Transcriptional Induction of Cholesterol 7a-Hydroxylase by Dexamethasone in L35 Hepatoma Cells Requires Sulfhydryl Reducing Agents*

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It is known that hepatic levels of reduced glutathione correlate with the activity of the liver-specific enzyme cholesterol-7a-hydroxylase. We examined the possibility that sulfhydryl reducing agents activate transcription of cholesterol 7a-hydroxylase. Adding dithiothreitol (DTT, 1 mM) and dexamethasone to L35 hepatoma cells increased the content of 7a-hydroxylase mRNA 3-fold above the levels observed with dexamethasone alone. Without dexamethasone, DTT had no affect. The addition of reduced glutathione to L35 cells demonstrated a similar potentiation of expression dependent on dexamethasone. Nuclear run-on assays showed that in the presence of both dexamethasone and DTT, the transcription of the 7a-hydroxylase gene was clearly increased. In contrast, by itself, dexamethasone did not cause a detectable increase in the transcription of the 7a-hydroxylase gene. Dexamethasone and DTT did not affect the transcription of ß-actin, suggesting a selective induction of the 7a-hydroxylase gene. DTT reversed repression of 7a-hydroxylase expression by insulin but not the repression by phorbol ester. Our data show for the first time that the sulfhydryl redox potential of the hepatocyte (i.e. level of reduced glutathione) has a marked influence on the transcription and expression of the liver-specific gene 7a-hydroxylase.

The conversion of cholesterol to bile acids is the major quantitative pathway through which cholesterol is removed from mammals (1, 2). The initial step controlling bile acid synthesis is catalyzed by cholesterol 7a-hydroxylase (EC 1.14.13.17). This cytochrome P-450 enzyme is expressed only in the liver (3). Hepatic expression of 7a-hydroxylase provides this organ with the unique ability to take up cholesterol ester-rich lipoproteins from the plasma and to excrete cholesterol into bile in the form of bile acids and cholesterol (1, 2, 4). This liver-specific cholesterol excretory pathway may help to maintain cholesterol homeostasis. Recent studies of mice having a targeted deletion of a functional 7a-hydroxylase gene show that they have marked disruption of hepatic function, lipid metabolism, and premature death (5, 6). Many of these pathological effects could be relieved by supplementation with bile acids and fat-soluble vitamins, providing evidence for the essential function of bile acid production (5). Thus, it is clear that 7a-hydroxylase plays an essential role both in regulating cholesterol homeostasis and in digestion and absorption of lipids including fat-soluble nutrients.

Expression of 7a-hydroxylase varies extensively in response to diet (7, 8), hormones (9–11), diurnal variation, and the enterohepatic circulation (1, 2). Recent studies indicate that the expression of 7a-hydroxylase is regulated mainly through changes in gene transcription (7, 11–15).

We have used a unique line of rat hepatoma cells (L35 cells) to examine the molecular mechanisms regulating 7a-hydroxylase (9, 16). These cells show the unique ability to express 7a-hydroxylase in cultured cells at levels similar to those observed in vivo (9). Expression of 7a-hydroxylase by L35 cells is sensitive to dexamethasone (induced) and insulin (repressed) in a manner similar to that which occurs in vivo. In this study, we examine the mechanism responsible for these changes. The results show that by itself, dexamethasone induces 7a-hydroxylase mRNA expression mainly by a posttranscriptional mechanism (no detectable change in transcription). However, when presented in combination with the sulfhydryl reducing agent dithiothreitol (DTT), transcription of 7a-hydroxylase was increased, resulting in an increased mRNA expression that was greater than that observed with dexamethasone alone. Hepatic levels of reduced sulfhydryl reagents may have a significant influence on 7a-hydroxylase expression.

MATERIALS AND METHODS

All reagents used for biochemical techniques were purchased from Sigma, VWR, or Fisher. Enzymes for restriction or labeling of cDNA probes were purchased from New England Biolabs or Boehringer Mannheim. Cell culture medium was obtained from Life Technologies, Inc./BRL, and serum was from Gemini. DTT and glutathione (obtained from Sigma) were stored as powdered forms (at −20 °C, desiccated without exposure to light). Immediately prior to use, each agent was dissolved in culture medium to a final concentration of 1 mM. This concentration of sulfhydryl reductant was chosen based on previous studies by our laboratory and others showing that it affects the sulfhydryl redox state of the cell as shown by changes in gene expression, the secretion of specific proteins, and the expression of 7a-hydroxylase without causing toxicity (see "Discussion"). The cDNA probes used for hybridizations have been described (3, 9, 16).

