Cytochrome P450 2E1-derived Reactive Oxygen Species Mediate Paracrine Stimulation of Collagen I Protein Synthesis by Hepatic Stellate Cells

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To evaluate possible fibrogenic effects of CYP2E1-dependent generation of reactive oxygen species, a model was developed using co-cultures of HepG2 cells, which do (E47 cells) or do not (C34 cells) express cytochrome P450 2E1 (CYP2E1) with stellate cells. There was an increase in intra- and extracellular H$_2$O$_2$, lipid peroxidation, and collagen type I protein in stellate cells co-cultured with E47 cells compared with stellate cells alone or co-cultured with C34 cells. The increase in collagen was prevented by antioxidants and a CYP2E1 inhibitor. CYP3A4 did not mimic the stimulatory effects found with CYP2E1. Collagen mRNA levels remained unchanged, and pulse-chase analysis indicated similar half-lives of collagen I protein between both co-cultures. However, collagen protein synthesis was increased in E47 co-culture. Hepatocytes from pyrazole-treated rats (with high levels of CYP2E1) induced collagen protein in primary stellate cells, and antioxidants and CYP2E1 inhibitors blocked this effect. These results suggest that increased translation of collagen mRNA by CYP2E1-derived reactive oxygen species is responsible for the increase in collagen protein produced by the E47 co-culture. These co-culture models may be useful for understanding the impact of CYP2E1-derived ROS on stellate cell function and activation.

Hepatic fibrosis is a common response to chronic liver injury, regardless of its nature (viral infection, alcohol abuse, and metal overload) and is also characterized by excessive deposition of extracellular matrix components (1). Oxidative stress represents a common link between the different types of chronic liver injury including iron overload (2, 3), ethanol (4, 5), CCl$_4$ (6), and hepatitis C virus (7). In exploring the role of oxidative stress, it can be difficult to discriminate between the indirect effect of necrosis on stimulating fibrosis and a direct fibrogenic effect of free radical species. However, it is increasingly clear that in addition to an effect of necrosis, there is a direct stimulation of extracellular matrix deposition during the prenecrotic stage of liver injury (8). The mechanisms underlying the fibrogenic effects of oxidative stress are not clarified. In particular, does oxidative stress provoke release of fibrogenic mediators by hepatocytes, directly stimulate fibrogenesis, or both?

Hepatic stellate cells (HSC) are the primary fibrogenic cell type in liver (9). During liver injury, they become activated, developing a myofibroblast-like phenotype associated with increased proliferation and collagen synthesis (1). In vivo, fibrosis is associated with free radical production in hepatocytes located near HSC, either in iron overload-induced (3, 10) or in CCl$_4$-induced liver injury (11). In fact, when iron overload is localized only in reticuloendothelial cells, no activation of collagen gene expression can be detected (2). Svegliati-Baroni et al. (8) have demonstrated that conditioned medium from hepatocytes activates the Na$^+$/H$^+$ exchanger and stimulates HSC proliferation, and the effect is reversed by the Na$^+$/H$^+$ exchange inhibitor, amiloride. These findings suggest that the Na$^+$/H$^+$ exchanger could represent a common mediator of the different effects induced by oxidative stress on HSC such as proliferation and increased collagen type I. These data further suggest that paracrine signals from hepatocytes may stimulate HSC proliferation and collagen synthesis in vivo.

Cytochrome P450 2E1 (CYP2E1) has assumed an important role in alcohol-induced oxidative stress and liver injury. In the intragastric model of ethanol feeding, there is prominent induction of CYP2E1, a large increase in lipid peroxidation, and significant alcoholic liver injury in rats fed diets containing polyunsaturated fatty acids but not saturated fatty acids (6, 12, 13), and inhibitors of CYP2E1 block these responses (14). In a recently established HSC line overexpressing CYP2E1, there was an increase in collagen production by a mechanism dependent upon CYP2E1-derived intracellular ROS (15, 16). In vivo, however, most CYP2E1 in the liver is localized in the hepatocyte, and there is little information about potential paracrine stimulation by hepatocyte-derived ROS of HSC fibrogenesis, either in vivo or in culture.

