Regulation of the Action of Steroid/Thyroid Hormone Receptors by Medium-chain Fatty Acids*

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Triiodothyronine (T₃) causes a 30-fold increase in transcription of the malic enzyme gene in chick embryo hepatocytes; medium-chain fatty acids (MCFAs) inhibit this increase. T₃ action is mediated by T₃ receptors (TRs) that bind to T₃ response elements (T₃REs) in this gene’s 5′-flanking DNA. In transiently transfected hepatocytes, fragments of 5′-flanking DNA of the malic enzyme gene or artificial T₃REs that conferred T₃ stimulation also conferred MCFA inhibition to linked reporter genes. Thus, MCFA inhibition may be mediated through cis-acting T₃REs and trans-acting TRs, distinguishing MCFA action from that of other fatty acids which act through unique sequence elements. Using binding assays and overexpression of TR, we showed that MCFAs inhibited the transactivating but not the silencing function of TR and did not alter binding of T₃ to TR or of TR to T₃RE. The C-terminal ligand-binding domain of TR was sufficient to confer stimulation by T₃, but not inhibition by MCFA. Inhibition of transactivation by MCFA was specific: ligand-stimulated transcription from T₃ or estrogen response elements was inhibited, but that from glucocorticoid or cyclic AMP response elements was not. We propose that MCFAs or metabolites thereof influence the activity of a factor(s) that interacts with the T₃ and estrogen receptors to inhibit ligand-stimulated transcription.

Malic enzyme (EC 1.1.1.40) catalyzes the oxidative decarboxylation of malate to pyruvate and CO₂, simultaneously generating NADPH from NADP⁺. In the livers of well fed birds, much of the NADPH generated by this reaction is used for de novo fatty acid synthesis. Malic enzyme activity, like that of other lipogenic enzymes, is regulated by nutritional state (1, 2). In the livers of starved birds (6, 7). Several T₃ response elements (T₃REs) have been localized in the 5′-flanking DNA of the chicken malic enzyme gene (8–10). Characterization of these T₃REs indicates that each contributes differentially to the overall response of the gene to T₃ (9).

Hexanoate (C6:0) and octanoate (C8:0) inhibit the T₃-induced increase in transcription of the malic enzyme gene in chick embryo hepatocytes within 30 min of their addition; inhibition is reversible upon removal of fatty acid. Inhibition by MCFAs is selective; they have no effect on total transcription in isolated nuclei or on transcription of the genes for glycerolaldehyde-3-phosphate dehydrogenase or β-actin. Inhibition is also specific: 4- and 10-carbon fatty acids and several modified fatty acids have little or no effect (7). These results suggest that the MCFA effect may be relevant biologically. What might that biological relevance be?

Levels of MCFAs in chicken plasma that are high enough to inhibit transcription have not been reported. During starvation, a process that inhibits transcription of the malic enzyme gene, however, fatty acid oxidation is increased. Concomitantly, production of hydroxylated fatty acids similar in chain length to the inhibitory MCFAs also is increased (11–13). In hepatocytes in culture, MCFAs may be converted to hydroxylated fatty acids or metabolites similar thereto, and these may be the intracellular mediators of the inhibition of transcription.

How might MCFAs inhibit transcription of the malic enzyme gene? Hydroxylated long-chain fatty acids activate transcription of the fungal gene for cutinase (14) by stimulating phosphorylation of a trans-acting factor that binds to a specific cis-acting enhancer element (15, 16). Polyunsaturated long-chain fatty acids (PUFAs) stimulate transcription of the acyl-CoA oxidase gene by interacting with the trans-acting factor, peroxisomal proliferator-activated receptor (PPAR) (17), or its adipocyte counterpart, fatty acid-activated receptor (FAAR).

1 The abbreviations used are: T₃, triiodothyronine; T₃RE, T₃ response element; T₃RU, T₃ reporter unit; TR, T₃ receptor; TRIP, TR-interacting protein; MCFA, medium-chain fatty acid; PFA, polyunsaturated long-chain fatty acid; PPAR, peroxisomal proliferator-activated receptor; ER, estrogen receptor; ERE, estrogen response element; GR, glucocorticoid receptor; GRE, glucocorticoid response element; RXR, retinoid X receptor; CAT, 5-chromaphenicol acetyltransferase; TK, thymidine kinase; CMV, cytomegalovirus; βGAL, β-galactosidase; RSV, Rous sarcoma virus; LUC, luciferase; ME, malic enzyme; CRE, CAMP response element; CREB, CRE-binding protein; DBA, direct repeat T₃RE with a 4-bp spacer; bp, base pair(s); CPT-cAMP, 8-(4-chlorophenylthio)adenosine 3′,5′-monophosphate; DBD, DNA-binding domain; LBD, ligand-binding domain.

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(18). These factors bind to peroxisomal proliferator response elements in the acyl-CoA oxidase gene. PUFAs also appear to inhibit expression of the S14 gene via a response element, the PUF response element; the mechanism for this is not known (19, 20). Long-chain fatty acids inhibit binding of T3 to nuclear TR in cells in culture (21, 22). Both long-chain fatty acids and their acyl-CoA derivatives inhibit binding of T3 to rat liver TR in vitro (23). In intact hepatocytes, however, MCFA's fail to inhibit binding of T3 to TR (7), suggesting that they act by some mechanism other than displacement of T3.