Cell Culture Lines and Conditions—L35 rat hepatoma cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 8% serum, as described (9, 16). Prior to each experiment, the medium was changed to DMEM containing 0.5% fetal bovine serum and 1% Nutridoma HU for 3 days. The medium was then changed to DMEM lacking serum but containing the indicated concentrations of additives (as indicated in the figure legends). When used, dexamethasone, dissolved in ethanol, was added to DMEM to a final concentration of 0.1 mM (9, 16). Control cells received ethanol only.

RNA Isolation and Quantitation—Cells were harvested, at the times indicated in the figure legends, by removing the culture medium and
RESULTS

**DTT Potentiates the Induction of 7α-Hydroxylase mRNA by Dexamethasone but Has No Effect by Itself**—When incubated with L35 cells for 48 h together with DTT (1 mM), dexamethasone increased the expression of 7α-hydroxylase to levels that were 2-3-fold greater than the levels obtained by incubating with dexamethasone alone (Fig. 1). In contrast, DTT by itself had no effect on the expression of 7α-hydroxylase mRNA. The increased abundance of 7α-hydroxylase mRNA caused by the combination of dexamethasone and DTT was specific, as demonstrated by no significant effect on the expression of β-actin (Fig. 1). Furthermore, the effect of DTT was not associated with any sign of toxicity. Protein synthesis, cell viability, and growth were unaffected (data not shown). These data suggest that the sulffhydryl reducing agent, DTT, potentiates the induction of 7α-hydroxylase caused by dexamethasone.

**Induction of 7α-Hydroxylase Expression by Dexamethasone Is Also Potentiated by Reduced Glutathione**—Results similar to those obtained using DTT were obtained using the natural sulffhydryl reducing agent glutathione (Fig. 2). In the presence, but not in the absence, of dexamethasone, reduced glutathione (1 mM) induced the expression of 7α-hydroxylase mRNA up to 4-fold greater than the level obtained with dexamethasone alone. In the absence of dexamethasone, reduced glutathione did not induce 7α-hydroxylase mRNA above that observed with culture medium alone (Fig. 2). Furthermore, the increased abundance of 7α-hydroxylase mRNA caused by glutathione was specific, as demonstrated by no significant effect on the expression of β-actin.
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7α-hydroxylase can be reversed by either insulin or phorbol esters (16). Studies by others show that insulin (20) and phorbol esters (21) both repress 7α-hydroxylase by blocking transcription. In the absence of DTT, insulin repressed the expression of 7α-hydroxylase but had no effect on the expression of β-actin (Fig. 4). However, in the presence of DTT, insulin had no significant effect on the expression of 7α-hydroxylase or β-actin (Fig. 4). Moreover, either in the presence or absence of insulin, DTT in combination with dexamethasone caused the same degree of induction of 7α-hydroxylase (Fig. 4). DTT did not affect the ability of insulin to increase protein synthesis (as determined by the incorporation of [35S]methionine into trichloroacetic acid-precipitable protein (data not shown)). Similar data were obtained using reduced glutathione (1 mM) (data not shown).

In contrast to the blocking insulin repression of 7α-hydroxylase, DTT had no effect on the repression caused by phorbol esters (Fig. 5). Both in the absence and presence of DTT, phorbol esters repressed the expression of 7α-hydroxylase (Fig. 5).

**DISCUSSION**

Our results show that DTT affects the ability of dexamethasone and insulin to alter the expression of 7α-hydroxylase. In contrast, DTT by itself has no effect on 7α-hydroxylase expression. Furthermore, DTT did not affect the ability of phorbol esters to repress 7α-hydroxylase. Additional data demonstrated that the effect of DTT could be recapitulated with the endogenous sulfhydryl reducing agent glutathione (22). Together, our data indicate that in the unique hepatoma cell line (L35 cells), the relative concentration of sulfhydryl reducing agents selectively affects the ability of hormones to alter the expression of 7α-hydroxylase mRNA. Previous studies show that the changes in 7α-hydroxylase mRNA levels in L35 cells correspond to parallel changes in enzyme activity (9). Moreover, in the presence or absence of insulin, DTT in combination with dexamethasone caused the same degree of induction of 7α-hydroxylase (Fig. 4). DTT did not affect the ability of insulin to increase protein synthesis (as determined by the incorporation of [35S]methionine into trichloroacetic acid-precipitable protein (data not shown)). Similar data were obtained using reduced glutathione (1 mM) (data not shown).

