increase in liver mitochondrial HMG-CoA synthase activity. Extracts of liver mitochondria from fed transgenic mice had HMG-CoA synthase activity that was 3-fold higher than that from fed control animals (Fig. 4). Serum analysis indicated that levels of ketone bodies increased 3-fold in transgenic mice (Table I). Control and transgenic mice had similar concentrations of serum glucose and triglycerides. However, transgenic mice showed a significant decrease in free fatty acid levels (Table I). These results suggest that ketone bodies are overproduced in transgenic mice, which may lead to a decrease in the rate of lipolysis in adipose tissue.

To extend our study of the hormonal regulation of the expression of mitochondrial HMG-CoA synthase, hepatocytes from control and transgenic mice were cultured. RNA was isolated from hepatocytes treated with a combination of β2cAMP and dexamethasone, which enhances the expression of the PEPC promoter (18, 20). Fig. 5 shows that the expression of the transgene increased to the same extent as the endogenous gene. A 7-fold increase in the 2.5-kb mRNA transcript was observed after 4 h of treatment with cAMP plus dexamethasone. The expression of the endogenous HMG-CoA synthase gene was

![Fig. 1. Schematic representation of the PEPC/K/HMG-CoA synthase chimeric gene used to create transgenic mice. The strategy of introducing β-globin sequences and SV40 enhancer was used to ensure expression of the HMG-CoA synthase cDNA in transgenic mice.](image-url)
Mitochondrial HMG-CoA Synthase

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8, control mice.

Experimental Procedures." Southern blots were hybridized with the probe as indicated under "Experimental Procedures." Genomic mice from the F1 generation of the PHMG-1 line; restriction fragments (1.43, 1.17, and 0.6 kb) were obtained when from tail samples and digested with EcoRI restriction enzyme. Three and 2 copies/genome, respectively; 2.0 and 2.5 kb, were detected in transgenic mice, resulting from the different polyadenylation signals used in the termination of the transgene. A 2.0-kb band in the transgenic lanes.

Fed control and transgenic mice. Values obtained from mice are indicated with different symbols. Fed control mice (Con), n = 10; fed transgenic mice (Tg), n = 10. Also induced by Bt2cAMP and dexamethasone, as described elsewhere (7, 8).

Since the transgene was expressed in short term cultured hepatocytes, ketone body production was further studied in both isolated and cultured hepatocytes. Isolated and cultured hepatocytes were incubated in DMEM for up to 90 min and 24 h, respectively, and at different times an aliquot of the medium was obtained and ketone body production was measured. In both isolated and cultured hepatocytes the overexpression of mitochondrial HMG-CoA synthase caused a 3-fold increase in the production of ketone bodies (Fig. 6). These results suggest that mitochondrial HMG-CoA synthase is a key enzyme regulating ketogenesis in hepatocytes.

Therefore, the results obtained in this study demonstrate that mitochondrial HMG-CoA synthase can be overexpressed

FIG. 2. Analysis by Southern blot of the presence of the PEPCk/HMG-CoA synthase chimeric gene. DNA was obtained from tail samples and digested with EcoRI restriction enzyme. Three restriction fragments (1.43, 1.17, and 0.6 kb) were obtained when Southern blots were hybridized with the probe as indicated under "Experimental Procedures." Lanes 1 and 7, control DNA (equivalent to 5 and 2 copies/genome, respectively); lanes 2, 3, 4, 9, 10, and 11, transgenic mice from the F1 generation of the PHMG-1 line; lanes 5, 6, and 8, control mice.

FIG. 3. Expression of the PEPCk/HMG-CoA synthase chimeric gene. The expression of the chimeric gene was analyzed by Northern blot from RNA isolated from liver of both fed and 24-h starved mice, as indicated under "Experimental Procedures." Two mRNA transcripts, of 2.0 and 2.5 kb, were detected in transgenic mice, resulting from the different polyadenylation signals used in the termination of the transcription of the transgene. The endogenous gene also contributed to the 2.0-kb band in the transgenic lanes. Con, fed control mice; Tg, fed transgenic mice; Stv-Con, starved control mice; Stv-Tg, starved transgenic mice.

FIG. 4. Mitochondrial HMG-CoA synthase activity in liver of fed control and transgenic mice. The activity of mitochondrial HMG-CoA synthase was determined as indicated under "Experimental Procedures." Values obtained from mice are indicated with different symbols. Fed control mice (Con), n = 10; fed transgenic mice (Tg), n = 10.

FIG. 5. Expression of the PEPCk/HMG-CoA synthase chimeric gene in hepatocytes in primary culture. The concentration of mitochondrial HMG-CoA synthase mRNA was determined in cultured hepatocytes from fed control (lanes 1 and 2) and transgenic mice (lanes 3 and 4). Hepatocytes were cultured for 4 h in DMEM alone (Con) (lanes 1 and 3) or in DMEM supplemented with 0.5 mM Bt2cAMP plus 1 μM dexamethasone (cAMP + Dex) (lanes 2 and 4). RNA was isolated and analyzed as indicated under "Experimental Procedures."

TABLE I

Metabolic parameters in serum of fed control and transgenic mice

| Parameter                  | Control          | Transgenics         |
|----------------------------|------------------|---------------------|
| β-Hydroxybutyrate (mmol/l) | 0.11 ± 0.02      | 0.29 ± 0.03         |
| Acetocetate (mmol/l)       | 0.06 ± 0.01      | 0.14 ± 0.02         |
| Glucose (mg/dl)            | 190 ± 20         | 198 ± 22            |
| Triglycerides (mg/dl)      | 84.2 ± 8.1       | 80.7 ± 8.6          |
| Free fatty acids (mmol/l)  | 0.64 ± 0.5       | 0.41 ± 0.6          |

FIG. 6. Ketone production by isolated and cultured hepatocytes. Ketone bodies were measured in the incubation medium of isolated (A) and cultured (B) hepatocytes from fed control and fed transgenic mice as indicated under "Experimental Procedures." Hepatocytes were incubated in DMEM alone. At the indicated times, an aliquot of the medium was removed and ketone body concentration was determined. Results are mean ± S.E. of four independent experiments. Con, fed control mice; Tg, fed transgenic mice.
in the liver of transgenic mice and that the regulation of the transgene is similar to that of the endogenous mitochondrial HMG-CoA synthase gene. This higher expression is accompanied by an increase in enzyme activity. In the absence of free fatty acid mobilization, transgenic mice have raised levels of ketone bodies in their serum. Our transgenic model presents a 3-fold increase in basal levels of blood ketone bodies when compared with controls, which is similar to that observed in non-insulin-dependent diabetes mellitus patients (31). Thus, transgenic mice expressing the PEPCK/HMG-CoA synthase chimeric gene are a model that may be used to study long term effects of chronic hyperketonemia in the development of diabetic alterations, since these mice have increased plasma levels of ketone bodies and normal concentration of glucose. These conditions are often difficult, if not impossible, to separate in the animal models available to date.

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