Regulation of hepatic neutral cholesteryl ester hydrolase by hormones and changes in cholesterol flux

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Regulation of hepatic neutral cholesteryl ester hydrolase by hormones and changes in cholesterol flux. Am. J. Physiol. 274 (Gastrointest. Liver Physiol. 37): G662–G668, 1998.—To understand molecular events in regulation of hepatic neutral cholesteryl ester hydrolase (EC 3.1.1.13; CEH), catalytic activity, protein mass, and mRNA levels were measured in rats with various perturbations of hepatic cholesterol metabolism. Cholesterol feeding decreased activity (56 ± 2%, mass (44 ± 2%), and mRNA (14 ± 3%). The cholesterol precursor mevalonate also decreased activity (42 ± 6%), mass (76 ± 3%), and mRNA (23 ± 16%). Stimulation of cholesterol biosynthesis by lovastatin increased activity (65 ± 12%) and mRNA (31 ± 24%). Stimulation of cholesterol efflux by chronic biliary diversion increased activity (138 ± 34%), mass (29 ± 7%), and mRNA (146 ± 28%). Chenodeoxycholate feeding decreased activity (46 ± 6%) and mRNA (26 ± 12%). These data suggest rational regulation of CEH in response to changes in cholesterol flux through the liver. In primary hepatocytes, steady-state mRNA markedly decreased during 72-h cultures and addition of l-thyroxine and dexamethasone synergistically maintained mRNA levels near control values. Lovastatin increased mRNA levels by 103 ± 15%. Taurocholate and phorbol 12-myristate 13-acetate suppressed mRNA (61 ± 4% and 49 ± 13%, respectively), suggesting that protein kinase C mediated effects of bile acids on CEH mRNA levels. These data suggest regulation of CEH by hormones and signal transduction in addition to changes in cholesterol flux.

THE LIVER PLAYS A CENTRAL role in both the maintenance of whole body cholesterol homeostasis and the regulation of plasma lipoprotein concentrations. Whereas de novo cholesterol synthesis and uptake of dietary cholesterol (as lipoproteins) represent the two input pathways, conversion of hepatic cholesterol to bile acids and biliary secretion of cholesterol are the only significant output pathways. In response to changes in cholesterol influx or efflux, sterol balance across the hepatocyte is maintained by altering the flux of cholesterol through 1) endogenous cholesterol synthesis, 2) lipoprotein uptake, synthesis, and secretion, 3) conversion of cholesterol to bile acids, and 4) reversible conversion of excess cholesterol to cholesteryl esters. Whereas hepatic free cholesterol and cholesteryl esters are maintained in dynamic equilibrium, a regulated flux of cholesterol through these hepatic pools not only maintains free cholesterol levels within the hepatocyte but also influences the secretion of cholesteryl esters as components of plasma lipoproteins. Neutral cholesteryl ester hydrolase (CEH) is the key enzyme required for releasing the pool of metabolically active free cholesterol from intracellular stores of cholesterol esters, providing substrate for bile acid synthesis and for biliary secretion of cholesterol. A second free cholesterol pool is derived from the de novo synthesis of cholesterol, which is regulated at the level of the rate-limiting enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCoAR). It has been suggested that newly synthesized cholesterol is the preferred substrate for cholesterol 7α-hydroxylase (C7αH), whereas free cholesterol released by the hydrolysis of cholesteryl esters is the proximal source of biliary cholesterol (31, 24). Treatments that affect cholesterol flux through the liver (e.g., cholesterol, bile acid, or cholestyramine feeding or lovastatin infusion) lead to compensatory changes in the expression of C7αH and HMGCoAR (16, 27, 35). Although C7αH appears to be regulated largely by changes in the mRNA levels and the contribution of posttranscriptional mechanisms is minimal (30), regulation of HMGCoAR is known to occur at multiple levels (15). However, few data are available concerning the regulation of CEH expression by changes in the cholesterol flux. Indeed, cholesterol-enriched diets decreased CEH activity in rats (16), and a consistent increase in activity is observed in response to cholestyramine feeding (13). CEH activity is also shown to be sensitive to hormonal stimuli in adrenal cortex (33), testis (10), corpus luteum (2), and aorta (17). Although hormonal regulation of hepatic enzymes involved in glycogen and glucose metabolism is widely studied (4), limited information is available on the modulation of hepatic CEH by hormones. Gandarias et al. (11) correlated increases in hepatic cholesterol content in response to estradiol treatment with decreases in hepatic neutral CEH activity after a single injection of estradiol. Thyroid hormone effectively lowers serum cholesterol levels (34), and Day et al. (9) correlated this reduction in plasma cholesterol with enhanced biliary secretion of cholesterol. These studies suggest a shift in the balance between free cholesterol and cholesteryl esters by thyroxine, probably mediated by CEH. However, a direct effect(s) of l-thyroxine on hepatic CEH has not been reported.

