Thyroid Hormone-mediated Transcriptional Activation of the Rat Liver Malic Enzyme Gene by Dehydroepiandrosterone

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Dehydroepiandrosterone (DHEA), a naturally occurring steroid secreted from the adrenal, has been reported to decrease the body weight gain in rodents without suppressing food intake and to stimulate malic enzyme activity in liver (Tepperman, H. M., de la Garza, S. A., and Tepperman, J. (1968) Am. J. Physiol. 214, 1126-1132). Herein, we demonstrate that DHEA induces hepatic malic enzyme activity by increasing the rate of transcription of the malic enzyme gene. This transcriptional activation of the malic enzyme gene is dose dependent, i.e. the treatment of euthyroid male rats with daily doses of 17.5 and 35 mg of DHEA/100 g of body weight for 7 days elevated the rate of malic enzyme gene transcription in liver above the basal levels 4-5- and 8-9-fold, respectively. The levels of nuclear malic enzyme RNA, cytoplasmic malic enzyme mRNA, and enzyme activity were increased correspondingly. Malic enzyme stimulation by DHEA was liver specific, i.e. malic enzyme activity in brain, heart, kidney, and testis was unchanged. Thyroid hormone is required for the induction of hepatic malic enzyme activity by DHEA since in hypothyroid animals, DHEA was without effect. However, stimulatory effects of thyroid hormone and DHEA on malic enzyme expression are additive in euthyroid rat livers at both levels of gene transcription and enzyme activity.

In our laboratory, we have cloned a cDNA for rat liver malic enzyme (t-malate:NAD\(^+\)-oxidoreductase (decarboxylating), EC 1.1.40) (14), which generates NADPH, and studied the molecular mechanism(s) involved in the induction of malic enzyme message following thyroid hormone treatment or carbohydrate feeding. We have shown that in rat liver, thyroid hormone activates transcription of the malic enzyme gene (3-4-fold) and decreases the rate of degradation of premRNA coding for this enzyme with an overall 10-12-fold elevation in malic enzyme mRNA in cytoplasm (15-17). By contrast, a high carbohydrate diet increases malic enzyme activity (7-8-fold) by stabilizing mRNA in the cytoplasm (18).

In this report, we wish to analyze molecular mechanisms underlying augmented activity of rat liver malic enzyme after DHEA treatment. Our results demonstrate that DHEA induces malic enzyme activity by stimulating the rate of transcription of the gene only in liver. An 8-9-fold stimulation in gene transcription fully accounts for parallel increases in the levels of mRNA and malic enzyme activity. Interestingly, thyroid hormone is absolutely required for the induction of hepatic malic enzyme by DHEA, whereas growth hormone has only a slight effect.

**EXPERIMENTAL PROCEDURES**

Materials—Remington iodine-deficient test diet and low iodine test diet were purchased from Teklad Diets. Calcium-L(+)-lactate, guanidine monothiocyanate, formamide, and formaldehyde were from Fluka. Dehydroepiandrosterone, MOPS, NADP, L(-)-malic acid, 3,5,3'-triiodothyronine (T\(_3\)), oligo(dT)-cellulose, bovine serum albumin (Pentax, fraction V), salmon sperm DNA, 2-mercaptoethanol, polyvinylpyrrolidone, Ficoll, phenylmethylsulfonyl fluoride, p-sorbitol, dithiothreitol, Nonidet P-40, and spermidine were obtained from Sigma. Rat growth hormone (NIDDK-rGH-B-10) was a gift from the NIDDK National Hormone and Pituitary Program located at the University of Maryland School of Medicine. Agarose, phenol, and proteinase K were purchased from Bethesda Research Laboratories. Restriction endonucleases were from New England BioLabs. Ribonuclease triphosphates and pUC19 were obtained from Pharmacia LKB Biotechnology Inc. DNase I was purchased from Copper-Worthington. RNasin was from Promega Biotech. [\(\alpha\)-\(32\)P]UTP (800 Ci/mmol) and [\(\alpha\)-\(32\)P]UTP (800 Ci/mmol) were obtained from Du Pont-New England Nuclear. BA-85 nitrocellulose filters were purchased from Schleicher & Schuell. All other chemicals were reagent grade. For experiments involving RNA, buffer solutions were autoclaved or filtered through 0.2-\(\mu\)m membranes, and all glassware was baked at 200°C for at least 2 hr.