The aim of this study was to develop a co-culture model in order to evaluate how CYP2E1-dependent generation of ROS impacts activation of HSC, their antioxidant defense system, and extracellular matrix production. The model is based on the co-incubation of the previously established HepG2 cell lines, which do (E47 cells) or do not (C34 cells) express the human CYP2E1 (17, 18), with an immortalized rat HSC line. Some

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1 The abbreviations used are: HSC, hepatic stellate cell(s); COL1A1, a1 collagen type I; COL1A2, a2 collagen type I; CYP2E1, cytochrome P450 2E1; GPX, glutathione peroxidase; MMP, metalloproteinase; SOD, superoxide dismutase; ROS, reactive oxygen species; TBARS, thiobarbituric acid-reactive substances; TIMP, tissue inhibitor of metalloproteinase; MEM, minimal essential medium.
experiments were carried out using primary hepatocytes from either control or pyrazole-treated rats, in which there is an increase in CYP2E1 expression, co-cultured with freshly isolated HSC. These co-culture systems would more closely resemble the in vivo situation, where cell-to-cell communication may play a critical role in provoking fibrosis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Most reagents, unless specified, were from Sigma. 2′,7′-dichlorodihydrofluorescein diacetate and carboxyfluorescein were from Molecular Probes (Eugene, OR).

**Cell Culture**—The model used in most of the experiments described below is based on the co-culture of HepG2 cell lines that either do (E47 cells) or do not (C34 cells) express the human CYP2E1 (17, 18) with an immortalized rat stellate cell line (T6-HSC) (Fig. 1) (19, 20). The T6-HSC contain intracellular filaments typical of primary HSC, express desmin, α-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 (19). Catalytic activity of CYP2E1 in the E47 cells was monitored by the oxidation of p-nitrophenol to p-nitroacetylphenol after treatment with H2O2.

**Assays**—H2O2 concentration as well as lipid peroxidation products in the culture medium were measured using the Launch assay (Cayman Chemical) as previously described (15)

**Production of ROS (including H2O2 using dichlorodihydrofluorescein diacetate and carboxyfluorescein) was measured at the beginning and at the end of the co-culture experiments.**

**In situ Synthesis of Protein**—The amount of newly synthesized protein was measured using the EXPRE-35S mix (Amersham Pharmacia Biosciences) for 0, 1, 2, 4, 8, and 12 h to study the rate of collagen synthesis. Following the pulse, the cells were washed in 1× phosphate-buffered saline and lysed at the indicated time points with 150 μl of 200 × 104 HepG2 (or hepatocytes)/HSC of 5:1 ratio (Fig. 2). The T6-HSC were co-cultured with rat hepatocytes. Hepatocytes were isolated from saline-treated or pyrazole-treated rats (200 mg/kg of body weight/day for 2 days, followed by an overnight fast) as described by Wu and Cedarbaum (22). Pyrazole was used to elevate the level of CYP2E1 in the hepatocytes, whose content and activity was measured at the beginning and at the end of the experiments. Primary HSC were isolated from male Sprague-Dawley rats (600 ± 5 g) (Charles River Laboratories, Wilmington, MA) by in situ liver perfusion with brefeldin, pronase and collagenase followed by density gradient centrifugation with Nycodenz according to published protocols (23). Cell viability (95%) was assessed by the trypan blue exclusion method. Purity of the HSC (97%) was determined as described previously (23). Cells were co-cultured using cell culture inserts (3-μm pore size) to separate both cell populations; the HSC are plated on the bottom, and the HepG2 cells or hepatocytes are plated on the insert to create a gradient of the released mediators. A ratio of HepG2 (or hepatocytes)/HSC of 5:1 was chosen, since it is representative of the ratio of parenchymal/nonparenchymal cells in liver (24).

After overnight incubation of HSC alone in MEM supplemented with 10% fetal bovine serum and essential amino acids, the HSC medium was discarded, the cell culture inserts containing the overnight incubated C34 or E47 cells were transferred from the C34 or E47 cells were added to the co-culture systems. At this time, all additions were made (t = 0 h). In experiments using hepatocytes, they were directly plated on top of the freshly isolated HSC. For experiments in which conditioned medium was used, C34 or E47 cells were grown in flasks until 90% confluence without replacing the medium, typically 5 days; the medium was collected, centrifuged, and filtered through a 0.2-μm syringe filter before it was added to the HSC. General Methodology—Plasmid DNA preparation as well as transfection procedures, Northern and Western blot analysis, intracellular production of ROS (including H2O2 using dichlorodihydrofluorescein and intracellular lipid peroxidation products using cis-pyruvamic acid), the activities of antioxidant enzymes, GSH levels, and cell viability assays were described in previous publications (15, 16, 23).