Because there is little published information on the regulation of hepatic CEH, we have examined the regulation of CEH expression (activity, protein mass, and mRNA levels) in rats in response to cholesterol feeding and drugs or metabolites that perturb cholesterol biosynthesis. Hepatic cholesterol flux was also
altered by perturbing bile acid metabolism with chronic biliary diversion or bile acid feeding. To confirm and simplify interpretation of results from in vivo studies, we have also examined the direct effects of hormones and signal transduction pathways on CEH mRNA levels in primary rat hepatocyte cultures. Our results indicate complex regulation of CEH at multiple levels.

**EXPERIMENTAL PROCEDURES**

Materials. Cholesterol, cholesteryl oleate, chenodeoxycholic acid, taurocholic acid, dexamethasone, dextran sulfate, phorbol 12-myristate 13-acetate (PMA), 4α-PMA, and N-lauroylsarcosine were purchased from Sigma Chemical (St. Louis, MO). Lovastatin was a generous gift from Merck Sharp & Dohme (Rahway, NJ). Guanidine isothiocyanate and cesium chloride were purchased from Fisher Scientific (Springfield, N.J.). Williams’ medium E and the nick-translation kit were obtained from Gibco-BRL (Gaithersburg, MD). GeneScreen membrane and radiolabeled probes (32PdCTP and cholesteryl-[1-14C]oleate) were purchased from NEN (Boston, MA). All other reagents were of the highest quality available commercially.

Experimental design. Male Sprague-Dawley rats (Charles River, Cambridge, MA) weighing 250–350 g were housed under controlled lighting conditions on a 12:12-h light-dark cycle (0600–1800 light phase). Groups of age- and weight-matched animals were used for all studies. The treatments were divided into two categories. 1) To change intracellular cholesterol levels, rats were either fed 2% cholesterol mixed with powdered lab chow or infused with mevalonate (180 μmol/h for 48 h) or lovastatin (2 mg·kg⁻¹·h⁻¹ for 24 h) intravenously. 2) To increase cholesterol efflux and perturb bile acid metabolism, rats either had biliary diversion as described below or were fed 1% chenodeoxycholic acid mixed with powdered chow. Animals were pair fed with modified diets as described above for 14 days. Rats were killed by decapitation, and blood was collected for measurement of serum aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase levels as indicators of normal liver function. Livers were harvested, and two 1-g pieces were removed. One piece was used to prepare cytosol (12) and the other to isolate RNA.

Chronic biliary diverted rat model. Under brief methoxyflurane anesthesia, biliary fistula and intraduodenal cannulas were placed as described previously (19). After surgery, rats were placed in individual metabolic cages with free access to water and Purina laboratory chow. All animals received continuous intraduodenal infusion of glucose-electrolyte replacement solution. Dietary intake, activity, and bile flow of the rats were carefully monitored. After 72 h of chronic biliary diversion, rats were killed by decapitation and blood was collected to assess the normal liver function as described above. Livers were harvested and processed as described above for the preparation of RNA and cytosol.