Animals—Male Sprague Dawley rats (Taconic Farms) weighing 120-150 g were used in all experiments. The stimulation by DHEA of hepatic malic enzyme was performed by subcutaneous injection of rats of different thyroidal states with previously suggested doses (4-9) of either 17.5 or 35 mg of DHEA (suspended at 250 mg/ml in sesame oil by brief sonication)/100 g of body weight between 9 and 10 a.m. daily for the length of time indicated in the text. Control animals received an equivalent volume of sesame oil. Serum levels of DHEA, DHEA sulfate, T\(_3\), T\(_4\), and thyroid-stimulating hormone at the time of death were determined by the Hazeltor. Laboratories, Vienna, VA. All euthyroid animals were maintained on a regular chow diet. In some experiments, hypothyroidism was induced by surgical thyroidectomy followed by feeding the rats a low iodine diet and 1% calcium lactate. A hypothyroid state was confirmed by the cessation of

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The abbreviations used are: DHEA, 3\(\beta\)-hydroxy-5-androsten-17-one; dehydroepiandrosterone; T\(_3\), 3,5,3'-triiodothyronine; T\(_4\), 3,5,3',5'-tetraiodothyronine; MOPS, 3-(N-morpholino)propanesulfonic acid.
of weight gain for at least 2 consecutive weeks and by the measurement of serum T₃, T₄, and thyroid-stimulating hormone levels (average 34 ng/dl, 1.4 μg/dl, and 32 ng/ml, respectively). Stimulation of hypothyroid rats with T₃ was achieved by a daily intraperitoneal injection of 50 μg of T₃/100 g of body weight for 7 days. T₃ was dissolved in 0.1 N NaOH at a concentration of 3.75 mg/ml and diluted 10 times with saline (0.85% NaCl). Treatment of hypothyroid rats with growth hormone (dissolved in 0.03 M NaHCO₃, 0.15 M NaCl, pH 10.8, at a concentration of 1.0 mg/ml) was performed by intra-peritoneal injection (daily dose of 100 μg/100 g of body weight) for 9 days beginning 2 days prior to the onset of DHEA treatment (35 mg of DHEA/100 g of body weight each day for 7 days). Control animals for T₃ and growth hormone stimulation received the same volume of the appropriate vehicle. Animals were killed between 9 and 11 a.m. by CO₂ inhalation.

Malic Enzyme Assays—Cytosolic malic enzyme activity was measured according to the method of Hu and Lardy (19) as described elsewhere (15). One unit of enzyme activity is the amount that reduces 1 nmol of NADP⁺ in 1 min. The concentration of protein present in the assay was measured by using the Bio-Rad protein assay dye reagent with bovine serum albumin as standard (20).

Preparation and Radiolabeling of DNA Probes for Hybridization—The full length malic enzyme cDNA probe (≈5 kb long) was prepared by digesting the plasmid pMBB with BamHI and BglII as described previously (17). The malic enzyme-specific intron probe MEI-C was a 1.5-kilobase BamHI-BamHI restriction fragment excised from the malic enzyme genomic clone λ18E (17). MEI-C did not contain repetitive sequences or other malic enzyme exon sequences and did not hybridize with any liver cytoplasmic poly(A)⁺ RNA sequences. Rat β-actin cDNA (21) was used as an internal reference in RNA blots. All DNA probes were 32P-labeled using a nick translation kit purchased from Bethesda Research Laboratories to a specific activity of ≈2 × 10⁶ cpm/μg of DNA.

Preparation of Cellular Poly(A)⁺ RNA—The RNA extraction method described by Cathala et al. (22) was used to prepare total cellular RNA from livers of euthyroid or DHEA-stimulated rats. Poly(A)⁺ selection was carried out by chromatography on oligo(dT)-cellulose (23).