We report here that inhibition of T3-stimulated transcription of the chicken malic enzyme gene caused by MCFA’s is mediated by the receptor that binds to T3RE's. This mechanism is distinct from that of fatty acids that act through unique sequence elements. Rather than displacing T3 from TR or displacing TR from T3RE, MCFA’s inhibit the transactivation function of TR. MCFA’s also inhibit estrogen-stimulated transcription through an estrogen response element and the estrogen receptor (ER), but have no effect on ligand-stimulated transcription mediated by glucocorticoid or cAMP response elements.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes were obtained from Life Technologies, Inc., New England Biolabs Inc., U. S. Biochemical Corp., or Boehringer Mannheim. Other enzymes were obtained from the indicated sources: RQ-DNase I (Promega), T4 polynucleotide kinase and Klenow fragment of Escherichia coli DNA polymerase I (Boehringer Mannheim), DNA polymerase from Thermus aquaticus (Promega), and Bst polynucleosome (Bio-Rad). Nucleotides were purchased from Sigma, Amskam Pharmacia Biotech, or Life Technologies, Inc. Radiolabeled nucleotides, Hyperfilm-HF, ECL Western blotting kits, and donkey anti-rabbit IgG conjugated to horseradish peroxidase (secondary antibody) were purchased from Amersham Pharmacia Biotech. n-three-(dichloroacetyl)-1,2,14C)Chloramphenicol was purchased from NEN Life Science Products. LipofectACE, Waymouth MD 705/1 medium, and E. coli cells of strain DH5α were obtained from Life Technologies, Inc. Seakem LE agarose, NuSieve GTG agarose, and DNA isolation columns (SpinBind) were purchased from FMC Corp. Hormones, fatty acids (as sodium salts), and heparin columns were purchased from Sigma. Polyclonal antiserum to chicken TRβ was purchased from Santa Cruz Biotechnologies (cross-reacts with chicken TRβ). Monoclonal antibody to chicken RXR was provided by Pierre Chambon (Université Louis Pasteur/IGBMC, Illkirch, France). Nitrocellulose membranes were purchased from Millipore Corp.

Plasmids—Expression plasmids containing 5'-flanking DNA of the chicken malic enzyme gene inserted upstream of the CAT gene in pRK7-CAT were constructed as described (10). Synthesis of DNA constructs containing fragments of chicken malic enzyme DNA inserted into the multiple cloning site 5' of the promoter of the herpes simplex virus thymidine kinase (TK) gene in pBLCAT2 (24) also has been described (10). Plasmid DR4×5-TKCAT was made by inserting five copies of annealed oligonucleotides encoding a direct repeat element with a 4-bp spacer (AGGTCA/mannoAGGTCA) into the multiple cloning site of pBLCAT2. Structures of plasmid DNA's were confirmed by restriction enzyme mapping and partial sequence analysis.

Construction of pRSV-Lac was described (10). Plasmid CMV-βGAL (25) was obtained from Richard Maurer (Oregon Health Sciences University). Plasmid [ME-3474/+31]CAT was provided by F. Bradley Hillgarter (West Virginia University). Marc Montminy (Salk Institute) provided pRSV-CREB. Bruce Luckow and Gunter Schutz (German Cancer Research Center, Heidelberg, Germany) provided pBLCAT2. The expression vector for GALa, pG5424, contained sequences encoding amino acids 1–147 of the GAL4 DNA-binding domain. Plasmid SG424 and the expression plasmid for the GAL4-binding element, pMCl10, were obtained from Mark Ptashne (Harvard University). Herbert H. Samuels (New York University) provided the DNA for chicken TR's (cloned into the PET5b expression vector) and pGAL4-CR (containing the sequences encoding amino acids 120–408 of the ligand-binding domain of chicken TR's cloned into pSG424). Ronald Evans (Salk Institute) and Bert W. O'Malley (Baylor College of Medicine) gave us pTRE-pal-TKCAT and pERE-pal-TKCAT, respectively. Expression vector for human ER (pHEO) was obtained from Geoffrey L. Greene (University of Chicago). Expression plasmids for the G4RE linked to TKCAT DNA (pΔGTCO) and rat GR (pVARO) were gifts from Keith R. Yamamoto (University of California, San Francisco). Ganes Sen (Cleveland Clinic Foundation) provided pCRE-TKCAT.

Cell Culture and Transient Transfection—Livers of 19-day-old chick embryos were removed, chopped, and treated with collagenase (26). Isolated hepatocytes were with 5 µg of human red blood cells; resuspended in Waymouth medium MD 705/1 supplemented with penicillin (60 µg/ml), streptomycin (100 µg/ml), insulin (50 nM), and corticosterone (1 µM); and incubated in 35-mm tissue culture dishes in an atmosphere of 5% CO2 in air at 40 °C. Twenty hours after the cells were plated, they were transiently transfected using 40 µg of LipofectACE/well. Each 35-mm dish was transfected with 0.2 µg of plasmid DNA: pME-5800/−31CAT (10.7 kilobase pairs, 2.5 µg) or a molar equivalent of other test constructs, pCMV-βGAL or pRSV-LUC (0.5 µg), and pBluescript KS+ (balance). Two 35-mm plates were used for each experimental condition. After 24 h in the transfection medium, the medium was removed by aspiration and replaced with Waymouth medium supplemented with or without 1.6 µM T3, and with or without 1 or 5 mM sodium hexanoate (26). Both of these hexanoate concentrations caused similar degrees of inhibition (data not shown).

CAT, β-Galactosidase, and Luciferase Assays—Forty-eight hours after adding T3, the cells were harvested; lysed by three cycles of freezing and thawing; and analyzed for soluble protein content (27) and denatured protein was removed by centrifugation. CAT activity was determined by incubating a portion of cell lysate with acetyl-CoA and [14C]chloramphenicol for 15 h at 37 °C. Incubation mixtures were then extracted with ethyl acetate and subjected to thin-layer chromatography. Conversion to the acetylated product was detected by liquid scintillation spectrophotometry or direct autoradiography using the Packard InstantImager.

Gel Electrophoretic Mobility Shift Assay—Each oligonucleotide probe contained a 5'-extension and was labeled by a fill-in reaction catalyzed by the Klenow fragment of E. coli DNA polymerase I. Other procedures and the preparation of nuclear extracts were described previously (9). D1Nase I Footprint Analysis—Nuclear extracts were prepared from chick embryo hepatocytes incubated with insulin plus corticosterone plus T3, with or without hexanoate (1 mM). Other procedures were as described previously (10).

