Tissue-specific control of rat malic enzyme activity and messenger RNA levels by a high carbohydrate diet

(BEATRICE DOZIN, JOSEPH E. RALL, AND VERA M. NIKODEM*)

Clinical Endocrinology Branch, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892

Contributed by Joseph E. Rall, March 14, 1986

ABSTRACT In euthyroid rats fed a high carbohydrate fat-free diet for 10 days, the mass of cellular malic enzyme mRNA in liver is increased 7- to 8-fold above the basal level. Malic enzyme activity is stimulated to the same extent. This effect does not result from an increase either in the transcriptional activity of the malic enzyme gene, as determined by nuclear run-off transcription assay, or in the content of intranuclear malic enzyme RNA sequences. Mathematical modeling shows that this increase in cytoplasmic mRNA is compatible with retarded degradation of cytoplasmic mRNA. Regulation of malic enzyme by carbohydrates is liver-specific, since no response is observed in the following nonhepatic tissues: brain, heart, spleen, kidney, testis, and lung. Furthermore, the amplitude of the response in liver depends on the thyroid state of the animals, being lower (by a factor of ~4) in hypothyroidism and higher (12- to 15-fold) when normal animals are injected simultaneously with a daily dose of 15 µg of triiodothyronine per 100 g of body weight for 10 days. Since thyroid hormone regulates liver malic enzyme synthesis predominantly at the nuclear level and carbohydrates at the cytoplasmic level, the additive effect of triiodothyronine and a high carbohydrate diet on the activity of malic enzyme is readily explicable.

A diet high in carbohydrates (CHO) stimulates malic enzyme [ME; (S)-malate:NADP+ oxidoreductase (decarboxylating) activity in rodent (1–3) and avian (4, 5) liver, while starvation or a low CHO diet has the opposite effect. This stimulation by CHO has also been demonstrated in vitro when isolated hepatocytes were cultured in medium enriched in glucose (6). In both mammals and birds, the increase in liver ME activity has been shown to reflect a proportionate increase in the relative rate of enzyme synthesis (7–9). Furthermore, in geese and mice, the rate of ME synthesis correlates positively with the concentration of ME mRNA in cytoplasm, as determined by hybridization analyses with ME-specific cDNAs (10, 11). In rat liver, such a pretranslational control has been suggested by in vitro translation assays followed by immunoprecipitation with ME antibody (9).

CHO have also been shown to interact with thyroid hormones in modulating ME activity in hepatocytes (12). We have recently reported that triiodothyronine (T3) by itself controls ME synthesis in thyroid hormone-responsive tissues at the nuclear level through both an increase in the rate of specific gene transcription and a decrease in degradation of nuclear ME RNA sequences, the latter site of control being liver specific (13; unpublished data). In the present study, we attempted to define precisely the level at which CHO regulates the concentration of ME. Hence, we investigated the effects of a high CHO fat-free diet on the rate of transcription of the ME gene and on the levels of nuclear and cytoplasmic ME RNA sequences. Our data suggest that, contrary to thyroid hormone, CHO stimulates ME synthesis predominantly by stabilizing the cytoplasmic mRNA encoding the protein. Furthermore, the effect is tissue specific, since none of the nonhepatic tissues tested was responsive to CHO feeding. Therefore, ME activity in rat liver is controlled by at least three of the known mechanisms: gene transcription rate, nuclear RNA stabilization, and rate of degradation of cytoplasmic mRNA.

MATERIALS AND METHODS

Animals. Female Sprague–Dawley rats weighing 120 g were used in all experiments. To induce ME, a high CHO fat-free diet (ICN) was provided ad libitum to either euthyroid or hypothyroid animals. All experiments were started between 7:00 and 9:00 p.m., at which time the animals were feeding. Hypothyroidism was achieved by surgical thyroidectomy (performed by the supplier, Taconic Farms, Germantown, NY) followed by feeding the rats a low-iodine diet (Teklad Diet). A hypothyroid state was confirmed by the cessation of weight gain for at least 2 consecutive weeks and by thyroid stimulating hormone and T3 serum level measurements at sacrifice (>15 µg/dl and 38 ng/dl, respectively). In some experiments, further induction of ME was obtained by injecting the animals with a daily dose of 15 µg of T3 per 100 g of body weight simultaneously with CHO feeding. For both euthyroid and hypothyroid states, control rats were maintained on a regular chow diet. The animals were sacrificed by CO2 inhalation at the time intervals indicated in the tables and figures.