**Results**—The effects of CYP2E1 on the rate of collagen production in HepG2 cells (or hepatocytes) and HSC were studied using primary hepatocytes from saline-treated or pyrazole-treated rats (200 mg/kg of body weight/day for 2 days, followed by an overnight fast) as described by Wu and Cedarbaum (22). Pyrazole was used to elevate the level of CYP2E1 in the hepatocytes, whose content and activity was measured at the beginning and at the end of the experiments. Primary HSC were isolated from male Sprague-Dawley rats (600 ± 5 g) (Charles River Laboratories, Wilmington, MA) by in situ liver perfusion with brefeldin, pronase and collagenase followed by density gradient centrifugation with Nycodenz according to published protocols (23). Cell viability (95%) was assessed by the trypan blue exclusion method. Purity of the HSC (97%) was determined as described previously (23). Cells were co-cultured using cell culture inserts (3-μm pore size) to separate both cell populations; the HSC are plated on the bottom, and the HepG2 cells or hepatocytes are plated on the insert to create a gradient of the released mediators. A ratio of HepG2 (or hepatocytes)/HSC of 5:1 was chosen, since it is representative of the ratio of parenchymal/nonparenchymal cells in liver (24).

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**Characterization of the Co-culture Model**—Indirect evidence suggests a paracrine mechanism by which hepatocytes stimulate HSC proliferation and collagen synthesis in vivo (3, 10). CYP2E1, which cells (E47 cells) or do not (C34 cells) overexpress CYP2E1, were incubated in the presence of HSC using a dual chamber apparatus that separates the cell types and allows for rapid exchange of soluble mediators (Fig. 1). Cells were plated at a ratio of HepG2 cells (or hepatocytes)/HSC of 5:1 (24). HSC were cultured alone for 16 h, after which the medium was discarded; then the cell culture inserts containing either HepG2 cells or hepatocytes (or no cells) were transferred with the culture medium to the plates containing the
HSC; this defined the beginning of the co-culture period (t = 0 h). Samples of either cell lysate from HSC or culture medium were then collected at different time points. Preliminary experiments were performed mixing both cell types directly to determine if cell-cell contact was necessary to stimulate collagen production; effects were identical to those described below for the dual chamber model. Therefore, the latter system was used exclusively, because the experiments allowed 1) determination whether there is a gradient/efflux of H$_2$O$_2$ from the E47 to the HSC that could explain the increase in HSC intracellular H$_2$O$_2$ in the HSC/E47 co-culture, we measured H$_2$O$_2$ concentration in the incubation medium and found a 40% increase in the HSC/E47 co-culture as compared with HSC incubated alone or co-cultured with C34 cells (Fig. 2A). To confirm that effects were due to H$_2$O$_2$, 5 µM commercial H$_2$O$_2$ was added as a positive control. The addition of catalase to the incubation medium or transfecting with catalase (pZeocat) lowered the levels of endogenous H$_2$O$_2$ or exogenous added H$_2$O$_2$. Note that since the E47 cells have higher activity of catalase, glutathione transferase, and GSH than the C34 cells (28), the addition of commercial H$_2$O$_2$ to the HSC/E47 co-culture resulted in greater metabolism of the added H$_2$O$_2$. These data suggest that some of the elevated H$_2$O$_2$ generated by the E47 cells increases the intracellular concentration of H$_2$O$_2$ in the HSC following its diffusion.