Statistical Analysis—Statistical significances of differences between matched pairs were determined by the Wilcoxon matched-pairs, signed-rank test (31). S.E. values are provided to indicate the degree of variability in the data.

RESULTS

Identification of the cis-Acting Element Involved in Mediating Inhibition by MCFA DNA constructs containing deletions from the 5'-end of the 5'-flanking DNA of the chicken malic enzyme gene were transiently transfected into chick embryo hepatocytes in culture in an effort to localize the inhibitory effect of MCFA's (hexanoate). Cells transfected with the longest construct (pME-5800/−31CAT) responded to T3 with a 14-fold increase in CAT activity, and hexanoate inhibited T3-stimulated CAT activity by 83% (Fig. 1). We have previously reported that 5800 bp of 5'-flanking DNA of the malic enzyme gene contained the sequence element(s) required for stimulation of transcription by T3 (10). The sequence element(s) necessary for inhibition by hexanoate were contained in the same DNA.

Deletions to −5200, −4135, −3845, and −3474 bp resulted in 16-, 17-, 9-, and 7-fold stimulations by T3, respectively. The degree of inhibition by hexanoate was the same for cells transfected with each of the above constructs. Cells transfected with constructs containing 5'-deletions to −2715 bp or to shorter end points did not respond to either T3 or hexanoate. These results confirmed the location of T3 response units (TR's) between −4135 and −3845 bp and between −3845 and −2715 bp (9, 10). The upstream T3RU contains four functional direct T3RE's at −3883 to −3858, −3833 to −3808, −3809 to
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Fig. 1. Effects of $T_3$ and hexanoate on CAT activity of hepatocytes transfected with constructs containing deletions from the 5'-end of the 5'-flanking DNA of the chicken malic enzyme gene. Chick embryo hepatocytes were transiently transfected using LipofectACE (40 μg/plate), pME−5800/+31CAT (2.5 μg/plate or an equimolar amount of the other constructs), pCMV-βgal (0.5 μg/plate), and pBluescript DNA (sufficient to balance DNA/plate to 5.0 μg) and treated with or without $T_3$ and with or without hexanoate (C6 (5 mM), except that 1 mM C6 was used in two experiments). Each point represents the mean ± S.E. of five to eight independent sets of hepatocytes, using at least two independently prepared batches of each plasmid. CAT and β-galactosidase activities of extracts from $T_3$-treated hepatocytes transfected with pME−5200/+31CAT were 1.6 ± 0.5 percentage of conversion/15 h/μg of protein and (4.4 ± 1.8) × 10⁻⁴ A₅₅₀ units/min/μg of protein, respectively. Left, DNA constructs used in these experiments; middle, effects of $T_3$, expressed as fold change in CAT activity caused by $T_3$ (+$T_3$−$T_3$); right, effects of hexanoate. Relative CAT activity of cells treated with $T_3$ and hexanoate is expressed as a percentage of that in cells treated with $T_3$ alone. Statistical significance between means within a column is indicated as follows: a, versus pME−2715/+31CAT (p < 0.05); b, versus pME−2715/+31CAT (p < 0.02). c, versus pME−3845/+31CAT (p < 0.02).
T3 and hexanoate (Figs. 4 and 5), indicating that T3RE2 by itself is sufficient to confer both stimulation by T3 and inhibition by hexanoate.

We next tested constructs containing artificial T3REs, the malic enzyme gene T3RU, and T3RE2 to determine if the response to hexanoate was specific to natural T3REs of the malic enzyme gene. Chick embryo hepatocytes were transiently transfected with the indicated constructs as described in the legend to Fig. 1 and treated with or without T3 (1.6 μM) and with or without hexanoate (C6, 5 mM). The results are expressed as described in the legend to Fig. 1; each value is the mean ± S.E. of six to eight independent experiments using at least two independently prepared batches of each plasmid. CAT and β-galactosidase activities of extracts from T3-treated hepatocytes transfected with p[ME–3903/–3703]TKCAT were 4.7 ± 1.7 percentage of conversion/15 h/μg of protein and (3.9 ± 1.8) × 10−4 A420 units/min/μg of protein, respectively. Left, DNA constructs used in these experiments; middle, effects of T3, expressed as -fold change in CAT activity caused by T3; right, effects of hexanoate. Relative CAT activity of cells treated with T3 and hexanoate is expressed as a percentage of that in cells treated with T3 alone. Statistical significance between means within a column is indicated as follows: a, versus pTKCAT (p = 0.02); b, versus pTKCAT (p = 0.05); c, versus p[ME–3903/–3703]TKCAT (p = 0.05).

Fig. 2. Effects of T3 and hexanoate on CAT activity of hepatocytes transfected with constructs containing 5'- and 3'-deletions in the upstream T3 response region of the 5'-flanking DNA of the chicken malic enzyme gene. Chick embryo hepatocytes were transiently transfected with the indicated constructs as described in the legend to Fig. 1 and treated with or without T3 (1.6 μM) and with or without hexanoate (C6, 5 mM). The results are expressed as described in the legend to Fig. 1; each value is the mean ± S.E. of six to eight independent experiments using at least two independently prepared batches of each plasmid. CAT and β-galactosidase activities of extracts from T3-treated hepatocytes transfected with p[ME–3903/–3703]TKCAT were 4.7 ± 1.7 percentage of conversion/15 h/μg of protein and (3.9 ± 1.8) × 10−4 A420 units/min/μg of protein, respectively. Left, DNA constructs used in these experiments; middle, effects of T3, expressed as -fold change in CAT activity caused by T3; right, effects of hexanoate. Relative CAT activity of cells treated with T3 and hexanoate is expressed as a percentage of that in cells treated with T3 alone. Statistical significance between means within a column is indicated as follows: a, versus pTKCAT (p = 0.02); b, versus pTKCAT (p = 0.05); c, versus p[ME–3903/–3703]TKCAT (p = 0.05).

