Ethanol and Arachidonic Acid Increase α2(I) Collagen Expression in Rat Hepatic Stellate Cells Overexpressing Cytochrome P450 2E1

ROLE OF H2O2 AND CYCLOOXYGENASE-2*

Received for publication, February 18, 2000, and in revised form, April 11, 2000
Published, JBC Papers in Press, April 17, 2000, DOI 10.1074/jbc.M001422200

Natalia Nieto‡, Patricia Greenwel§§, Scott L. Friedman§, Fan Zhang¶, Andrew J. Dannenberg‡, and Arthur I. Cederbaum‡ **

From the ‡Departments of Biochemistry and Molecular Biology and §§Medicine and Liver Diseases, Mount Sinai School of Medicine, New York, New York 10029 and the ¶Departments of Cardiothoracic Surgery and ¶¶Medicine, Weill Medical College of Cornell University and Strang Cancer Prevention Center, New York, New York 10021

The ability of ethanol and arachidonic acid (AA), as inducers of oxidative stress and key factors in alcoholic liver disease, to up-regulate α2 collagen type I (COL1A2) gene expression was studied in a hepatic stellate cell line overexpressing the ethanol-inducible cytochrome P450 2E1 (CYP2E1) (E5 cells). A time- and dose-dependent induction in COL1A2 mRNA by ethanol or AA was observed that was prevented by diallylsulfide, a CYP2E1 inhibitor. Nuclear run-on experiments showed transcriptional activation of the COL1A2 gene by ethanol and AA. Catalase abrogated the increase in COL1A2 mRNA suggesting an H2O2-dependent mechanism. Cyclooxygenase-2 (COX-2) levels and production of prostaglandin E2 upon addition of AA were elevated in the E5 cells. Incubation with NS-398, a COX-2 inhibitor, blocked the effect of AA, but not of ethanol, on COL1A2 expression suggesting that CYP2E1 activates COX-2 expression, and the oxidation of AA by COX-2 is responsible for the increase in COL1A2. Activity of a reporter construct driven by ~378 base pairs of the proximal promoter region of the COL1A2 gene increased in E5 but not control cells and was further increased by ethanol or AA. These experiments link CYP2E1-dependent oxidative stress to induction of COX-2 and the actions of ethanol and AA on activation of collagen gene expression in hepatic stellate cells.

Hepatic stellate cells (HSCs) are central to the fibrotic response to liver injury as these cells undergo activation with an increase in extracellular matrix deposition during fibrogenesis (1). Induction of collagen type I gene expression is a key component of increased fibrogenesis by stellate cells (2). Reactive oxygen species and lipid peroxidation have emerged as important stimuli to collagen gene induction in HSCs (3).

CYP2E1 is an important source of reactive oxygen species in alcohol-induced liver injury and fibrosis, generating superoxide (O2·-) and hydrogen peroxide (H2O2) (4–6). To examine the role of intracellular generation of reactive oxygen species in inducing the expression of COL1A2, we previously established an HSC line that overexpresses CYP2E1 and found, in the absence of exogenous substrate, a 3.5- to 4-fold increase in COL1A2 mRNA compared with control cells (7). This increase was related to CYP2E1-derived reactive oxygen species, because it was prevented by antioxidants (7). In this study, we hypothesized that ethanol could further increase COL1A2 mRNA by a CYP2E1-dependent mechanism, because 1) CYP2E1 metabolizes ethanol to acetaldehyde (8) or reactive radical species such as the 1-hydroxethyl radical (9), and 2) ethanol elevates CYP2E1 levels by increasing the stability of the protein (10, 11).

In addition to effects of ethanol metabolism, recent evidence implicates dietary fat as contributing to the severity of alcoholic liver disease. In animal models, for example, diets containing saturated fatty acids protect against alcohol-induced liver injury, whereas polyunsaturated fatty acids enhance the toxic potential of ethanol as measured by fatty liver, inflammation, necrosis, and fibrosis (12). At the molecular level, saturated dietary fat combined with ethanol diminishes lipid peroxidation, the activity of CYP2E1, and the synthesis of vasoactive and inflammatory eicosanoids by cyclooxygenase (COX) (13, 14). In contrast, polyunsaturated fatty acids such as linoleic acid are a requirement for the development of alcoholic liver disease; the conversion of linoleic acid to arachidonic acid (AA) and its subsequent metabolism may play an integral role in the pathogenesis of alcoholic liver disease (13, 14). AA, as a component of cell membranes, is a target for autodestruction and it is susceptible to lipid peroxidation (13, 14), and lipid peroxidation-derived products such as malondialdehyde and 4-hydroxynonenal can increase collagen expression (15).