cDNA Clones and [3H]cRNA + Synthesis. Blot hybridization assays for ME were performed with the 1250-base-pair HindIII/Pvu II restriction fragment excised from the recombinant DNA prME described previously (14) and nick-translated (15) with [α-32P]dCTP (5000 mCi/mmol; 1 Ci = 37 GBq; New England Nuclear) to a specific activity of 1–2 x 106 cpm per µg of DNA. For in vitro transcription assays, the ME cDNA probe was the 1627-base sequence excised from the λgt11 recombinant DNA λME-X7 (16) and subcloned into the Sst I and Kpn I sites of M13 mp18. The albumin cDNA probe was an 1800-nucleotide sequence, obtained in our laboratory, subcloned into the Sal I and Xba I sites of pUC13, and whose identity had been confirmed by sequencing. [3H]cRNA + complementary to a Pst I/EcoRI restriction fragment excised from prME was synthesized as described by Mueckler et al. (17) in the presence of 250 µCi of [3H]ATP (46.2 mCi/mmol; New England Nuclear). The product gave a specific activity of 2 x 107 cpm per µg of RNA.

ME Activity. ME activity was assayed according to the procedure of Hsu and Lardy (18) as described (19). Total

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.
protein concentration was measured by the method of Bradford (20) with bovine serum albumin as standard.

**Total and Poly(A)⁺ Cellular RNA Preparation.** Total RNA was extracted from the tissues by the lithium chloride/urea procedure of Auffray and Rougeon (21). Poly(A)⁺ RNA was purified by chromatography on oligo(dT)-cellulose (22).

**Nuclear Poly(A)⁺ RNA Purification.** Liver was perfused in situ with Dulbecco's phosphate-buffered saline containing 200 µg of heparin per ml. The tissue was homogenized in 5% (wt/vol) 20 mM Tris-HCl, pH 7.6/100 mM KCl/10% (vol/vol) glycerol/0.1% Triton X-100. Nuclei were pelleted by centrifugation at 4000 × g for 10 min and washed four times with the same buffer except that Triton X-100 was omitted during the last two washes. The lack of contamination of the nuclear preparation with cytoplasm by this method has been demonstrated by assessing the content of ME mRNA remaining in the supernatants of the four successive washes. Total nuclear RNA was extracted as described by Cathala et al. (23). Poly(A)⁺ RNA was purified as indicated above.

**Dot-Blot Hybridization.** Dot-blot hybridization assays were performed according to the procedure of Thomas (24). Total cellular RNA samples (150 µg each) were blotted on 490-mm² nitrocellulose filters (BA 85, Schleicher & Schuell), whereas poly(A)⁺ RNA samples (5 µg each) were blotted on nitrocellulose membrane by using the Schleicher & Schuell 96-well manifold. All assays were performed in duplicate. Hybridization with the 32P-labeled ME cDNA probe was carried out as described (14, 19). After the filters were washed, the radioactive signal in individual dots was quantitated by β liquid scintillation counting.

**In Vitro Run-Off Transcription Assay.** Liver was perfused as described above. The tissue was homogenized in 10% (wt/vol) buffer A (10 mM Tris-HCl, pH 7.6/40% (vol/vol) glycerol/10 mM MgCl₂/10 mM NaCl) containing 0.1% Triton X-100, and precooled at −20°C. Nuclei were pelleted by centrifugation at 4000 × g for 10 min at −15°C, washed twice with the same buffer without Triton X-100, repelleted by centrifugation at 4°C at 150,000 × g for 65 min through a 2.1 M sucrose cushion made in 10 mM Tris-HCl, pH 7.6/10 mM MgCl₂, and stored in buffer A at −70°C at a concentration of 1.5 to 4 × 10⁸ nuclei per ml. In vitro transcription assays were performed by the method of Clayton and Darnell (25) with the following modifications: nuclei were adjusted to 50 absorbance units at 260 nm (determined in 1% sodium dodecyl sulfate) and the reaction medium contained 1 mM each ATP, GTP, and CTP; RNasin ribonuclease inhibitor at 1 unit/ml (Promega Biotec, Madison, WI); and 250 µCi of [α-32P]UTP (3000 mCi/mmol; New England Nuclear). Incubation was carried out at 30°C for 20 min and the reaction was terminated by digestion with DNase I and proteinase K (100 µg/ml each) in the presence of 10 mM CaCl₂ (26). After yeast tRNA was added to a final concentration of 25 µg/ml, RNA was extracted with phenol/chloroform (1:1) and precipitated with 0.5 vol of 7.5 M ammonium acetate and 2.5 vol of 95% ethanol. RNA hybridization to the ME and albumin cDNAs described above was carried according to McKeown and Palmiter (27). The specific cDNAs (5 µg each per assay) were immobilized on nitrocellulose filters. Nonspecific hybridization was estimated by blotting in parallel equal amounts of the respective vectors, M13 mp18 and pUC. All plasmid DNAs were denatured prior to binding to the filters. Hybridization efficiency was monitored by supplementing the medium with 9 × 10⁶ cpm of ME [3H]cRNA. Since transcription rates of albumin and ME genes were determined simultaneously in the same hybridization assay, hybridization efficiency for albumin RNA was assumed to be similar to that estimated for ME RNA. After hybridization, the filters were washed and digested with RNases A and T₁ (1 µg/ml and 10 units/ml, respectively) (25). The [32P]- and [3H]RNAs were eluted (27) and the radioactivity in individual dots was quantitated in Beckman Ready Solv HP. The relative rates of gene transcription were expressed as parts per million (ppm) as follows: for the ME gene, [cpm (ME-X7 filter – M13 mp18 filter)/cpm × 10⁻⁶ in total transcription] × 100/hybridization efficiency; for the albumin gene, [cpm (albumin filter – pUC filter)/cpm × 10⁻⁶ in total transcription] × 100/hybridization efficiency.