Isolation and culture of primary rat hepatocytes. Hepatocytes were isolated from male Sprague-Dawley rats (250–300 g) using the collagenase perfusion technique of Bissell and Guzelian (3). Before plating, cells were judged to be >90% viable using trypsin blue exclusion. Parenchymal cells (2.5 × 10⁷) were plated onto 150-mm petri dishes previously coated with rat tail collagen. Cells were incubated in Williams’ medium E (20 ml/plate) supplemented with insulin (0.25 U/ml) and penicillin (100 U/ml) at 37°C in a 5% CO₂ atmosphere. The medium was changed every 24 h and supplemented with various hormones where indicated. Cells were routinely harvested after 48–72 h.

Measurement of CEH activity and protein. CEH activity was measured in the cytosol by the radiometric assay described previously (12). The specificity of activity is expressed as nano moles of oleate released per hour per milligram of protein. Relative CEH protein mass was determined by densitometric analysis of the Western blots (23). Protein was estimated using the Pierce bicinchoninic acid protein assay kit (Rockford, IL).

Preparation of total RNA. A one-gram piece of liver was homogenized using a Dounce homogenizer in a solution of 4 M guanidine isothiocyanate, 10 mM tris(hydroxymethyl)-aminomethane-HCl, pH 7.4, and 7% 2-mercaptoethanol. N-lauroylsarcosine was added to a final concentration of 2%, and the homogenate was passed through a 23-gauge needle. This suspension was passed through a 23-gauge needle and underlaid with 5.7 M cesium chloride containing 10 mM EDTA (pH 7.4). The total RNA pellet obtained after centrifugation at 100,000 × g for 16 h was washed twice with 100% ethanol, dissolved in diethyl pyrocarbonate (DEPC)-treated water, and precipitated with 2 vol of 100% ethanol in the presence of 0.10 vol of 3 M sodium acetate, pH 5.0, and stored at −20°C for overnight precipitation (5). Total RNA was pelleted by centrifugation at 12,000 × g for 30 min, redissolved in DEPC-treated water, and quantified by measuring the absorbance at 260 nm. For RNA preparation from cultured hepatocytes after incubation, the media were aspirated and cells washed with 5 ml of phosphate-buffered saline (PBS). After the last traces of PBS were removed, the cells were harvested in 7.5 ml of guanidine isothiocyanate solution, and N-lauroylsarcosine was added to a final concentration of 2%. The mixture was vortexed to lyse the cells completely, passed through a 23-gauge needle, and processed as described above.

Determination of CEH mRNA levels. The methods for determination of CEH mRNA levels have been described previously (23). In brief, 10 μg of total RNA were electrophoresed on a 1% agarose gel in the presence of formaldehyde, transferred to GeneScreen membrane, and hybridized with 32P-labeled full-length cDNA probe for hepatic CEH according to the manufacturer’s instructions. The blots were washed under high stringencies (0.2 × SSC plus 0.1% SDS at 65°C; 1 × SSC is 0.15 M NaCl and 0.0015 M sodium citrate, pH 7.0). Positive hybridization was detected by exposure to Kodak XAR-2 films for 18 h at −70°C (14). Radioactivity associated with each band was quantified using the personal densitometer from Molecular Dynamics. Rat cyclophilin was used as the internal control (8).

Statistical analyses. Data were analyzed by Student’s t-test. P < 0.05 was considered significant.

**RESULTS**

Effects of treatments known to change intracellular cholesterol levels. Cellular cholesterol metabolism was perturbed by cholesterol feeding and by intravenous infusion of mevalonate or lovastatin. As shown in Fig. 1, cholesterol feeding significantly decreased CEH activity (56 ± 2%, P < 0.001), protein mass (44 ± 2%, P < 0.001), and mRNA levels (14 ± 3%, P < 0.005) compared with the pair-fed controls. Intravenous infusion of mevalonate, which increases cholesterol biosynthesis, led to a compensatory decrease in CEH activity (42 ± 6%, P < 0.005) and protein mass (74 ± 3%, P < 0.001). The mRNA levels also showed a similar decreasing trend (22 ± 16%); however, unlike with cholesterol
feeding, the changes did not reach statistical significance. Infusion of the HMGCoAR inhibitor lovastatin, on the other hand, significantly increased CEH activity (65 ± 612%, P < 0.001). Although CEH mRNA levels also showed an increasing trend after lovastatin infusion, this difference did not reach statistical significance (31 ± 624%) and no significant change was seen in CEH protein mass (Fig. 1).