Cell culture models have been developed to explore the relationships between ethanol, AA, and CYP2E1 in mediating liver cell injury by oxidant stress. By overexpressing CYP2E1 in HepG2 cells, for example, AA can lead to oxidant stress-dependent toxicity (16). To date, however, no studies have explored the impact on fibrogenesis of oxidant stress generated by metabolism of ethanol or AA by CYP2E1. In this work, the oxidant stress derived from metabolism of ethanol or AA in CYP2E1-expressing HSC was found to be directly fibrogenic. Mechanistic studies reveal a critical role for H2O2 in the up-regulation of COL1A2 expression by ethanol and AA; moreover,
COX-2 mediates the AA-mediated induction of COL1A2 expression.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection Experiments**—This study employed a rat stellate cell line (HSC-T6) (17). The cells contain intracellular filaments typical of primary HSCs, express desmin, alpha smooth muscle actin, vimentin, and glial fibrillary acidic protein, take up and esterify retinol and retinol-binding protein, and are otherwise similar to primary stellate cells (17). A stable cell line that overexpresses CYP2E1 (E5), as well as stable cell lines transfected with either the empty vector pCI-neo (D21) or CYP2E1 in the antisense orientation (F11), were previously established (7). Cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, 2 mM glutamine, and 0.5 mg/ml of G-418 in a 5% CO2 humidified atmosphere. The amount of fetal bovine serum was reduced to 5% following transfections and/or when cells were incubated with ethanol or AA. Cell viability assays were performed for each treatment using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay as described previously (7). Protein concentration was determined by the method of Lowry et al. (18).

Reporter DNA constructs containing upstream sequences of the COL1A2 gene were provided by Dr. Francesco Ramirez (Mount Sinai School of Medicine) (19). In these constructs, COL1A2 sequences span from −3500 to +58 base pairs (−3500COL1A2-CAT), from −772 to +58 base pairs (−772COL1A2-CAT), or from −378 to +58 base pairs (−378COL1A2-CAT). Parallel transfections of the corresponding empty vector pEMBL8-CAT at equivalent concentrations were performed in all experiments. Plasmid DNA for transfection was prepared by the alkaline lysis method using the EndoFreeTM Maxiprep kit (Qiagen, Valencia, CA) followed by double CsCl-ethidium bromide gradient centrifugation (20).

Cells were trypsinized 16 h prior to transfection and seeded at a density of 2.5 × 10^4 cells per 10-cm dish. Fresh medium was added 2 h before the transfection. Complexes containing FuGENE 6 (Roche Molecular Biochemicals) and plasmid DNA were prepared according to the manufacturer’s instructions with a final concentration of plasmid DNA for each of the chimeric COL1A2 DNA constructs of 0.5 μg/ml. Parallel co-transfections with 0.01 μg/ml of the control pRL-null and TK-luciferase vectors (Promega, Madison, WI) containing the cDNA encoding the Renilla or firefly luciferase enzymes were performed in all the experiments to normalize for the transfection efficiency. The cells were incubated in the presence of the transfection mix for 6 h, after which the media was replaced. Two h later, 50 mM ethanol or 20 μM AA were added to some of the cells. Cells treated with ethanol (and controls) were incubated in paraffin-sealed plates. After overnight incubation, a second dose of ethanol or AA was added. CAT activity was determined 48 h after the transfection using a commercial kit (Promega). An extract of cytosolic microsomal fraction (2 or 10 μg) by three cycles of thawing in 0.25 M Tris buffer, pH 7.5, was used in each assay, and the CAT reaction was carried out for either 6 min or 1 h. After extraction with ethyl acetate, the samples were loaded onto TLC plates, and the acetylated chloramphenicol was separated from the remaining chloramphenicol by liquid chromatography in chloroform:methanol (97:3). The intensity of the signal was detected using a Molecular Dynamics phosphorimager. Peroxidase activity was assayed using the EndoFreeTM Maxiprep kit (Qiagen, Valencia, CA) as the primary antibody and IgG conjugated to horseradish peroxidase as the secondary antibody.

**Statistics**—Results refer to mean ± S.E. and are average values from three to six values per experiment; experiments were repeated at least twice. Comparisons among groups were made using the Student’s t test.

**RESULTS**

**Ethanol and AA Are Not Toxic to Rat HSC**—To evaluate the effect of ethanol and AA on collagen expression under oxidative stress conditions, HSC-T6 (17) cells were transfected with the human CYP2E1 cDNA in the sense and antisense orientation as well as with the empty vector (pCI-neo), and stable cell lines referred to as E5, F11, and D21 cells, respectively, were developed (7). The clone expressing the highest level of CYP2E1 (E5 cells) was used in all the experiments described below. CYP2E1 expression was validated before each experiment by assaying for p-nitrophenol oxidation and by Western blot using human liver microsomes as a positive control. To evaluate for toxicity, 15 × 10^6 cells were seeded onto 24-well plates and incubated overnight. In some wells, 25, 50, or 100 mM ethanol or 10, 20, or 30 μM AA was added to the culture medium for 0, 6, 12, and 24 h, and the viability after each treatment was assessed by the MTT assay. The average values of cells grown in regular medium were considered as the 100% viability; no toxicity greater than 10% was observed in any of the ethanol or AA treatments at any of the indicated time or dose points (data not shown).