Fig. 3. Effects of T3 and hexanoate on CAT activity in hepatocytes transfected with DNA constructs containing block mutations in T3RE half-sites. A, natural and mutant sequences of the T3RE half-sites. The numbers refer to the half-sites identified in the T3RU of the chicken malic enzyme gene (10). MUT1 to MUT6 refer to the number of the half-site that was mutated within the 5'-flanking DNA of each construct. Right, chick embryo hepatocytes were transiently transfected as described in the legend to Fig. 1 and treated with or without T3 (1.6 mM) and with or without hexanoate (C6, 5 mM). The results are expressed as described in the legend to Fig. 1; each value is the mean ± S.E. of seven to nine independent experiments using at least two independently prepared batches of each plasmid. CAT and β-galactosidase activities of extracts from T3-treated hepatocytes transfected with p[ME–3903/–3703]TKCAT were 8.8 ± 2.6 percentage of conversion/15 h/μg of protein and (4.4 ± 1.4) × 10−4 A420 units/min/μg of protein, respectively. Left, DNA constructs used in these experiments; middle, effects of T3, expressed as -fold change in CAT activity caused by T3; right, effects of hexanoate. Relative CAT activity of cells treated with T3 and C6 is expressed as a percentage of that in cells treated with T3 alone. Statistical significance between means within a column is indicated as follows: a, versus pTKCAT (p = 0.01); b, versus pTKCAT (p = 0.05); c, versus p[ME–3903/–3703]TKCAT (p = 0.01); d, versus p[ME–3903/–3703]TKCAT (p = 0.05).

T3 and hexanoate (Figs. 4 and 5), indicating that T3RE2 by itself is sufficient to confer both stimulation by T3 and inhibition by hexanoate.
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A

| Mutant Identifier | Half-site 2U | Half-site 2D |
|------------------|-------------|-------------|
| A                | -3883       | -3858       |
| B                | -3883       | -3858       |
| C                | -3883       | -3858       |
| D                | -3883       | -3858       |

Fig. 4. Effects of T₃ and hexanoate on CAT activity in hepatocytes transfected with constructs containing wild-type and mutant versions of a T₃ response region of the chicken malic enzyme gene linked to TKCAT. A, block mutations in half-sites 2U and 2D. Fragment A has the wild-type sequence; fragments B, C, and D contain block mutations in positions II–IV of one (B and C) or both (D) half-sites. B, chick embryo hepatocytes were transiently transfected as described in the legend to Fig. 1 and treated with or without T₃ (1.6 μM) and with or without hexanoate (C6, 5 mM). Left, DNA constructs used in these experiments. Middle, effects of T₃, expressed as fold-change in CAT activity caused by T₃. The results are expressed as described in the legend to Fig. 1 and represent the mean ± S.E. of six to nine independent experiments using at least two independently prepared batches of each plasmid. CAT and β-galactosidase activities of extracts from T₃-treated hepatocytes transfected with p[ME−3903/−3703]TKCAT were 8.1 ± 2.7 percentage of conversion/15 h/μg of protein and (5.7 ± 1.8) × 10⁻⁴ A₄₂₀ units/min/μg of protein, respectively. Right, effects of hexanoate. Relative CAT activity of cells treated with T₃ and C6 is expressed as a percentage of that in cells treated with T₃ alone. Statistical significance between means within a column is indicated as follows: a, versus pTKCAT (p < 0.01); b, versus pDR4-TKCAT (p < 0.02); c, versus p[ME−3883/−3858]TKCAT (p < 0.02); d, versus p[ME−3883/−3858]TKCAT (p < 0.01).

B

Fig. 5. Effects of T₃ and hexanoate on CAT activity in hepatocytes transfected with constructs containing different kinds and numbers of T₃REs. Chick embryo hepatocytes were transiently transfected as described in the legend to Fig. 1 and treated with or without T₃ and with or without hexanoate (C6, 1 mM). Left, DNA constructs used in these experiments. First four columns, relative CAT activities are expressed as described in the legend to Fig. 1; each value is the mean ± S.E. of six to nine independent experiments using at least two independently prepared batches of each plasmid. Relative CAT activities were calculated by setting the CAT activities for T₃-treated hepatocytes transfected with pTKCAT to 1.0 and adjusting all other activities proportionately. CAT and β-galactosidase activities of extracts from T₃-treated hepatocytes transfected with pTKCAT were 0.21 ± 0.02 percentage of conversion/15 h/μg of protein and (7.1 ± 1.3) × 10⁻⁴ A₄₂₀ units/min/μg of protein, respectively. Fifth column, effects of T₃ are expressed as -fold change in CAT activity caused by T₃. Sixth column, the effect of hexanoate is expressed as -fold change in CAT activity in cells treated with T₃ plus hexanoate divided by that in cells treated with T₃ alone × 100. The boxed region A is wild-type T₃.RE2. TREpal contains two copies of a palindromic T₃.RE, and DR4 contains five copies of a DR4 T₃.RE, both linked to TKCAT. Statistical significance between means within a column (p < 0.05) is indicated as follows: a, versus pTKCAT; b, versus pDR4-TKCAT.