Preparation of Nuclear Total RNA—Nuclei were prepared from livers of control or DHEA-treated euthyroid rats according to the procedure of Blobel and Potter (24). The nuclear pellet was briefly vortexed in buffer containing Triton X-100 (10 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 0.32 mM sucrose, 0.1% Triton X-100) and centrifuged immediately. After two successive washes in detergent-free buffer, nuclear RNA was prepared employing the guanidine monothiocyanate/lithium chloride method of Cathala et al. (22).

Slot Blot Hybridization—Slot blots were made with cellular poly(A)⁺ RNA (0.5 μg) or total nuclear RNA (5 μg) using a Schleicher & Schuell Minifold II slot blotter. Hepatic RNA samples purified from sesame oil- or DHEA-injected rats were denatured by heating at 55–60°C for 15 min in 14 × SSC, 2.2 M formaldehyde (1 × SSC = 0.15 M sodium chloride and 0.15 M sodium citrate) and cooled on ice before spotting on a BA-85 nitrocellulose membrane that had been presoaked in water and equilibrated in 20 × SSC. All nitrocellulose blots were baked at 80°C for 2 h in a vacuum oven.

Prehybridization, hybridization, and high stringency washes of RNA blots were carried out according to Maniatis et al. (23). Radioactive DNA probes were added at 2 × 10⁶ cpm of 32P/μl of hybridization solution. Autoradiograms were scanned with an LKB 2202 Ultrascan laser densitometer.

Transcriptional Activity of the Malic Enzyme Gene—Sucrose-purified rat liver nuclei (24) were further washed twice in homogenization buffer containing 0.5% Nonidet P-40 and twice in the buffer without Nonidet P-40. In vitro run-off transcription assays were performed as described by McKnight and Palmiter (25) and modified by Santiesteban et al. (26) except that the blots were not treated with RNase during washes. The relative effect of T₃ on transcription rates was the same whether RNase was used in the washes or not (16, 17). The extent of hybridization was measured by densitometric scanning.

RESULTS

Cytosolic malic enzyme activity has been reported to be increased in liver following DHEA administration (4–8). Since malic enzyme is present in a variety of rat tissues (27), we examined whether malic enzyme activity in nonhepatic tissues is also inducible by DHEA treatment. For this experiment, we injected Sprague-Dawley euthyroid male rats every morning with DHEA (35 mg/100 g of body weight) for 7 days and measured cytosolic malic enzyme activity in brain, heart, kidney, and testis as well as in liver. As summarized in Table I, none of the nonhepatic tissues showed stimulation of malic enzyme activity with DHEA administration, whereas there was a 9-fold increase in liver enzyme activity. Euthyroid male rats that received 17.5 mg of DHEA/100 g of body weight each day for 7 days showed a 4–5-fold increase in hepatic malic enzyme activity.

The time course of induction of hepatic malic enzyme activity during DHEA treatment showed a 2-fold stimulation after 1 day of treatment with 35 mg of DHEA/100 g of body weight (Fig. 1). The maximum elevation (8–9-fold) occurred after 7 days, and induction was half-maximal at ≈3 days. Serum concentrations of DHEA and DHEA sulfate at the

| Tissue treatment | Malic enzyme activity | Ratio versus control |
|-----------------|-----------------------|----------------------|
|                 | units/mg protein      |                      |
| Liver           | Control                | 10.8 ± 0.3            | 1.0 |
|                 | DHEA                   | 94.8 ± 3.7            | 8.8 |
| Brain           | Control                | 6.5 ± 0.1             | 1.0 |
|                 | DHEA                   | 7.1 ± 0.9             | 1.1 |
| Heart           | Control                | 10.2 ± 0.8            | 1.0 |
|                 | DHEA                   | 10.7 ± 0.5            | 1.0 |
| Kidney          | Control                | 10.0 ± 0.2            | 1.0 |
|                 | DHEA                   | 10.8 ± 0.1            | 1.1 |
| Testis          | Control                | 27.7 ± 0.9            | 1.0 |
|                 | DHEA                   | 24.9 ± 0.5            | 0.9 |