**Oxidative Stress in E5 Cells**—Ethanol induction of CYP2E1 may contribute to the generation of a state of intracellular oxidative stress in the liver (25–30). The production of H2O2 in the E5 cells was evaluated in the presence or absence of 50 mM ethanol or 20 μM AA. Endogenous production of reactive oxygen species such as H2O2 was monitored by the increase in the green fluorescence spectrum of dichlorofluorescein, a product of H2O2 peroxidation after chronic ethanol consumption (26, 27, 30–32). Therefore, the levels of lipid peroxidation in the E5 cells incubated in the presence or absence of ethanol or AA was evaluated by studying the quenching of fluorescence of dichlorofluorescein. Ethanol treatment of E5 cells increased H2O2 production by 30% when compared with the control group (p < 0.05), whereas no significant changes were observed in the presence of AA (Fig. 1A). Several studies have emphasized the important role of CYP2E1 in the enhanced microsomal lipid peroxidation after chronic ethanol consumption (26, 27, 30–32). Therefore, the levels of lipid peroxidation in the E5 cells incubated in the presence or absence of ethanol or AA was evaluated by studying the quenching of fluorescence of cis-parinaric acid. A 15% increase in the peroxidative degradation of cis-parinaric acid was detected in the presence of AA when compared with the non-treated cells (p < 0.05, Fig. 1B). Ethanol did not promote lipid peroxidation, although it did increase H2O2 production. The E5 cells, in the absence of ethanol or AA, were previously shown to display high rates of reactive oxygen production compared with the D21 and F11 cells (7). Hence, the modest effects of ethanol or AA occur in cells already producing high levels of reactive oxygen species.

**Ethanol and AA Induce COL1A2 mRNA in a Time- and Dose-dependent Manner in E5 Cells**—We previously demon-
strated that overexpression of CYP2E1 in HSC increased COL1A2 mRNA levels even in the absence of any added substrate (7). To evaluate whether ethanol and AA could further increase COL1A2 mRNA levels, time course and dose-response experiments were carried out. Northern blots were performed in the presence or absence of 25, 50, and 100 mM ethanol or 10, 20, and 30 mM AA, and mRNA levels were quantified as the ratio of COL1A2:s14 (Fig. 2, A and C). COL1A2 mRNA did not change with any of the treatments in the D21 or F11 cells; the COL1A2:s14 ratio for the untreated D21 cells was assigned a value of 1. As described previously (7), the E5 cells had a 3.5- to 4-fold increase of COL1A2 mRNA levels over values for the D21 or F11 cells. When E5 cells were treated with 25, 50, and 100 mM ethanol for 24 h there was a further increase in the COL1A2 mRNA levels (Fig. 2A). After incubation for 24 h with 50 mM ethanol, there was a 2-fold increase in COL1A2 mRNA. Similarly, AA had no effect on COL1A2 mRNA levels in the D21 or F11 cells but produced a concentration-dependent increase in E5 cells (Fig. 2C). Incubation for 24 h with 20 mM AA produced a 2-fold increase in COL1A2 mRNA expression. To study the effect of the time of incubation, cells were treated with 50 mM ethanol and 20 mM AA for 0, 6, 12, and 24 h. Time-dependent increases of COL1A2 mRNA levels by ethanol or AA were only observed in HSC where CYP2E1 was overexpressed (Fig. 2, B and D). COL1A2 mRNA expression was increased about 8-fold at 24 h with ethanol and AA treatment in the E5 cells compared with the D21 cells. Because ethanol or AA induced no measurable changes in COL1A2 mRNA levels at any time or dose in the D21 or F11 cells, subsequent experiments described below were carried out only in E5 cells.

**Antioxidants Inhibit the Ethanol- or AA-mediated COL1A2 mRNA Induction**—As described above, ethanol treatment increased H$_2$O$_2$ production, whereas AA treatment produced a modest increase in lipid peroxidation in E5 cells; increases in reactive oxygen species production by CYP2E1 metabolism of ethanol or AA were likely to be responsible for the increase of COL1A2 mRNA. To evaluate whether antioxidants prevented the induction of collagen mRNA, dose-response curves were performed by preincubating the cells for 6 h prior to 50 mM ethanol or 20 mM AA treatment in the presence or absence of antioxidants. Catalase led to a concentration-dependent inhibition of COL1A2 mRNA levels in the E5 cells (Fig. 3A). Catalase also prevented the increase in COL1A2 mRNA produced by ethanol or by AA, whereas catalase inactivated by boiling had no effect (Fig. 3A). Vitamin E is the principal lipid-soluble antioxidant in biological tissues and is widely used to prevent the onset of cell damage consequent to the induction of lipid peroxidation (32). However, vitamin E did not affect the stimulation of COL1A2 mRNA by ethanol (Fig. 3B), which is consistent with the lack of increase in lipid peroxidation by ethanol (Fig. 1B). To our surprise, vitamin E did not prevent or decrease the stimulation of COL1A2 mRNA by AA (Fig. 3B). Trollox, a synthetic vitamin E analogue, also did not prevent the increase in COL1A2 mRNA by AA (data not shown).

**N-t-butyl-$\alpha$-phenyl-nitrone (PBN),** a spin-trapping reagent that reacts with many radicals, including superoxide, hydroxyl, lipid, and 1-hydroxylethyl radicals (33), produced a concentration-dependent decrease in COL1A2 mRNA levels in the E5 cells and completely prevented the increase by AA (Fig. 3C). Ebselen, a seleno-organic drug with anti-inflammatory properties that mimics the glutathione peroxidase reaction (34), as well as the phospholipid hydroperoxide glutathione peroxidase reaction (35), lowered COL1A2 mRNA levels in the E5 cells (higher concentrations than 25 mM were toxic to the cells and could not be studied) and completely prevented the increase by AA (Fig. 3C) (these concentrations of PBN and ebselen were not toxic).