Effects of increased cholesterol efflux and bile acid feeding. Chronic biliary diversion, which drains hepatic cholesterol through both biliary cholesterol and bile acid synthesis, increased CEH activity (138 ± 34%, P < 0.025) and mRNA (146 ± 28%, P < 0.05) relative to sham-operated controls (Fig. 2). The CEH protein mass also increased (29 ± 7%, P < 0.025; data not shown). In contrast, chenodeoxycholate (fed as 1% of diet) decreased CEH activity by 46 ± 6% (P < 0.005). Although CEH mRNA levels were also decreased by chenodeoxycholate (26 ± 12%), this trend did not reach statistical significance. Cholic acid (1% of diet) and deoxycholic acid (fed at 0.25% to avoid toxicity) did not significantly change CEH activity and mRNA levels (data not shown).

Effects of L-thyroxine and dexamethasone on CEH mRNA levels in primary hepatocytes. Steady-state CEH mRNA levels were initially measured as a function of culture age in a chemically defined medium without added serum. In the absence of dexamethasone and L-thyroxine, a large decrease (98%) in CEH mRNA was observed compared with the levels in freshly isolated hepatocytes (Fig. 3). Addition of both hormones stabilized mRNA levels near those of fresh hepatocytes (Fig. 3). In the presence of 0.1 μM dexamethasone, L-thyroxine yielded a maximum response at concentrations between 1 and 10 μM, whereas 100 μM L-thyroxine reduced mRNA levels to 29% of controls (Fig. 4). In the presence of 1 μM L-thyroxine, a maximum response was seen with 0.1 μM dexamethasone (Fig. 5). Although the specific activity of CEH in freshly isolated hepatocytes is only 50% of that in liver cytosol, activity of CEH could not be measured in cultured hepatocytes, even in the presence of L-thyroxine and dexamethasone (data not shown).

Effects of modulators of signal transduction pathways on CEH mRNA levels in hepatocytes. The effects of dibutyryl-cAMP (DBcAMP), glucagon, and PMA on CEH mRNA levels were determined under optimal culture conditions (in the presence of insulin, 0.1 μM dexamethasone, and 1 μM L-thyroxine). As shown in Table 1, although addition of glucagon or DBcAMP had no effect on CEH mRNA levels, PMA decreased CEH mRNA levels by 49 ± 13% (P < 0.01). The response to PMA was time dependent with a maximum at 6 h (data not shown). The effects of PMA were specific to 4β-PMA, because incubation with the 4α-analog of PMA had no effect on CEH mRNA levels.
Effects of agents that perturb cholesterol metabolism on CEH mRNA levels in hepatocytes. Hepatic free cholesterol levels are coordinately regulated by HMGCoAR, C7αH, acyl-CoA:cholesterol acyltransferase, and CEH. To evaluate the molecular mechanisms underlying the compensatory role of CEH in cholesterol homeostasis in vivo, hepatocytes were treated with lovastatin, a competitive inhibitor of HMGCoAR, or taurocholate, a repressor of C7αH and the primary bile acid in rat, before measurement of CEH mRNA levels. Lovastatin increased CEH mRNA levels by 103±615% (P<0.001). Suppression of CEH mRNA levels by taurocholate was both time (Fig. 6A) and concentration dependent (Fig. 6B). The hydrophilic bile acid taurosodeoxycholate (50 µM), which does not suppress bile acid synthesis (27), had no effect on CEH mRNA levels (105% of controls with no additions).