enzyme gene or could be mediated by any element that responded to T₃ (Fig. 5). Constructs containing T₃RE2 (p[ME−3883/−3858]-TKCAT) or T₃RE2 within the entire T₃RU (p[ME−3903/−3703]TKCAT) bestowed 120- and 130-fold responses to T₃, respectively. These T₃-induced activities were decreased to 35 and 37%, respectively, by hexanoate. Transfection of constructs containing either two copies of the artificial palindromic T₃.RE or five copies of a consensus direct repeat T₃.RE linked to TKCAT DNA conferred robust responsiveness to T₃ and inhibition by hexanoate (Fig. 5). These results suggest that the inhibitory effect of hexanoate is not limited to sequences from the malic enzyme gene; inhibition by hexanoate is transduced by several kinds of T₃REs. Moreover, when sequences containing the T₃RUs were inserted into TKCAT in reverse orientation or were linked to the minimal promoter of the malic enzyme gene (−147 to +31 bp) in either orientation and transfected into hepatocytes, T₃ stimulation and hexanoate inhibition were conferred (data not shown). The minimal promoter of the malic enzyme gene did not confer responsiveness to T₃ or MCFA. Therefore, the T₃REs that act as cis-acting elements for the
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Fig. 6. Effects of T₃ and hexanoate on CAT activity in hepatocytes transfected with constructs containing wild-type and mutant versions of a T₃ response region and wild-type T₃RE2. Chick embryo hepatocytes were transiently transfected as described in the legend to Fig. 1 and treated with or without T₃ (1.6 μM) and with or without hexanoate (C₆, 1 μM). Left, DNA constructs used in these experiments. First four columns, relative CAT activities are expressed as described in the legend to Fig. 1; each value is the mean ± S.E. of seven independent experiments using at least two independently prepared batches of each plasmid. Relative CAT activities were calculated by setting the CAT activities for T₃-treated hepatocytes transfected with p[ME–3903/−3703]TKCAT to 1.0 and adjusting all other activities proportionately. CAT and β-galactosidase activities of extracts from T₃-treated hepatocytes transfected with p[ME–3903/−3703]TKCAT were 11.1 ± 3.3 percentage of conversion/15 h/μg of protein and (9.5 ± 3.9) × 10⁻³ A₆₅₃ units/min/μg of protein, respectively. Fifth column, effects of T₃ are expressed as -fold change in CAT activity caused by T₃. Sixth column, the effect of hexanoate on CAT activity is expressed as CAT activity in cells treated with T₃ plus hexanoate divided by that in cells treated with T₃ alone × 100. The boxed region A is wild-type T₃RE2; x indicates a mutation upstream of T₃RE2 (MUT1; see Fig. 3) that has little or no effect on T₃ responsiveness. No statistically significant differences were observed between means for samples lacking T₃, with or without hexanoate.

Table: Effects of T₃ and hexanoate on CAT activity in hepatocytes transfected with constructs containing wild-type and mutant versions of a T₃ response region and wild-type T₃RE2

| Construct | Relative CAT activity | Effect of T₃ | Effect of C₆ |
|-----------|-----------------------|--------------|--------------|
| -3903     | -T₃ | +T₃ | -C₆ | +C₆ | -T₃ | +T₃ | +C₆ | -C₆ | +C₆ | (-T₃+T₃) | (+C₆+C₆) |
| TKCAT     | 15 ± 0.4 | 17 ± 0.3 | 100 | 49 ± 2.5 | 92 ± 18 | 49 ± 5 |
| A         | 10 ± 0.3 | 11 ± 0.2 | 88 ± 12 | 37 ± 6 | 140 ± 30 | 45 ± 6 |
| TKCAT     | 0.20 ± 0.06 | 0.3 ± 0.1 | 13 ± 2 | 7.0 ± 1.0 | 96 ± 20 | 54 ± 6 |
| A         | 10 ± 0.3 | 12 ± 0.2 | 16 ± 0.3 | 11 ± 0.2 | 20 ± 0.3 | 82 ± 10 |

The effect of hexanoate can vary greatly in their distance from the start site of transcription, gene of origin, and orientation with respect to the direction of transcription.

Identification of the Trans-acting Factor(s) Involved in Mediating Inhibition by MCFA. —TR is the trans-acting factor common to all the T₃REs that conferred MCFA inhibition in transfected cells, suggesting that it also may be the trans-acting factor that mediates the effects of MCIFAs. TR acts as a silencer of transcription in the absence of T₃ (33). Mutation or deletion of the major functional T₃RE (T₃RE2) from sequences linked to either a natural or a heterologous promoter causes an increase in basal activity in transfected cells (10). This increase is consistent with loss of the silencing action of TR. Nuclear receptors such as PPAR and chicken ovalbumin upstream promoter transcription factor inhibit T₃-stimulated transactivation by TR; they also inhibit TR-mediated silencing of basal activity (20, 34–36). Three constructs, each of which contained the major functional T₃RE of the malic enzyme gene, were tested for an effect of MCFA on basal activity (Fig. 6). Plasmid p[ME–3903/−3703]TKCAT contains the entire T₃RU; pMUT1-[ME–3903/−3703]TKCAT contains a block mutation in a non-functional direct repeat upstream of the major T₃RE of the upstream T₃RU; and p[ME–3883/−3858]TKCAT contains a single copy of the major T₃RE. When transfected into hepatocytes, all three constructs conferred robust responses to T₃ and strong inhibitory responses to hexanoate (Fig. 6). None of these constructs nor the vector itself conferred responsiveness to hexanoate in the absence of T₃. This result suggests that the factor that mediates responsiveness to hexanoate does not mediate the silencing action of unliganded TR.

To transactivate, liganded TR must bind to a T₃RE. Previous work suggested that MCIFAs do not inhibit transactivation by disrupting binding of T₃ to TR (7), suggesting that TR binding to the T₃RE or transactivation may be disrupted by MCIFAs. DNase I footprint and gel mobility shift analyses were used to test the effect of hexanoate on binding of TR to the T₃REs in the upstream T₃RU of the malic enzyme gene. Bacterially expressed (37) and partially purified (0.3 μg) TR bound to regions of the T₃RU corresponding to T₃RE2 and an extension of T₃RE-3 (10). Proteins present in nuclear extracts prepared from chick embryo hepatocytes in culture treated with T₃ and/or hexanoate for 24 h showed the same protection in these two regions (data not shown). Hexanoate added directly to binding reactions had no effect on binding of TR or other nuclear proteins, indicating that these fatty acids did not have a direct effect on binding of TR to the T₃RE. Gel mobility shift analyses indicated that, in nuclear extracts from cells treated with T₃, RXR/TR heterodimers bound to the major functional T₃RE in a manner similar to that of those in nuclear extracts made from hepatocytes treated with T₃ plus hexanoate (data not shown). Thus, hexanoate does not disrupt binding of TR to the T₃RE, nor does it disrupt the interaction between TR and its heterodimerization partner, RXR.