**Diallylsulfide (DAS), a CYP2E1 Inhibitor, Prevents the Induction of COL1A2 mRNA by Ethanol and AA**—To confirm that the effect of both ethanol and AA on COL1A2 mRNA in the E5 cells is mediated by CYP2E1, cells were preincubated in the presence or absence of 1 mM DAS (7) 12 h before the addition of ethanol or AA. Cells were further incubated for 24 h, and COL1A2 mRNA expression was analyzed by Northern blot analysis. DAS, an effective inhibitor of CYP2E1 (36), reduced the COL1A2 mRNA levels in the E5 cells and completely abolished the increase in COL1A2 mRNA produced by ethanol or AA (Fig. 4). These data indicate that CYP2E1 plays a role in the enhanced COL1A2 expression in the E5 cells and in the further increase produced by either ethanol or AA.

**Ethanol and AA Increase COL1A2 mRNA at the Transcriptional Level**—To determine the mechanisms responsible for the increase in COL1A2 mRNA by ethanol or AA, nuclear run-on experiments were performed with nuclei isolated from the E5 cells. The rate of transcription of the collagen gene was compared with that of the S14 ribosomal protein gene as a control. Equal amounts (10$^{5}$ cpm/ml) of nascent transcripts of nuclei from cells treated in the presence or absence of ethanol or AA were hybridized to cDNAs immobilized onto nylon membranes. Cells incubated with ethanol or AA had a transcriptional rate of the (COL1A2 gene that was 75 or 100% greater than that of the non-treated cells (Fig. 5A) (p < 0.001). These increases are similar to the increased level of COL1A2 mRNA produced by ethanol or AA (about 2-fold). Previous observations demonstrated that the CYP2E1-dependent increase in COL1A2 mRNA.
FIG. 2. Ethanol and AA induce COL1A2 mRNA in a time- and dose-dependent manner in E5 cells. $5 \times 10^5$ HSCs transfected with either the empty vector (D21, - - -), CYP2E1 (E5, ---), or CYP2E1 in the antisense orientation (F11, ... ... ) were seeded onto 10-cm dishes for 24 h. The cells were treated with 0, 25, 50, or 100 mM ethanol for 24 h (A); 50 mM ethanol for 0, 6, 12, and 24 h (B); 0, 10, 20, or 30 mM AA for 24 h (C); and 20 mM AA for 0, 6, 12, and 24 h (D). Total RNA was isolated and Northern blots to detect COL1A2 and S14 ribosomal protein mRNAs were carried out. Results are expressed as relative COL1A2 mRNA expression with the empty vector group (D21) assigned a value of 1 and refer to mean ± S.E. (n = 3 for all the experiments). In all cases, no significant changes by ethanol or AA were observed in cells transfected with the empty vector or CYP2E1 in the antisense orientation. All values were corrected for differences in loading using the S14 ribosomal protein signal. ***, $p < 0.001$ when compared with values of the no ethanol or no AA-treated E5 cells.

FIG. 3. Effect of antioxidants on COL1A2 mRNA levels in E5 cells. Experiments were carried out with untreated E5 cells or E5 cells treated with 50 mM ethanol or 20 mM AA for 24 h in the absence or presence of the indicated antioxidants (Catalase, Vitamin E, PBN, and Ebselen). Concentrations of the various additions are listed at the top of the blots. The antioxidants were added to the cells 6 h prior to the addition of medium (labeled as -), ethanol, or AA. Total RNA was isolated after incubation for an additional 24 h, and Northern blots to detect COL1A2 and S14 mRNA were carried out using 5 μg of total RNA. All values were corrected for the differences in loading using the S14 ribosomal protein signal. Results are expressed as relative COL1A2 mRNA expression, with the untreated E5 cells (labeled as -) assigned a value of 1.
mRNA levels, in the absence of any added substrate, involved both transcriptional activation of the COL1A2 gene and stabilization of the mRNA (7).

To further validate that the increased COL1A2 mRNA produced by ethanol or AA was due to increased synthesis of the mRNA, the effect of actinomycin D was evaluated. Previous studies of mRNA stability (7) showed that at 24 h, COL1A2 mRNA decayed by 50% in the D21 cells, whereas no degradation was observed in the E5 cells, consistent with increased mRNA stability. When E5 cells were preincubated with 10 μg/ml of actinomycin D for 2 h prior to ethanol or AA treatment, no increases in mRNA levels were observed when compared with the non-treated E5 cells (Fig. 5B); thus, actinomycin D blocked the increase in COL1A2 mRNA produced by ethanol or AA. To confirm that actinomycin D had indeed blocked transcription, the same membrane was probed for S14 ribosomal protein, which has a half-life of about 3 h; as expected, this mRNA could not be detected in cells incubated with actinomycin D for 24 h (data not shown). The addition of actinomycin D did not change the mRNA level of the untreated E5 cells, consistent with the stabilization of COL1A2 mRNA found in these cells. Thus, the nuclear run-on and actinomycin D results collectively suggest that the effect of ethanol and AA on COL1A2 mRNA expression in the E5 cells involves transcriptional activation and new mRNA synthesis.