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**FIG. 3.** In the presence of dexamethasone, DTT induces transcription of the 7α-hydroxylase gene in L35 cells. L35 cells were treated as described in the legend to Fig. 1. After 48 h, nuclei were isolated and transcribing RNA labeled with the addition of a [3H]UTP. The labeled RNA was hybridized to blots containing excess cDNA fragments of 7α-hydroxylase and β-actin.

**FIG. 4.** DTT blocks the insulin repression of 7α-hydroxylase mRNA. L35 cells were plated and grown and then switched to serum-free DMEM, and the indicated reagents were added: dexamethasone (Dex, 100 μM), DTT (1.0 mM), or insulin (INS, 0.1 μg/ml). After 48 h, cells were harvested, and poly(A) containing RNA was purified and subjected to gel electrophoresis and Northern blotting.

**FIG. 5.** DTT does not block the inhibition of 7α-hydroxylase mRNA by phorbol 12-myristate 13-acetate. L35 cells were cultured and induced with dexamethasone (Dex). After 48 h, phorbol 12-myristate 13-acetate (PMA, 1.0 mM) and DTT (1.0 mM) were added for 3 h, after which poly(A) RNA was purified and subjected to Northern analysis. A similar experiment in which DTT was added with dexamethasone for 48 h prior to phorbol 12-myristate 13-acetate addition gave similar results (data not shown).
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cells, DTT can activate the transcription factor NF-κB (32). Interestingly, in these cells, DTT had no affect on SP1 (32). NF-κB also responds to reduced glutathione levels and a number of different thiols in T cells (33). These effects of reducing agents on gene expression are thought to be related to physiologic responses, in at least T cells (33) and the liver (29), via the relative levels and of intracellular reduced glutathione. DTT and the cytokine, tumor necrosis factor, were antagonistic to each other in the regulation of NF-κB activity in HeLa cells, with tumor necrosis factor activating and DTT inhibiting NF-κB (34). Regulation of NF-κB by sulfhydryl agents is mediated via IκB phosphorylation, dissociation, and proteolysis (35–37).

The expression of 7α-hydroxylase has been shown to be negatively regulated by phorbol esters (16, 21) and various cytokines including tumor necrosis factor and interleukin-1 (38). Phorbol esters also decreased the level of intracellular thiols (35). Although based on these findings, it might be possible that phorbol esters repress 7α-hydroxylase via a mechanism associated with reduced intracellular sulfhydryl agents; however, our findings that DTT and glutathione could not reverse the phorbol ester repression of 7α-hydroxylase in L35 cells argues against this.

An alternative mechanism that might account for reduced sulfhydryl reagents affecting the transcription of 7α-hydroxylase is through an indirect effect mediated by the secretory pathway. The activation of NF-κB occurs through proteolysis of the regulatory factor IκB in the endoplasmic reticulum (35–37). DTT can alter the retention, degradation, and secretion of some proteins containing disulfide bridges by interfering with their ability to fold properly (39). Because NF-κB can be regulated by the level of unfolded proteins in the endoplasmic reticulum (34), it is possible that DTT induction of 7α-hydroxylase might be mediated through a signal derived from the secretory pathway. This signal could affect IκB proteolysis and/or NF-κB itself or the content of other transcription factors or gene products. NF-κB does not always act positively on its target genes; at least one gene that NF-κB represses has been identified (40).

Our findings showing that sulfhydryl reducing agents have a marked influence on 7α-hydroxylase transcription and expression are analogous to those reported on the transcriptional regulation of CYP1A1 and CYP1A2 (41). In cultured hepatoma cells, all three hepatic cytochrome 450s (CYP7, CYP1A1, and CYP1A2) are repressed by insulin in a manner that is reversed by sulfhydryl reducing agents. CYP1A1 and CYP1A2 are also repressed by inflammation (41). Previous studies in C57BL/6 and BALB/c inbred mice show that CYP7 is repressed by inflammation (41). Previous studies in C57BL/6 mice (42). This inflammatory response is associated with reduced intracellular sulfhydryl agents; however, the ability of insulin to repress 7α-hydroxylase has been shown to be negatively regulated on the level of insulin (42). An appreciation of the molecular events through which the transcription of this important gene product is altered in response to diet and physiologic state.

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