FIG. 1. Time course of induction of hepatic malic enzyme activity by DHEA. Euthyroid male rats were treated with 35 mg of DHEA/100 g of body weight each day, and malic enzyme activity in liver cytosol was measured. The results of two separate experiments, each performed in duplicate, were used to calculate the fold-induction (bar representing average ± S.D.) of enzyme activity.
time of death were gradually increased from 90 and 500 µg/dl (after 1 day of treatment) to 200 and 630 µg/dl (after 7 days), respectively. Control levels of DHEA and its sulfate conjugate were less than 0.1 pg/dl and less than 5 pg/dl, respectively. After 7 days of treatment of euthyroid rats with a daily dose of 35 mg of DHEA/100 g of body weight, serum concentrations of T3 and T4 were not substantially changed from control levels.

To investigate the mechanism whereby DHEA increases hepatic malic enzyme activity, we measured the cellular malic enzyme mRNA concentration, the nuclear malic enzyme RNA level, and the rate of transcription of the malic enzyme gene after the administration of DHEA.

Slot blots of cytoplasmic mRNA were hybridized with either 32P-labeled malic enzyme cDNA (Fig. 2A, left) or β-[^32P]actin cDNA for normalization (data not shown). Densitometric scanning of autoradiograms showed that the cytosolic malic enzyme mRNA level increased 4–5 and 8–9 times after 7 days of DHEA administration at a daily dose of 17.5 and 35 mg of DHEA/100 g of body weight, respectively. These results indicated that the DHEA-stimulated hepatic malic enzyme activity was due to the elevated concentration of malic enzyme mRNA in the cytoplasm. As observed earlier, when hepatic malic enzyme mRNA was induced by T3 (14, 15), DHEA also stimulated equally the two poly(A)+ RNA species (21 and 27 S) (data not shown).

To determine the cellular site of malic enzyme RNA induction by DHEA and to investigate whether DHEA affected nuclear processing of malic enzyme pre-mRNA, we measured the level of nuclear malic enzyme RNA using a malic enzyme intronic probe, MEI-C (17) (Fig. 2A, right). The level of nuclear malic enzyme RNA following DHEA treatment for 7 days at two different daily doses (17.5 and 35 mg of DHEA/100 g of body weight) increased to the same extent as cytosolic malic enzyme mRNA (4–5- and 7–9-fold).

Nuclear in vitro run-off transcription assays were performed to determine whether this nuclear induction of malic enzyme activity was due to the increased transcriptional activity of the malic enzyme gene or to stabilization of the malic enzyme transcript in the nucleus. When the amount of 32P-labeled nascent RNA complementary to malic enzyme cDNA was compared with that hybridizable with β-actin cDNA (Fig. 2B), the rate of transcription of the hepatic malic enzyme gene was increased 4–5- and 9–9-fold above the control after 7 days of treatment with 17.5 and 35 mg of DHEA, respectively. Similar results were obtained when the same experiment was performed using MEI-C (data not shown).

We have reported recently (17) that treatment of euthyroid rats with T3 activates transcription of malic enzyme gene in liver ~3–4-fold. It was of interest to investigate whether simultaneous injection of T3 and DHEA to euthyroid rats affects the rate of transcription by independent mechanisms in an additive fashion. Male euthyroid rats were treated for 7 days with both T3 (daily intraperitoneal injection of 50 µg of T3/100 g of body weight) and DHEA (daily subcutaneous injection of 35 mg of DHEA/100 g of body weight). Nuclear in vitro run-off transcription assays were performed with nuclei isolated from livers of euthyroid and stimulated rats. When the relative rate of malic enzyme gene transcription was estimated using β-actin gene transcription as an internal reference, 11–14-fold elevation above the euthyroid level was observed (Fig. 2C). The same liver samples showed ~20-fold increase in malic enzyme activity (11.2 ± 0.9 versus 221.4 ± 11.3 units/mg of protein).