**RESULTS**

In rat liver, the increase in ME activity in response to a high CHO diet has been shown by in vitro translation assay to result from a proportionate change in the template activity of the mRNA encoding the protein (9). This observation was consistent either with a modification in the translation efficiency of preexisting mRNA or an authentic increase in the mass of cytoplasmic ME mRNA. To distinguish between these two possibilities, we performed hybridization assays with the ME cDNA prepared in our laboratory (14) after feeding euthyroid rats a high CHO fat-free diet for different time intervals. The results of these experiments are summarized in Fig. 1 and indicate that CHO promotes the accumulation of cellular ME mRNA to the same extent as the enzyme activity. After 3–10 days of treatment, the levels of induction were ~7.3-fold for ME mRNA and ~7-fold for enzyme activity. Thus, CHO regulates the synthesis of hepatic ME at a pretranslational level by increasing the amount of the specific mRNA in cytoplasm.

Additional experiments provided some insight into the interaction between CHO and thyroid hormones. ME activity was not detectable in hypothyroid rats on a regular chow diet, as reported previously (19), but reached 31 units per mg of protein after 10 days of CHO feeding. Under the same conditions, ME mRNA levels were increased ~10-fold compared with those in euthyroid CHO-fed rats. In contrast, CHO did not affect ME mRNA levels in the euthyroid rats, even though the ME activity increased nearly 5-fold. These results suggest that CHO stimulates ME mRNA levels through a mechanism that is independent of thyroid hormone action.

**FIG. 1.** Effects of CHO on the levels of ME activity and cellular ME mRNA in liver under various thyroid states. Euthyroid (EU) and hypothyroid (HYP) rats were fed a high CHO fat-free diet for the time intervals indicated, ranging from 3 to 10 days. For additional induction, some euthyroid animals were simultaneously injected with a daily dose of 15 µg of T₃ per 100 g of body weight for the same periods of time. ME activity (A) and ME mRNA levels (B) were assayed in parallel. The limit of detection of ME activity was 1.2 units per mg of protein. The hybridized cpm values were corrected by subtracting the filter and machine background (135 cpm). Individual values of two separate determinations, each performed in duplicate, are indicated. Cₑ, untreated euthyroid; Cₜₑ, untreated hypothyroid; CHO, carbohydrate-treated; CHO + T₃, carbohydrate- and T₃-treated; ND, not detectable. Numbers (3, 6, and 10) indicate days of treatment.
conditions, the amount of ME mRNA was increased ∼4.4-fold (Fig. 1).

We have reported that T₃ treatment of euthyroid rats led to a proportionate stimulation of ME activity and ME mRNA ∼10-fold above the basal levels (19). When both CHO and T₃ were combined, ME mRNA accumulated to a greater extent (12- to 15-fold above the basal value; Fig. 1) than under administration of either stimulus alone. ME activity was induced to the same degree, which suggests that the interaction between CHO and thyroid hormones occurs at a pretranslational level.