**Increased Levels of COX-2 and Prostaglandin E2 Production in E5 Cells**—Because only a small increase in lipid peroxidation was observed after AA treatment in the E5 cells (15% when compared with non-treated E5 cells) and neither vitamin E nor trolox were effective in preventing the AA-induced increase in COL1A2 mRNA, we considered the hypothesis that other AA metabolic pathways could be responsible for the increase in the steady-state levels of COL1A2 mRNA after AA treatment. An increase in COX levels has been described in non-parenchymal cells in experimental alcoholic liver disease (37). Studies have shown that oxidant status can be a determinant of both the rate of transcription of COX-2 and its enzymatic activity (38). The amount of COX-2 was markedly increased as assessed by Western blot in E5 cells compared with T6, D21, and P11 cells (Fig. 6A). To determine whether this enzyme was functional, synthesis of prostaglandin E2 was measured. As shown in Fig. 6B, when AA was supplemented, E5 cells produced 22-fold more prostaglandin E2 than did D21 cells. The observed increase in prostaglandin E2 synthesis by E5 cells was blocked by NS-398, a known specific inhibitor of COX-2 (39).

**NS-398, a Selective COX-2 Inhibitor, Blocks the Increase in COL1A2 mRNA Levels Produced by AA**—To evaluate a role for COX-2 in regulating COL1A2 mRNA levels, cells were preincubated in the presence of either 1 or 10 μM NS-398 for 2 h and then treated with or without ethanol or AA for 24 h. To validate that NS-398 did not affect the endogenous COL1A2 mRNA, cells transfected with the empty vector were also incubated in the presence of the COX-2 inhibitor. NS-398 had no effect on
COX-2

COX-2

AA

T6

D21

E5

F11

E5+NS-398

Fig. 6. A, CYP2E1 induces COX-2 expression. Cellular lysate (30 μg of protein) was resolved in a 10% SDS polyacrylamide gel followed by Western blot using rabbit polyclonal anti-COX2 antibody. Lane 1, HSC-T6 (T6) cells; lane 2, HSC transfected with the empty vector (D21); lane 3, HSC transfected with CYP2E1 (E5); lane 4, HSC transfected with CYP2E1 in the antisense orientation (F11). B, CYP2E1 overexpression enhances the production of prostaglandin E2. 5 × 10^5 D21 or E5 cells were seeded, and after overnight incubation, fresh minimum essential medium containing 5% fetal bovine serum with (+) or without (-) 10 μM AA was added. Some of the E5 cells were incubated in the presence of NS-398 2 h prior to AA treatment. After 30 min, the medium was collected for analysis of prostaglandin E2 by enzyme immunoassay. Results are expressed as pg/μg of protein and refer to mean ± S.E. (n = 4); ***, p < 0.001 when compared with D21 cells incubated with AA; ●●●, p < 0.001 when compared with the NS398-treated E5 cells.

Identification of the Minimal Sequences of the COL1A2 Promoter Required for CYP2E1-mediated Activation and Response to Ethanol and AA—Transient transfection experiments with chimeric constructs harboring progressive 5' deletions of the COL1A2 promoter linked to the CAT reporter gene were performed to identify the promoter regions of the COL1A2 gene required for CYP2E1-mediated activation and the further up-regulation by ethanol and AA. D21 and E5 cells were transfected with the constructs shown in Fig. 8A or with the parental empty vector pEMBL8-CAT. As shown in Fig. 8B, the basal percentage of acetylation of chloramphenicol in E5 cells transfected with either one of the COL1A2 promoter-driven vectors was significantly higher than activity found in transfected D21 cells. These data are consistent with our previous findings that CYP2E1-dependent COL1A2 activation is exerted, at least in part, at the transcriptional level (7). Interestingly, the activities of the −3500COL1A2-CAT and −378COL1A2-CAT plasmids in E5 cells were very similar (52 and 56% acetylation of chloramphenicol, respectively). On the other hand, the activity of the −772COL1A2-CAT vector was significantly lower (5.6% in E5 cells; Fig. 8B). These data are in agreement with previous results reported by other investigators using fibroblasts or HSC, which have shown that the −772 to −378 region of the COL1A2 gene contains negative regulatory elements (19, 40). Addition of either 50 mM ethanol or 20 μM AA further increased the activity of the −378COL1A2-CAT plasmid in E5 cells (5-fold; Fig. 8C). In contrast to these results, activities of the −3500COL1A2-CAT and −772COL1A2-CAT vectors remained unchanged. Overall, these results suggest that in E5 cells, the −378 to +58 region of the COL1A2 gene is essential for increased basal COL1A2 expression, as well as for their responsiveness to ethanol and AA. For comparative purposes, Fig. 8C also shows the effect of TGF-β, a well known stimulus to collagen synthesis, on the expression of the different chimeric constructs in E5 cells. TGF-β increased COL1A2 mRNA levels in E5 cells (data not shown). As shown in Fig. 8C, the extent of the response to TGF-β in cells transfected with the −378COL1A2-CAT vector is very similar to that exerted by ethanol or AA. TGF-β increased approximately 2-fold the activity of the −3500COL1A2-CAT vector.