The results of these experiments and our previous observations (15–17) are summarized in Table II. They clearly indicate the additive effects of T3 and DHEA on the induction of malic enzyme activity and gene transcription rate in euthyroid rat liver. It should be noted that the difference between the increase in rate of gene transcription and the increase in enzyme activity observed in T3-treated rats is likely due to the nuclear stabilization of the malic enzyme primary transcripts as we have reported previously (17).

The level of malic enzyme gene expression is very low in hypothyroid rats (15). We carried out experiments to investigate whether T3 is required for the stimulation of malic enzyme by DHEA. The results are summarized in Table III.
TABLE II
Fold elevation of hepatic malic enzyme gene transcription and enzyme activity by T3 and DHEA in euthyroid rats

Euthyroid male rats were treated with a daily dose of 50 μg of T3 or 35 mg of DHEA or both in the appropriate vehicle/100 g of body weight for 7 days. Euthyroid values were set to 1 for the estimation of fold increase. At least triplicate results were used for calculations.

| Treatment                  | Malic enzyme gene transcription | Malic enzyme activity |
|----------------------------|---------------------------------|----------------------|
| Euthyroid                  | 1                               | 1                    |
| Eu + T3                    | 3-4                             | 10-12                |
| Eu + DHEA                  | 8-9                             | 8-9                  |
| Eu + T3 + DHEA             | 11-14                           | 18-20                |

*Eu, euthyroid.

TABLE III
Stimulation of hepatic malic enzyme activity by DHEA in hypothyroid rats

Hypothyroid male rats were treated with a daily dose of 50 μg of T3 or 35 mg of DHEA or 100 μg of growth hormone in the appropriate vehicle/100 g of body weight for 7 days as described under "Experimental Procedures." Growth hormone injections were started 2 days prior to the onset of DHEA administration. Results are the mean ± S.D. of the number of livers in parentheses.

| Treatment                          | Malic enzyme activity units/mg protein |
|------------------------------------|---------------------------------------|
| Vehicle for T3                     | <1 (4)                                 |
| T3                                 | 96.2 ± 5.9 (6)                         |
| Sesame oil                         | <1 (4)                                 |
| DHEA                               | <1 (8)                                 |
| T3 + sesame oil                    | 97.5 ± 6.3 (6)                         |
| T3 + DHEA                          | 184.4 ± 10.2 (6)                       |
| Vehicle for growth hormone         | <1 (2)                                 |
| Growth hormone                     | <1 (2)                                 |
| Growth hormone + sesame oil        | <1 (2)                                 |
| Growth hormone + DHEA              | 9.5 ± 3.0 (4)                          |

When hypothyroid rats were treated with T3 (daily dose of 50 μg of T3/100 g of body weight for 7 days), malic enzyme activity was increased from a nondetectable level (less than 1 unit/mg of protein) to 96.2 ± 5.9 units/mg of protein. Surprisingly, after DHEA (35 mg/100 g of body weight each day) administration for 7 days to hypothyroid rats, no malic enzyme activity was detected. However, when hypothyroid rats were treated simultaneously with T3 and DHEA, hepatic malic enzyme was stimulated to a level ≥2-fold greater than with T3 alone. These data indicated that T3 is absolutely required for the enhancement of malic enzyme activity by DHEA. Since thyroid hormone has been reported to increase the rate of transcription of the growth hormone gene in cultured rat pituitary cell lines (28, 29), we treated hypothyroid rats with growth hormone and 2 days later with DHEA to evaluate the mechanism of the T3-mediated DHEA effect. As shown in Table III, growth hormone also triggered malic enzyme stimulation by DHEA although the extent of activation was much less than that observed with T3 and DHEA (9.5 ± 3.0 versus 184.4 ± 10.2 units/mg of protein). Growth hormone alone did not induce malic enzyme activity. Thus, it appears that growth hormone plays a minor role in the stimulation of hepatic malic enzyme following administration of T3 and DHEA to hypothyroid rats.