The ubiquitous distribution of ME and ME mRNA has been demonstrated by immunological analysis with ME antibody (28) and hybridization assays with ME cDNA (19). To gain some information about the tissue specificity of ME regulation by nutritional factors, we assessed the levels of ME activity and ME mRNA after CHO feeding in the following tissues: brain, lung, testis, kidney, spleen, and heart. The ratios of CHO-treated versus control samples were as follows: 1.03, 1.08, 0.95, 1.06, 0.98, 1.15, and 0.93, 1.29, 1.1, 0.91, 0.93, 1.0, respectively. Hence, no response in either enzyme activity or mRNA could be detected in any of these tissues.

To define more precisely the site of action of a high CHO diet on ME mRNA, we measured the rate of transcription of the ME gene under CHO stimulation. In vitro run-off transcription assays with nuclei isolated from control and CHO-fed rat livers were performed in which RNA chains initiated in vivo were allowed to elongate in the presence of [32P]UTP. ME RNA sequences were then detected within the nuclear RNA population by hybridization to the complementary single-stranded ME cDNA described in Materials and Methods. An albumin cDNA was included simultaneously as an internal control. The concentrations of cellular ME mRNA were estimated in parallel. The results of three separate determinations are summarized in Table 1. For each assay, the level of incorporation of [32P]UTP into total RNA was not affected by the nutritional state of the animals, which excludes a general effect of CHO on overall RNA synthesis. Likewise, the rates of transcription of the ME gene in control and CHO-treated rat liver nuclei were not significantly different, ranging from 7.8 to 8.8 cpm, whereas the amount of cellular ME mRNA was increased 6.7- to 8.5-fold above the basal level. The rate of transcription of the albumin gene serving as a control for the technique averaged 340 cpm, which is in good agreement with the values reported by others (29).

Since the relative rate of transcription of the ME gene was not altered by CHO, a posttranscriptional control had to be invoked to account for the accumulation of ME mRNA in cytoplasm. Among possible mechanisms were alterations in the processing of the nuclear primary transcript or changes in the rate of degradation of the nuclear and/or cytoplasmic RNA sequences specific for ME. Table 1 shows the results of dot-blot hybridizations with nuclear poly(A)⁺ RNA from control and CHO-treated rat livers, performed simultaneously with cellular mRNA assays. As can be seen, the level of nuclear ME RNA sequences was not modified by the dietary treatment, which caused a 7.2-fold increase in the mass of cytoplasmic ME mRNA. This suggested that the effect of CHO was on the stability of ME mRNA in cytoplasm, and this was tested further by evaluating changes in the degradation of ME mRNA upon CHO treatment. Euthyroid rats were fed the high CHO diet, and the level of ME mRNA was determined at the time intervals indicated in Fig. 2. ME mRNA accumulated rapidly to reach a maximal concentration ∼7 times higher than under regular chow diet after 17 hr of treatment (Fig. 2). The rate of accumulation of ME mRNA during this period was exponential with a half-time value of 3–4 hr, as calculated from the semilogarithmic plot (30, 31). After CHO feeding for 48 hr, the level of ME mRNA remained at its maximal value for at least 4 hr after shifting to regular chow diet; subsequently, it decreased, eventually reaching the basal value at 22 hr. A simple model to describe these events is shown in Fig. 3A. Two hypotheses were considered for examining the effect of a high CHO diet. These hypotheses are based on the biochemical assumption that a high CHO diet causes a change (increase or decrease) in some factors that affect either (i) the rate of degradation of cytoplasmic ME mRNA or (ii) the proportionation of nuclear ME RNA between degradative pathways and transport into the cytoplasm.

These hypotheses assume that the transcription rate measured in vitro accurately reflects in vivo transcription and is therefore unchanged by diet. These changes were incorporated into the models shown in Fig. 3 B and C. In Fig. 3B, λ,r,2, the rate constant of degradation of cytoplasmic mRNA, was abruptly slowed when the high CHO diet began, and it returned to a slightly lower value than control shortly after return of the animals to a regular diet. The simulation of the model in Fig. 3B is shown in Fig. 2 as a solid line. In Fig. 3C, the ratio between the fraction of nuclear ME RNA degraded to that transferred to the cytoplasmic pool was changed. The sum of the rate of degradation and the rate of transfer remained constant for model simulation. The relative values

Table 1. Effects of CHO on ME gene transcription, and total cellular and nuclear ME RNA sequences in euthyroid rat liver