If inhibition by hexanoate were mediated by a factor that competed with TR or RXR/TR dimers for binding to the T₃RE, inhibition by hexanoate should be decreased in cells that overexpress TR. We tested p[ME–3868/−3703]TKCAT and p[ME–3474/+31]CAT for responsiveness to T₃ and hexanoate in the absence and presence of overexpressed TRα (Fig. 7). Cells transfected with these constructs have higher basal CAT activities than those transfected with constructs containing an intact T₃RE or the entire upstream T₃RU. Overexpression of chicken TRα caused 62- and 100-fold increases in T₃ responsiveness of cells transfected with p[ME–3868/−3703]TKCAT and p[ME–3474/+31]CAT, respectively. Hexanoate inhibited the response to T₃ to a similar extent whether or not chicken TRα was overexpressed. As noted earlier, hexanoate had no effect on CAT activity in the absence of T₃. Thus, the factor that mediates the MCFA response may not compete with TR for binding to the T₃RE.

Many TR-associated proteins regulate transactivation by liganded TR (38–40); MCIFAs could inhibit transactivation by interacting with such TR-associated proteins. Some of these proteins also interact with ER, but do not interact with GR. To gain insight into the specificity of action by MCFA and to narrow the potential list of factors that might mediate inhibition by MCFA, we tested the effects of hexanoate on cells transfected with constructs that bestow responsiveness to estrogen and glucocorticoids. Single copies of elements that bind these receptors were linked to TKCAT DNA and transfected into hepatocytes with and without overexpression of the cognate receptor. Plasmid GRE-TKCAT conferred 30- and 37-fold responses to β-estradiol in the absence and presence of overexpressed ER, respectively (Fig. 8). Plasmid GRE-TKCAT conferred 220- and 160-fold responses to corticosterone without and with overexpression of GR, respectively. Therefore, endogenous forms of both ER and GR were functional on these cis-acting elements. Hexanoate inhibited function of the ERE, but not the GRE, with or without overexpression of the cognate receptors. A hormone-activated transcription factor that does...
not belong to the steroid/thyroid hormone receptor superfamily also was tested. The cis-acting element was a CRE linked to TKCAT DNA. Cells transfected with the CRE construct showed 10- and 23-fold responses to CPT-cAMP (a nonmetabolizable analog of cAMP) with and without overexpressed CRE-binding protein (CREB), respectively; they did not respond to hexanoate. These results suggest that inhibition by hexanoate has specificity with respect to the involved transcription factor. The actions of TR and ER are inhibited; those of GR and CREB (or other CRE-binding protein) are not inhibited. This distinction may be due to the interaction of TR and ER with a factor that mediates the effect of MCFA; GR and CREB may not interact with that factor.

Members of the family of proteins that interact with TR and ER, but not GR, bind to the ligand-binding domains of these receptors (40). These proteins were identified in yeast two-hybrid screens (41). The factor that mediates the effect of MCFA may be unique to TR and ER, or it may be due to the interaction of TR and ER with a factor that mediates the effect of MCFA; GR and CREB may not interact with that factor.

Fig. 7. Effects of T3 and hexanoate on CAT activity of hepatocytes transfected with constructs containing part of the T3RU linked to TKCAT or part of the 5' flanking DNA containing the downstream T3RU linked to ME-147/+31CAT, with and without overexpression of chicken TR. Chick embryo hepatocytes were transiently transfected as described in the legend to Fig. 1 with or without overexpression of pRSV-cTrα (0.2 μg/plate) and treated with or without T3 and with or without hexanoate (C6, 1 mM). Left, DNA constructs used in these experiments. First four columns, relative CAT activities are expressed as described in the legend to Fig. 1; each value is the mean ± S.E. of six to seven independent experiments using at least two independently prepared batches of each plasmid. Relative CAT activities were calculated by setting CAT activities for T3-treated hepatocytes transfected with p[ME–3868–3703]TKCAT DNA minus overexpressed TR equal to 100 and adjusting all other activities proportionately. CAT activities of extracts from T3-treated hepatocytes transfected with p[ME–3868–3703]TKCAT were 4.9 ± 0.8 percentage of conversion/15 h/μg of protein and (2.0 ± 0.3) × 10−4 A420 units/min/μg of protein, respectively. Fifth column, effects of T3 are expressed as -fold change in CAT activity caused by T3. Sixth column, the effect of hexanoate on CAT activity is expressed as CAT activity in cells treated with T3 plus hexanoate divided by that in cells treated with T3 alone × 100. Statistical significance between means within a column (p < 0.05) is indicated as follows: a, versus pTKCAT; b, versus p[ME–147/+31]CAT.