DISCUSSION

HSCs play a key role in the fibrotic response to liver injury (41). Activation of stellate cells with an accompanying increase in collagen production and other biochemical and structural changes is mediated by cytokines, reactive oxygen species, aldehydes such as acetaldehyde, and lipid peroxidation-derived products (42). Because oxidative stress can stimulate stellate cell proliferation and collagen synthesis in vitro, we established a rat HSC line, which overexpresses the CYP2E1 isoform of cytochrome P450 (E5 cells) (7). Yamada et al. (43) reported that CYP2E1 is present in rat HSC at levels 21% of those found in hepatocytes, whereas Oninen et al. (44) observed immunoreactive CYP2E1 at levels of about 4% of that found in hepatocytes. Casini et al. (45) did not observe expression of CYP2E1 in human stellate cells. CYP2E1 was detectable in T6 cells transfected with the empty vector (D series of cell lines, Fig. 1; see Ref. 7) and the parental T6 cells. However, levels were relatively low, about 2% of that of human liver microsomes. Therefore, to evaluate the effects of CYP2E1 on collagen production in HSC cultures at reasonable time points and concentrations of ethanol and AA, HSCs overexpressing CYP2E1 were established. The E5 cells stably and constitutively express CYP2E1 at levels of about 10 to 13% of those found in hepatocytes. Whereas it is recognized that these levels of CYP2E1 are higher (about 5- to 7-fold) than those present in the HSC-T6, overexpression of CYP2E1 in HSC lines provides a unique model to study the direct effects of intracellular oxidative stress on COL1A2 regulation in response to ethanol or AA. Such studies set the stage for more physiologic models utilizing co-cultures of hepatocytes and HSC by precisely defining relevant pathways and mediators. The E5 cells produced increased reactive oxygen species and a 3.5- to 4-fold increase in COL1A2 mRNA because of transcriptional activation of the COL1A2 gene as well as stabilization of its mRNA (7). The present work was undertaken to examine the ability of ethanol and AA as inducers of oxidative stress and key factors in the development of liver disease to up-regulate COL1A2 gene expression in E5 cells.

Ethanol and AA produced concentration- and time-dependent increases in COL1A2 mRNA in E5 cells. Both compounds stimulated another 2-fold increase in COL1A2 mRNA over that produced by CYP2E1. DAS, a CYP2E1 inhibitor (36), decreased COL1A2 mRNA levels in the E5 cells and totally prevented the increase by ethanol and AA on COL1A2 mRNA expression thus validating the role of CYP2E1. In vivo administration of DAS
has been shown to partially protect against alcoholic liver disease in the intragastric model of chronic ethanol administration (29).

In view of the previously demonstrated stabilization of COL1A2 mRNA in the E5 cells (7), it is likely that the increase of COL1A2 mRNA by ethanol or AA is due to transcriptional activation of the COL1A2 gene. Indeed actinomycin D prevented the increase by ethanol or AA of COL1A2 mRNA indicating that this increase is due to new mRNA synthesis. The nuclear run-on experiments documented a 1.7- and 2-fold increase in synthesis of COL1A2 mRNA by ethanol and AA, respectively. This increase parallels that of COL1A2 mRNA. Consistent with these results, in transient transfection assays with E5 cells, ethanol and AA increased the activity of reporter constructs driven by different sequences of the COL1A2 promoter. Thus, enhanced COL1A2 mRNA expression by ethanol or AA in the E5 cells results from transcriptional activation of the COL1A2 gene.

Transient transfection of E5 cells with chimeric constructs driven by different sequences of the COL1A2 promoter clearly indicate that the −378 to +58 region is essential for CYP2E1-mediated high basal COL1A2 expression and for ethanol and AA responsiveness. Interestingly, this region of the promoter contains TGF-β1- and acetaldehyde-responsive elements that are also active in fibroblasts, as well as in HSC (46–49). This element binds several transcription factors including Sp1, AP1, and/or NF-1 (40, 48–51) whose transcriptional activity is regulated by changes in the redox status. Thus, it is conceivable that CYP2E1-induced changes in the redox potential in E5 cells enhances the transcriptional activity of these factors, which in turn transactivate COL1A2 gene expression. Recent studies have implicated H₂O₂ in stimulating collagen gene expression by acetaldehyde (52) and TGF-β (53). We now demonstrate that H₂O₂ also plays a critical role in the stimulation of COL1A2 expression by CYP2E1. Hence, acetaldehyde, TGF-β, and CYP2E1, factors known to be produced or increased by chronic ethanol treatment, may induce collagen gene expression via common oxidant-dependent mechanisms and at common sites in the COL1A2 promoter.

Several mechanisms may be involved in ethanol- and AA-mediated COL1A2 up-regulation. With respect to ethanol, the increase in COL1A2 mRNA may result from the metabolic effects of ethanol. Indeed, the hepatic oxidation of ethanol results in the production of acetaldehyde (8, 54). Acetaldehyde increases collagen production via an H₂O₂-dependent mechanism (52, 55). Redox changes can also have an impact on collagen metabolism (54). Alternatively, the increase by ethanol in the E5 cells may be due to enhanced production of reactive oxygen species. The addition of ethanol to the E5 cells elevated H₂O₂ levels by 30%. The ability of catalase to totally prevent the ethanol-induced increase in COL1A2 mRNA indicates that H₂O₂ plays a central role in this increase by ethanol. It is uncertain whether H₂O₂ itself or products derived from it such as the hydroxyl radical, perferryl species, or the 1-hydroxyethyl radical are ultimately responsible for the increase in COL1A2 mRNA. Because ethanol can be oxidized to acetaldehyde by the peroxidative activity of catalase (56), the decrease in COL1A2 mRNA by catalase suggests that ethanol-derived acetaldehyde is not responsible for the increase observed in this model. The inability of vitamin E to prevent the ethanol-induced increase suggests that lipid peroxidation also does not mediate this effect, consistent with the lack of an
effect on lipid peroxidation by ethanol in the E5 cells. Catalase also decreased basal COL1A2 mRNA levels in the untreated E5 cells suggesting that CYP2E1-derived reactive oxygen species such as H2O2 play a key role in the enhanced expression of COL1A2 by CYP2E1.