<table>
<thead>
<tr>
<th>Total RNA, ME gene, ME mRNA, cpm × 10⁻⁶</th>
<th>Ratio vs. control</th>
<th>Albumin, cpm per 150 μg of RNA</th>
<th>Ratio vs. control</th>
<th>ME nuclear RNA, cpm per 5 μg of poly(A)⁺ RNA</th>
<th>Ratio vs. control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 40 7.8 1.0 326 1.0 210 1.0 520 1.0</td>
<td>Control 38 7.9 1.0 398 1.21 1407 6.7 509 0.97</td>
<td>Control 30 8.5 1.0 — — 307 1.0 492 1.0</td>
<td>Control 32 8.3 0.9 — — 2610 8.5 542 1.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 21 8.1 1.0 324 1.0 286 1.0 570 1.0</td>
<td>Control 40 7.8 1.1 297 0.92 2088 7.3 654 1.15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Euthyroid rats were fed a high CHO fat-free diet for 10 days. Relative rates of transcription for the ME and albumin genes were estimated as described and are expressed as cpm. Each assay was performed in duplicate with nuclei isolated from two livers and pooled. Individual values of three separate experiments are given. Hybridization efficiency averaged 30%. Non-specific hybridization to M13 mp18 and pUC was 3 ppm and 7 ppm, respectively. Total cellular ME mRNA and nuclear ME RNA were quantitated in parallel by dot-blot hybridization with 150 μg of total RNA and 5 μg of poly(A)⁺ RNA, respectively, per assay. Values are means of two separate determinations, each performed in duplicate with RNA extracted from two livers and pooled. Hybridized cpm values were corrected by subtracting the filter and machine background (107 cpm).
Fig. 2. Kinetics of accumulation and decay of ME mRNA in rat liver upon CHO feeding and deprivation. The animals were sacrificed at the time intervals indicated. Total RNA was extracted and 150-μg samples were blotted on nitrocellulose filters and hybridized with the 32P-labeled ME cDNA probe. Each point represents the mean of two separate experiments, each performed by testing individual RNA preparations in duplicate. The hybridized cpm were corrected by subtracting the filter and machine background (630 cpm).

of these rate constants are shown in Fig. 3C, and the model simulation appears as a dashed line in Fig. 2.

**DISCUSSION**

In the present study, we have attempted to understand the biochemical mechanism(s) underlying the regulation of ME synthesis by CHO in the rat. We have demonstrated that this dietary control is pretranslational and tissue specific, since proportionate increases in ME activity and cellular ME mRNA were observed only in the liver. Since the rate of transcription of the ME gene and the level of nuclear ME RNA sequences were unchanged by a high CHO diet, we suspected that a decrease in the rate of degradation of cytoplasmic ME mRNA was the cause for the increase in cytoplasmic ME mRNA.

Mathematical modeling of the data is compatible with the effect of CHO being due solely to a decrease in the rate of degradation of ME mRNA in cytoplasm, although a more complicated mechanism involving changes in both the rate of degradation of nuclear ME RNA and its transport to cytoplasm will also fit the data. We favor stabilization of ME mRNA, since the data are fitted somewhat better with this simulation and because a change in the ratio of pre-mRNA between degradation and transport to the cytoplasm requires two mechanisms. In other systems, notably when specific proteins are increased by hormones, stabilization of mRNA has been suggested (32-34). In the present case, proof that a high CHO diet increases the stability of cytoplasmic ME mRNA will require more direct biochemical data.

The nutritional regulation of ME in avians has also been studied. A pretranslational control in goose uropygial gland and liver has been reported by Winberry et al. (10). Using in vitro transcription assays in isolated nuclei, Goldman et al. (31) recently demonstrated that in duck liver, ME synthesis is regulated by CHO not only through a decrease in the rate of degradation of cytoplasmic ME mRNA but also by a stimulation of the transcriptional rate of the ME gene. The existence of this additional nuclear control, which we did not observe in rat liver, may be related to the type of species analyzed.

ME synthesis in rat liver is known to be regulated also by several hormonal factors acting either together with or independently of the nutritional state of the animal (35). A stimulating interaction between thyroid hormones and CHO has been reported by Towle et al. (9), who demonstrated that the combination of both effectors promoted a higher response in enzyme activity and translatable ME mRNA levels than either stimulus alone. Our data support this observation, since T3 and a high CHO diet by acting at different sites should in combination give a greater effect than either agent alone. The molecular basis of the primary action of both thyroid hormones and CHO remains to be elucidated.

We are greatly indebted to Drs. Peter Grief and David Covell for discussion and for the computer modeling resulting in the curves shown in Fig. 2.

---