DISCUSSION

In the present study, we show that DHEA regulates the expression of malic enzyme gene in the liver of euthyroid rats by increasing the rate of gene transcription in a dose-dependent manner. Transcription increased 4-5- and 8-9-fold above the control level after 7 days of treatment with 17.5 and 35 mg of DHEA/100 g of body weight each day, respectively. This activation was followed by a comparable increase in the concentration of nuclear malic enzyme RNA, the amount of cytoplasmic mRNA, and the enzyme activity. Others have shown the malic enzyme-stimulating effect of DHEA in female rats (8). Malic enzyme activity in nonhepatic tissues (brain, heart, kidney, and testis) was unaffected by DHEA treatment.

Experiments with hypothyroid rats demonstrated that thyroid hormone is absolutely required for the induction of hepatic malic enzyme activity by DHEA. The degree of induction appears to be related to the level of thyroid hormone in blood since ≥2-fold stimulation by DHEA was detected in liver of T3-treated hypothyroid rats, whereas an 8-9-fold increase was observed when euthyroid rats were treated with DHEA. Treatment of euthyroid rats with both hormones demonstrated that they alter malic enzyme gene transcription in an additive fashion.

Malic enzyme, which is strongly induced by DHEA, is a member of the lipogenic enzymes because it furnishes reducing equivalent (NADPH) for lipid synthesis (30). However, DHEA has been indicated to be hypolipidemic in rats due to its effects on the suppression of body weight gain (4–9), the decrease of hepatic fatty acid synthetase activity (8), and the reductions of the serum triglyceride concentration and body fat (31). Also, when Casazza et al. (7) measured the ratio between [NADP+] and [NADPH] in control and DHEA-treated rat liver, they found no change, indicating that NADPH is rapidly converted to NADP+. Hence, the metabolic significance of hepatic malic enzyme activation by DHEA is not clear. Of interest is that this increase in malic enzyme activity does not correlate with liver lipogenic activity (9). These observations strongly suggest that increased malic enzyme activity and production of NADPH are linked with NADPH consumption in metabolic conditions different from those operating during lipogenesis. A hypolipidemic drug, clofibrate, has been reported to induce proliferation of peroxisomes (32, 33), increase fatty acyl-CoA-oxidizing activity (34), malic enzyme activity (35), and stimulate the transcriptional activity of the cytochrome P-450A1 gene (36) in liver. A similar mechanism of action for DHEA is not unlikely since DHEA treatment also has been shown to elevate liver peroxisomal fatty acyl-CoA oxidase activity (37) as well as malic enzyme activity (4–8). Work is in progress to identify the cytochrome P-450 isoform (NADPH-dependent mixed function oxidase) inducible by DHEA.

Ayala et al. (38) suggested that an elevated activity of malic enzyme accompanied by generation of NADPH could be a general response of the liver to administration of many drugs that are metabolized through detoxification processes utilizing NADPH. Thus, the mechanism by which DHEA elicits its action on body weight is probably different from that process, which consumes NADPH, following DHEA administration, both mechanisms being presently unclear. The molecular basis of transcriptional activation of malic enzyme gene expression after DHEA administration is also obscure. The receptor for DHEA is not known. Since DHEA is structurally similar to testosterone, it might be that the testosterone receptor-DHEA complex can activate malic enzyme gene transcription upon interaction with regulatory sequences recognizing this complex. Since the testosterone receptor affinity for DHEA would be low, a high concentration of DHEA would be required. However, this possibility seems unlikely since Tepperman et al. (4) found no changes in malic enzyme activity after testosterone treatment. DHEA could activate
malic enzyme gene expression through an unidentified receptor, or its effect could be secondary, involving modification of some transcription factors either through alteration of their transcription rate and/or through post-translational modifications.

In conclusion, we have described the mechanism whereby hepatic malic enzyme is induced by DHEA. The stimulation of malic enzyme by DHEA is regulated exclusively at the level of gene transcription. In contrast, thyroid hormone increases malic enzyme activity by stabilizing the primary transcripts of malic enzyme gene expression through an unidentified receptor, or its effect could be secondary, involving modification of malic enzyme mRNA in cytoplasm (17, 18). Thus, hepatic malic enzyme gene expression is regulated by different mechanisms depending on the inducing factors.

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