Results with AA proved to be more complex and somewhat surprising. An initial hypothesis was that lipid peroxidation, produced as a consequence of the interaction of CYP2E1-derived reactive oxygen species with AA, would play a key role in the transcriptional activation of COL1A2 by AA. However, only a small increase in lipid peroxidation was observed upon incubating the E5 cells with AA. More importantly, vitamin E and trolox did not prevent the increase in COL1A2 mRNA by AA; these agents were added at concentrations that strongly prevent lipid peroxidation and were previously shown to prevent AA toxicity to HepG2 cells expressing CYP2E1 (16). The increase by AA was inhibited by catalase, ebselen, and PBN, indicating that reactive oxygen species played a role in this increase. The inhibition by catalase (and ebselen) further suggested that H2O2 might mediate the stimulatory effect of AA on COL1A2 mRNA, as discussed above for ethanol. However, AA did not elevate H2O2 levels in the E5 cells. These results raised the possibility that other metabolic pathways involving AA might be activating the COL1A2 gene.

Cyclooxygenases (COX-1 and COX-2) catalyze the conversion of AA into prostaglandins and thromboxanes. COX-1 is constitutively expressed in a wide variety of tissues, including the liver, whereas COX-2 is a highly inducible gene that is expressed in response to many proinflammatory agents and cytokines (57). Reactive oxygen species play an important role in inflammation as mediators of injury and potentially in signal transduction. Several studies have linked reactive oxygen species to the signaling pathways that induce COX-2 expression (58). In cells incubated in the presence of H2O2, the COX-2 gene was transiently induced, whereas O2^- was a more potent inducer (38). In view of the activation of COX-2 transcription by oxidative stress, we evaluated the possible contribution of COX-2 to the mechanism by which AA and CYP2E1 increase COL1A2 mRNA levels in stellate cells. COX-2 and prostaglandin E2 synthesis were markedly increased in E5 cells compared with control cells. NS-398, a COX-2 inhibitor, did not modulate either basal or ethanol-induced levels of COL1A2.

**Fig. 8. Identification of the minimal sequences of the COL1A2 promoter required for CYP2E1-, ethanol-, or AA-mediated activation.** Transient transfection experiments with different chimeric constructs of the COL1A2 promoter linked to the CAT reporter gene, as well as the empty vector pEMBL8-CAT, were performed and cell extracts were collected as described under “Experimental Procedures.” A schematic representation of the CAT reporter vectors driven by various sequences of the COL1A2 promoter used for transfection experiments is shown in panel A. Panel B compares results of D21 and E5 cells to evaluate the minimal sequences required for CYP2E1-mediated activation of the COL1A2 gene. The CAT reaction was run with 10 µg of protein for 1 h, and the acetylated chloramphenicol (AcC) was separated from the remaining chloramphenicol (C) by thin layer chromatography. Results were corrected by transfection efficiency and are expressed as a percentage of acetylated chloramphenicol and refer to the mean ± S.E. ***, p < 0.001 when compared with D21 cells. Panel C shows the effects of ethanol, AA, or TGF-β in E5 cells. 2 h after transfection with the different chimeric constructs of the COL1A2 promoter linked to the CAT reporter gene, as well as the empty vector pEMBL8-CAT, cells were treated with 50 mM ethanol, 20 µM AA, or 8 ng/ml of TGF-β. After overnight incubation, a second dose of ethanol, AA, or TGF-β was added, and samples were collected 48 h after transfection. The CAT assay was run with 0.2 µg of protein for 6 min, in contrast to the conditions shown in panel B, to lower the high activity of the untreated E5 cells and avoid saturation of the system. A representative chromatogram is shown in which the last two lanes (+ and ―) refer to non-transfected E5 cells as a negative control and E5 cell extract incubated with purified CAT as a positive control. All the experiments were performed in quadruplicate (except for the TGF-β-treated cells), and values refer to mean ± S.E. after correcting for efficiency of transfection. ***, p < 0.001 when compared with the non-treated E5 cells transfected with the appropriate construct.
mRNA in D21 or E5 cells. By contrast, AA-mediated induction of COL1A2 expression was suppressed by NS-398. These results suggest that metabolites produced by the oxidation of AA by COX-2 are responsible for the increase in COL1A2 expression when AA is added to the E5 cells. A model can be proposed that links CYP2E1-dependent production of reactive oxygen species, sensitivity to antioxidants such as catalase, COX-2 induction, and COL1A2 expression (Fig. 9). CYP2E1-dependent production of reactive oxygen species such as H$_2$O$_2$, among others, activates COL1A2 expression, and this stimulation is catalase-sensitive and augmented by ethanol. COX-2 expression is also increased by CYP2E1-derived reactive oxygen species; however, in the absence of AA, there were only very modest increases in COX-2-derived metabolites such as prostaglandin E$_2$. Hence, the expression of COL1A2 in the E5 cells or its augmentation by ethanol is not sensitive to NS-398, although COX-2 itself is increased in the E5 cells (Fig. 6a). When AA is added, there is a marked increase in the production of COX-2-derived metabolites (Fig. 6b) in association with enhanced COL1A2 expression. The finding that NS-398 inhibits both prostaglandin E$_2$ production and the AA stimulation of COL1A2 expression directly links both events to a COX-2-dependent mechanism. The ability of catalase and ebselen to decrease COL1A2 expression may reflect the removal of H$_2$O$_2$ (or H$_2$O$_2$-derived oxidants) with the subsequent down-regulation of COL1A2 and possibly COX-2 expression. It is important to note that ebselen inhibits lipoxigenase and cyclooxygenase activity, in addition to removal of H$_2$O$_2$ and lipid hydroperoxides (59); PBN, besides scavenging a variety of free radicals, acts also as an anti-inflammatory agent because it decreases steady-state COX-2 mRNA levels and COX-2 catalytic activity in macrophages (60). Therefore, the prevention of the AA-induced increase in COL1A2 expression by ebselen and PBN may occur by several mechanisms.