Table 1. Changes in CEH mRNA levels in response to agents that perturb signal transduction pathways

<table>
<thead>
<tr>
<th>Addition</th>
<th>n</th>
<th>Concentration</th>
<th>Relative CEH mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBcAMP</td>
<td>4</td>
<td>50 µM</td>
<td>111±9.5</td>
</tr>
<tr>
<td>Glucagon</td>
<td>5</td>
<td>0.1 µM</td>
<td>111±6.7</td>
</tr>
<tr>
<td>PMA</td>
<td>6</td>
<td>500 nM</td>
<td>51±33.1*</td>
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Values are means ± SE. Hepatocytes were plated in presence of 0.1 µM dexamethasone and 1 µM T4-thyroxine. After 48 h, indicated additions were made. Duration of treatment was 6 h. Total RNA was extracted, and cholesteryl ester hydrolase (CEH) mRNA levels were determined by densitometric analysis of Northern blots. Data are expressed as % value for no addition controls (100%), harvested along with treated samples. PMA, phorbol 12-myristate 13-acetate. *Significantly different (P<0.001) from CEH mRNA levels in hepatocytes cultured without indicated additions.
DISCUSSION

We report here the novel observation of compensatory changes in hepatic neutral CEH activity, protein mass, and mRNA levels in response to changes in the cholesterol flux through the liver. To effect the necessary perturbations of cholesterol homeostasis, cholesterol input pathways were augmented by cholesterol feeding or mevalonate infusion or inhibited by lovastatin. Cholesterol output pathways were stimulated by chronic biliary drainage or suppressed by bile acid feeding.

The observed 56% decrease in CEH activity with cholesterol feeding (Fig. 1) is a rational compensatory response to the increased influx of cholesterol from a massive dietary challenge and is consistent with the reported 10- to 30-fold increase in hepatic cholesterol ester content with this high-cholesterol diet (1, 16, 30). With a similar diet, Spady and Cuthbert (30) reported complete inhibition of HMG-CoA reductase and a twofold activation of C7αH. Taken together, the observed changes in these three hepatic enzyme activities would stabilize hepatic free cholesterol in cholesterol-fed rats by decreasing hydrolysis of stored esters, decreasing de novo synthesis, and increasing the conversion of cholesterol to bile acids.

Comparable compensatory changes in CEH activity are seen in response to alterations in cholesterol synthesis (Fig. 1). Mevalonate infusion sufficient to provide 30 µmol/h of newly synthesized cholesterol (26) decreased CEH activity by 42%, whereas suppression of cholesterol synthesis by the HMG-CoA reductase inhibitor lovastatin increased CEH activity twofold. In each case, the change in CEH activity indicates a rational regulatory response that would tend to stabilize hepatic free cholesterol levels by shifting the balance between hydrolysis and esterification of cholesterol.

Conversion of cholesterol to bile acids accounts for ~80% of cholesterol eliminated from the rat. Thus changes in the rate of bile acid synthesis can markedly alter sterol balance across the liver. This output pathway can be stimulated by chronic biliary diversion or cholestyramine feeding. Preliminary results demonstrated a higher increase in CEH activity with biliary diversion than with cholestyramine feeding (138% vs. 56%). This difference probably reflects the total interruption of the enterohepatic circulation by biliary diversion, which prevents all reabsorption of biliary cholesterol as well as bile acids. These treatments are also reported to increase the activities of C7αH and HMG-CoA reductase (27, 35), suggesting concerted regulation of these three enzymes to maintain the circulating bile acid pool and to stabilize hepatic free cholesterol in response to activation of the output pathways. In contrast, suppression of bile acid synthesis by chenodeoxycholate (20) inhibited CEH activity by 46% (Fig. 2). Thus changes in CEH activity with bile acid feeding are consistent with regulation of hepatic free cholesterol in response to suppression of the output pathway.