Fig. 8. Effects of estradiol, corticosterone, cyclic AMP, and hexanoate on CAT activity of hepatocytes transfected with constructs containing single copies of the estrogen, glucocorticoid, and cyclic AMP response elements linked to TKCAT, with or without overexpression of the respective receptor or binding protein. Chick embryo hepatocytes were transiently transfected as described in the legend to Fig. 1 with or without overexpression of pHEO (ER), pVARO (GR), and pRSV-CREB (0.5, 1, and 0.5 μg/plate, respectively). Transfected cells were treated with or without β-estradiol (10 μM), corticosterone (1 μM), and CPT-cAMP (10 μM), respectively, with or without hexanoate (C6, 1 mM). Left, DNA constructs used in these experiments. First four columns, relative CAT activities are expressed as described in the legend to Fig. 1; each value is the mean ± S.E. of six to seven independent experiments using at least two independently prepared batches of each plasmid. Relative CAT activities were calculated by setting CAT activities for T3-treated hepatocytes transfected with p[ME–3868–3703]TKCAT DNA minus overexpressed TR equal to 100 and adjusting all other activities proportionately. CAT activities of extracts from T3-treated hepatocytes transfected with p[ME–3868–3703]TKCAT were 4.9 ± 0.8 percentage of conversion/15 h/μg of protein and (2.0 ± 0.3) × 10−4 A420 units/min/μg of protein, respectively. Fifth column, effects of T3 are expressed as -fold change in CAT activity caused by T3. Sixth column, the effect of hexanoate on CAT activity is expressed as CAT activity in cells treated with T3 plus hexanoate divided by that in cells treated with T3 alone × 100. Statistical significance between means within a row is indicated as follows: a, versus pTKCAT; b, versus p[ME–147/+31]CAT.
Hexanoate Inhibits Action of T<sub>3</sub> and Estrogen Receptors

Table 1. Effects of T<sub>3</sub> and hexanoate on CAT activity in hepatocytes transfected with chimeric GAL4-cTRα and a construct containing a GAL4-binding site. Chimeric GAL4-cTRα and the construct containing the GAL4-binding site were obtained as described under "Experimental Procedures." Chick embryo hepatocytes were transiently transfected as described in the legend to Fig. 1 and treated with or without T<sub>3</sub> (1.6 μM) and with or without hexanoate (C<sub>6</sub>, 1 mM). pGAL4-DBD (pSG424) and pGAL4-DBD/TR-LBD were cotransfected (0.2 μg each per plate) with the pGAL4-CAT (pMC110) reporter construct. Left, DNA constructs used in these experiments. First four columns, relative CAT activities are expressed as described in the legend to Fig. 1; each value is the mean ± S.E. of six independent experiments using at least two independently prepared batches of each plasmid. Relative CAT activities were calculated by setting CAT activities for T<sub>3</sub>-treated hepatocytes transfected with pME−3903/−3703/TKCAT (73RUT7R/TKCAT) to 100 and adjusting all other activities proportionately. CAT and β-galactosidase activities of extracts from T<sub>3</sub>-treated hepatocytes transfected with pME−3903/−3703/TKCAT were 22.7 ± 2.3 percentage of conversion/15 h/μg of protein and (6.5 ± 1.3) × 10<sup>4</sup> units/min/μg of protein, respectively. Fifth column, the effect of hexanoate on relative CAT activity is expressed as relative CAT activity in cells treated with T<sub>3</sub> plus hexanoate divided by that in cells treated with T<sub>3</sub> alone × 100.

| Receptors + DNA Construct | Relative CAT Activity | Effect of C<sub>6</sub> | Relative CAT activity | Effect of C<sub>6</sub> |
|----------------------------|----------------------|-----------------------|----------------------|-----------------------|
|                            | -T<sub>3</sub>         | +T<sub>3</sub>         | -C<sub>6</sub>         | +C<sub>6</sub>         | [+(T<sub>3</sub>+C<sub>6</sub>)−100] |
| TRU-RU CAT                  | 0.28±0.04            | N.D.                  | 100                   | 36±3                  | 36±3                  |
| GAL4-DBD +                  | 0.04±0.01            | 0.05±0.02             | 0.05±0.01             | 120±20                |
| GAL4-CAT                    | 0.006±0.002          | 0.005±0.001           | 0.005±0.01            | 21±2                  | 110±2                 |
| GAL4-DBD/TR-LBD +           | GAL4-CAT             |                       |                       |                       |                       |

FIG. 9. Effects of T<sub>3</sub> and hexanoate on CAT activity in hepatocytes transfected with chimeric GAL4-cTRα and a construct containing a GAL4-binding site. Chimeric GAL4-cTRα and the construct containing the GAL4-binding site were obtained as described under "Experimental Procedures." Chick embryo hepatocytes were transiently transfected as described in the legend to Fig. 1 and treated with or without T<sub>3</sub> (1.6 μM) and with or without hexanoate (C<sub>6</sub>, 1 mM). pGAL4-DBD (pSG424) and pGAL4-DBD/TR-LBD were cotransfected (0.2 μg each per plate) with the pGAL4-CAT (pMC110) reporter construct. Left, DNA constructs used in these experiments. First four columns, relative CAT activities are expressed as described in the legend to Fig. 1; each value is the mean ± S.E. of six independent experiments using at least two independently prepared batches of each plasmid. Relative CAT activities were calculated by setting CAT activities for T<sub>3</sub>-treated hepatocytes transfected with pME−3903/−3703/TKCAT (73RUT7R/TKCAT) to 100 and adjusting all other activities proportionately. CAT and β-galactosidase activities of extracts from T<sub>3</sub>-treated hepatocytes transfected with pME−3903/−3703/TKCAT were 22.7 ± 2.3 percentage of conversion/15 h/μg of protein and (6.5 ± 1.3) × 10<sup>4</sup> units/min/μg of protein, respectively. Fifth column, the effect of hexanoate on relative CAT activity is expressed as relative CAT activity in cells treated with T<sub>3</sub> plus hexanoate divided by that in cells treated with T<sub>3</sub> alone × 100.