In liver disease, antioxidant levels are depleted in whole liver. Depletion of antioxidants in stellate cells also occurs and could be related to their fibrogenic capacity (42). Reducing oxidative stress is a relatively practical avenue of intervention to prevent liver fibrosis (42). Antioxidants, such as vitamin E, suppress fibrogenesis in some but not all studies of experimential fibrogenesis (61). Recent studies have documented inhibition of stellate cell activation by resveratrol, quercetin, and N-acetylcysteine (62). Silibinin reduces collagen accumulation by 30% in secondary biliary fibrosis in rats via its antioxidant activity (63). The antifibrotic properties of these flavonoids rely on their antioxidant effects (63). The powerful inhibition of COL1A2 expression by catalase and ebselen in the CYP2E1 stellate cell model suggests that agents that efficiently remove H$_2$O$_2$ may be especially promising in preventing stellate cell activation and alcoholic liver disease.
Ethanol and Arachidonic Acid Induce COLIA2

48. Inagaki, Y., Truter, S., Tanaka, S., Di Liberto, M., and Ramirez, F. (1995) J. Biol. Chem. 270, 3353–3358
49. Inagaki, Y., Truter, S., and Ramirez, F. (1994) J. Biol. Chem. 269, 14828–14834
50. Rossi, P., Karsenty, G., Roberts, A. B., Roche, N. S., Sporn, M. B., and de Crombrugghe, B. (1988) Cell 52, 405–414
51. Chung, K.-Y., Agarwal, A., Uitto, J., and Mauviel, A. (1996) J. Biol. Chem. 271, 3272–3278
52. Greenwel, P., Dominguez-Rosales, J. A., Mavi, G., Rivas-Estilla, A. M., and Rojkind, M. (2000) Hepatology 31, 109–116
53. Garcia-Trevijano, E. R., Iraburu, M. J., Fontana, L., Dominguez-Rosales, J. A., Auster, A., Covarrubias-Pinedo, A., and Rojkind, M. (1999) Hepatology 29, 960–970
54. Lieber, C. S. (1999) Alcohol. Clin. Exp. Res. 6, 991–1007
55. Brenner, D. A., and Chojkier, M. (1987) J. Biol. Chem. 262, 17690–17695
56. Thurman, R. G. (1973) Mol. Pharmacol. 9, 670–675
57. Matsuura, H., Sakaue, M., Subbaramaiah, K., Kamitani, H., Eling, T. E., Dannenberg, A. J., Tanabe, T., Inoue, H., Arata, J., and Jetten, A. M. (1999) J. Biol. Chem. 274, 29138–29148
58. Feng, L., Xia, Y., Garcia, G. E., Hwang, D., and Wilson, C. B. (1995) J. Clin. Invest. 95, 1669–1675
59. Hurst, J. S., Paterson, C. A., Bhattacherjee, P., and Pierre, W. M. (1989) Biochem. Pharmacol. 38, 3357–3363
60. Kotake, Y., Sang, H., Miyajima, T., and Wallis, G. L. (1998) Biochem. Biophys. Acta 1448, 77–84
61. Pietrangelo, A., Gualdi, R., Casalgrandi, G., Montosi, G., and Ventura, E. (1995) J. Clin. Invest. 95, 1824–1831
62. Kawada, N., Seki, S., Inoue, M., and Kuroki, T. (1998) Hepatology 27, 1265–1274
63. Boigk, G., Stroedter, L., Herbst, H., Waldschmidt, J., Riecken, E. O., and Schuppan, D. (1997) Hepatology 26, 643–649
Ethanol and Arachidonic Acid Increase \( \alpha_2(I) \) Collagen Expression in Rat Hepatic Stellate Cells Overexpressing Cytochrome P450 2E1: ROLE OF H2O2 AND CYCLOOXYGENASE-2

Natalia Nieto, Patricia Greenwel, Scott L. Friedman, Fan Zhang, Andrew J. Dannenberg and Arthur I. Cederbaum

\[ J. \text{ Biol. Chem.} \ 2000, \ 275:20136-20145. \]
\[ \text{doi: 10.1074/jbc.M001422200 \ originally published online April 17, 2000} \]

Access the most updated version of this article at doi: 10.1074/jbc.M001422200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 62 references, 19 of which can be accessed free at http://www.jbc.org/content/275/26/20136.full.html#ref-list-1