As an indication of possible mechanisms for these compensatory changes in CEH activity, changes in mRNA trended toward changes in activity with every in vivo treatment (Figs. 1–3). Although differences in mRNA only reached statistical significance with cholesterol feeding and chronic biliary diversion, lovastatin stimulated CEH mRNA levels twofold in cultured hepatocytes (see RESULTS), roughly paralleling the increased activity seen in vivo. CEH mass also followed changes in activity and mRNA, except with lovastatin, which had no effect on mass (Fig. 1). Similar effects have been reported for HMG-CoA reductase. Spady and Cuthbert (30) observed near-complete inhibition of HMG-CoA activity with only a marginal decrease in mRNA levels in cholesterol-fed rats, similar to effects on CEH shown in Fig. 1. Nakanishi et al. (22) described a substantial decrease in protein mass for HMG-CoA reductase without a corresponding decrease in mRNA levels after mevalonate treatment, similar to the effects on CEH shown in Fig. 1. Lovastatin, on the other hand, increased HMG-CoA reductase protein mass without a parallel increase in mRNA levels (22), the reverse trends on CEH shown in Fig. 1. These similarities to HMG-CoA reductase suggest that, similar to HMG-CoA reductase, CEH may be regulated by both changes in mRNA levels and posttranscriptional mechanisms.

C7αH expression is regulated by bile acids in vivo (27, 25). C7αH transcription is also downregulated by taurocholate in primary rat hepatocytes (21). Stravitz et al. (32) have recently described an “indirect or second messenger-type model” involving protein kinase C catalyzed phosphorylation of a trans-acting factor required for C7αH transcription. Observed suppression of CEH mRNA levels by taurocholate (Fig. 6) and PMA in cultured hepatocytes suggests a similar protein kinase C-mediated effect of bile acids on CEH transcription. Support for such a mechanism has been provided by mapping a PMA-responsive regulatory element on the CEH promoter (23a). Unlike C7αH, CEH mRNA levels were not responsive to glucagon or cAMP in primary hepatocyte cultures (Table 1).

The direct effect(s) of hormones on CEH mRNA levels was examined in cultured primary hepatocytes. Steady-state CEH mRNA levels in cultured rat primary hepatocytes decreased with increasing culture age. These levels could be maintained close to those of freshly isolated hepatocytes by the addition of dexamethasone (0.1 µM) and L-thyroxine (1 µM). The concentrations of L-thyroxine (18) and dexamethasone (7) required to maintain CEH mRNA levels were slightly above the normal physiological ranges found in rat plasma. The effects of L-thyroxine and dexamethasone were synergistic and similar to those previously reported for these hormones with C7αH (21). Glucocorticoids increase C7αH mRNA levels and activity in primary hepatocyte cultures (21) but decrease the abundance of mRNA for HMG-CoA reductase by stimulating its degradation (29). L-Thyroxine increases the activity and mRNA levels for C7αH thereby increasing bile acid and biliary cholesterol secretion. This potential depletion in the regulatory cholesterol pools is apparently compensated by an increase in cholesterol synthesis mediated by an increase in the mRNA for HMG-CoA reductase (28). Simonet and
Ness (28) showed that this increase in mRNA for HMGCoAR is a result of stabilization of the mRNA, also induced by thyroid hormone. Although CEH mRNA levels are maintained in the presence of L-thyroxine and dexamethasone, the data presented do not distinguish between the stabilization effects and direct transcriptional activation of the CEH gene by these two hormones. Preliminary studies show a 30% decrease in CEH mRNA levels in thyroidectomized and hypophysectomized rats, indicating a direct transcriptional regulation of the CEH gene by hormones. Additional support for the direct effect of hormones on the CEH gene comes from the identification of functional glucocorticoid response elements and L-thyroxine response elements in the CEH promoter (23a).

Cholesterol balance across the liver is maintained by regulating the rates of cholesterol biosynthesis, lipoprotein uptake and secretion, and bile acid synthesis. In the rat, suppression of hepatic cholesterol synthesis and increased bile acid synthesis are described as the major physiological responses to an increasing dietary load of cholesterol (30). The present study establishes the role of CEH as an additional compensatory mechanism in cholesterol homeostasis. The regulation of CEH is apparently multivalent and similar to that of HMG-CoA reductase rather than C7H, which is predominantly transcriptional. The question of whether CEH mRNA stability and/or posttranscriptional regulatory mechanisms are affected under all these nutritional conditions will require additional study. Detailed characterization of the CEH promoter will define the functional regulatory elements involved in the transcriptional regulation of CEH.

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