**DISCUSSION**

The cis-acting elements in the 5′-flanking DNA of the chicken malic enzyme gene that confer inhibition by MCFAs co-localized with the T<sub>3</sub>REs in this DNA. Furthermore, artificial palindromic and direct repeat T<sub>3</sub>REs also conferred inhibition by MCFA. MCFA also inhibited transcription from an ERE, but had no effect on basal activity in the absence of T<sub>3</sub> or estradiol. Thus, the trans-acting factor that binds to T<sub>3</sub>REs or EREs and participates in the inhibitory effect of MCFA is TR or ER, respectively. The MCFA-regulated factor did not compete with TR for binding to the T<sub>3</sub>RE, but probably interacted with TR to influence its ability to transactivate linked promoters. Sequences N-terminal to the ligand-binding domain of TR are required for the action of MCFA, presumably because they interact with a MCFA-regulated factor. T<sub>3</sub>REs are found in many nutritionally regulated genes; T<sub>3</sub> stimulates and MCFAs or a compound derived therefrom may be similar in structure to the true inhibitor, the production of which may signal the starved state.

Modulation of the transactivation function of TR without displacement of T<sub>3</sub> from TR or from of TR from the TR-ERE has not been described previously as a mechanism by which fatty acids regulate transcription. Long-chain fatty acids, specifically polyunsaturated fatty acids, regulate transcription of some eucaryotic genes by binding to or modifying regulatory proteins, which, in turn, bind to unique DNA elements in the 5′-flanking regions of these genes (17–20). Hydroxy fatty acids also regulate transcription through a unique cis-acting element (14–16). By contrast, our results suggest that MCFAs differ from these other fatty acids in that their inhibitory actions are transduced through the TR-ERE-TR<sub>3</sub> complex, rather than by a complex unique to MCFAs.

Proximal promoter elements may play important roles in T<sub>3</sub>-regulated expression of some genes (41). This is unlikely to confound the interpretation of our results. First, T<sub>3</sub>REs linked to both TK and minimal malic enzyme promoters are inhibited by hexanoate; the two promoters have quite different structures (10). Second, both estrogen- and T<sub>3</sub>-stimulated transcription is inhibited by MCFA. Third, MCFAs have no effect on promoter activity in the basal state. Fourth, if hexanoate did regulate activity of a factor bound at a site common to both TK and malic enzyme promoters and that factor mediated both estrogen- and T<sub>3</sub>-regulated gene transcription, then one would expect fractional inhibition by hexanoate to decrease as T<sub>3</sub> responsiveness decreased; it did not.

The superfamily of steroid/thyroid hormone receptors is subdivided into two major classes. Class I includes GR and ER; these receptors are cytosolic until bound by ligand. Class II includes TR, RXR, and PPAR, receptors that are nuclear in the absence or presence of ligand (42). The interaction of estrogen with ER triggers the translocation of the complex to the nucleus, where estrogen-bound ER binds to an ERE present in the DNA (43). Because both TR and ER are affected by MCFA, it seems unlikely that MCFA acts cytosolically to disrupt the interaction of estrogen and ER, preventing ER from reaching the nucleus. The mechanisms by which MCFA inhibit estrogen- and T<sub>3</sub>-induced transcription are probably the same.

Inhibition of the action of ER by MCFA is consistent with the hypothesis that RXR and PPAR, a known heterodimerization partner for TR at T<sub>3</sub>REs of the malic enzyme gene and a potential partner for TR, respectively, are not targets for MCFA because ER dimerizes with itself and not RXR or PPAR (42, 43). Overexpression of TR should have increased the number of TR/TR dimers bound to T<sub>3</sub>REs. Overexpression of TR, however, failed to alter inhibition by MCFA, additional evidence that potential heterodimerization partners such as RXR
and PPAR did not mediate the inhibitory action of MCFA. Furthermore, fatty acids of carbon chain lengths shorter than 10 are poor activators of PPAR (44). The ligand-binding domains of both ER and TR interact with TR-interacting proteins (TRIPs); TRIPs do not interact with GR (41). The specificity of the inhibitory action of MCFA for ER or TR, but not GR, suggested that one or more of these TRIPs might be the target for MCFA; modification or displacement of a TRIP could decrease transactivation by ER or TR. TRIPs, however, bind to the ligand-binding domain of TR or ER, and this part of TR was not sufficient to confer inhibition by MCFA even though it was sufficient for T₃ responsiveness. TRIPs thus seem unlikely to be the MCFA-regulated factor that interacts with TR or ER.

T₃ responsiveness of hepatocytes transfected with constructs containing all or parts of the T₁₂₃R, mutant versions of T₃R, or artificial T₃R varied from <5-fold to >1500-fold. These differences in responsiveness are due to novel proteins that bind to the different T₃R and differentially influence responsiveness (9), differences in binding of RXR/TR to T₃R of different sequences (42), and artificial overexpression of TR. Despite the resulting broad range of promoter activities, MCFA always resulted in inhibitions of 50–80%. Within individual sets of experiments, the range for inhibition was even narrower, despite a wide range in T₃ responsiveness (e.g. Fig. 2).

Thus, MCFA may modify function of a factor that has the same relative regulatory effect on RXR/TR heterodimers bound at any T₃R, whether the intrinsic ability of that T₃R to bind to the different T₃R and differentially influence responsiveness (9), differences in binding of RXR/TR to T₃R of different sequences (42), and artificial overexpression of TR. Despite the resulting broad range of promoter activities, MCFA always resulted in inhibitions of 50–80%. Within individual sets of experiments, the range for inhibition was even narrower, despite a wide range in T₃ responsiveness (e.g. Fig. 2). Thus, MCFA may modify function of a factor that has the same relative regulatory effect on RXR/TR heterodimers bound at any T₃R, whether the intrinsic ability of that T₃R to bind to the different T₃R and differentially influence responsiveness (9), differences in binding of RXR/TR to T₃R of different sequences (42), and artificial overexpression of TR. Despite the resulting broad range of promoter activities, MCFA always resulted in inhibitions of 50–80%. Within individual sets of experiments, the range for inhibition was even narrower, despite a wide range in T₃ responsiveness (e.g. Fig. 2).
Regulation of the Action of Steroid/Thyroid Hormone Receptors by Medium-chain Fatty Acids
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