Increased Lipid Peroxidation—In addition to H$_2$O$_2$, lipid peroxidation-derived products are another possible mediator for the effects of CYP2E1 on HSC. Therefore, lipid peroxidation in each cell type within the co-cultures was measured using the cis-parinaric acid method (decreased fluorescence reflects increased lipid peroxidation). Increased peroxidation was observed in the HSC/E47 co-culture compared with HSC alone or co-cultured with C34 cells (p < 0.01, Fig. 3A). As expected, lipid peroxidation was increased in the E47 cells compared with the C34 cells, and this increase was not affected by co-culture with the HSC (E47 or HSC/E47) compared with C34 cells (Fig. 3A). Moreover, when lipid peroxidation-derived products in the incubation medium were measured, there was a 40% increase in TBARS in the HSC/E47 co-culture when compared with the HSC incubated alone or the HSC/C34 co-culture (p < 0.01) (Fig. 3B). Cells were also incubated in the presence of 45 µM arachidonic acid as a positive control in the presence or absence of 25 µM vitamin E. Arachidonic acid increased TBARS production especially in the co-cultures containing the E47 cells, and vitamin E prevented this increase. These results suggest that both H$_2$O$_2$ and lipid peroxidation products are increased in the HSC themselves and the incubation medium after co-culturing with CYP2E1-containing cells. We believe that the H$_2$O$_2$ and lipid peroxidation products are largely derived from the E47 cells, since the increase of these ROS was blocked by added catalase and vitamin E, analogous to the prevention of increased collagen type I protein (described below). However, the possibility that the E47-derived oxidative stress can elevate ROS by the HSC cells themselves cannot be ruled out, so that several possibilities contribute to the enhanced state of oxidative stress in the HSC/E47 co-culture.
CYP2E1-derived Mediators Increase Collagen I Protein but Not COLIA2 mRNA—Incubation of HSC with conditioned medium from the C34 cells grown in flasks for 5 days had no effect on the levels of collagen type I protein or COLIA2 mRNA levels (data not shown). However, there was a 4-fold increase in collagen type I protein expression in the HSC co-cultured with the E47 cells. This increase in collagen I protein was not associated with a corresponding change in the COLIA2 mRNA levels (not shown). Thus, these initial experiments validated the concept that the CYP2E1-expressing HepG2 cells released collagen type I protein expression in the HSC co-cultured with the E47 cells. This increased expression was not as pronounced at 12 and 24 h in the E47 co-culture than in the C34 co-culture (data not shown). This effect was slightly increased by treatment with 1-buthionine sulfoximine at the more prolonged incubation time of 24 h. Fig. 4 shows the 24-h co-culture data; there was enhanced type I collagen in co-cultures with E47 cells in the absence or presence of 0.1 mM 1-buthionine sulfoximine compared with the HSC co-cultured with C34 cells. Moreover, there was an increase in secretion of collagen I to the medium of the E47 co-culture as assessed by Western blot, which was also potentiated by 1-buthionine sulfoximine (Fig. 4). To ensure that the increase in collagen I protein in HSC by co-culture with E47 cells and treatment with 1-buthionine sulfoximine was not a general effect on HSP protein synthesis, the expression of catalase in the samples collected at 24 h was also determined; no change was detected (Fig. 4). However, under these conditions, co-culture of HSC with either C34 or E47 cells, in the absence or presence of 0.1 mM 1-buthionine sulfoximine (BSO), did not alter the expression of COLIA1 or COLIA2 mRNA or the ratio of COLIA1 or COLIA2 to the housekeeping S14 ribosomal protein mRNA (data not shown). This suggests that the stimulating effect of the E47 co-culture system on collagen I protein is not regulated at the mRNA levels but similar to those obtained with the conditioned medium experiments described above.

Since changes in collagen I protein may occur in association with altered collagenase activity, the expression of MMP-13 and TIMP-1 was determined by Western blot. The expression of MMP-13 and TIMP-1 protein in the HSC and in the extracellular medium remained unchanged at the different time points selected (data not shown) and was not altered by either co-culture with E47 cells compared with C34 cells or by 1-buthionine sulfoximine treatment (Fig. 4). Taken together, these data suggest that the increase by the E47 co-culture on collagen I protein levels is not due to changes in the expression of major proteases such as MMP-13 or its inhibitors. Experiments to determine the rate of collagen synthesis and degradation in both co-culture systems are described below.

Comparison between the Effect of CYP2E1- and CYP3A4-derived Diffusible Mediators on Collagen I Protein in the Co-culture Model—The C34 cells do not have significant amounts of cytochrome P450; therefore, it is not clear whether the increase in collagen I protein by the E47 co-culture is due to the presence of CYP2E1 or to the presence of any other P450. We therefore evaluated whether CYP3A4, another enzyme of the P450 family, produces the same effect as CYP2E1 on collagen I protein. HSC were cocultured with C34, E47, or HepG2 cells overexpressing CYP3A4 (kindly provided by Dr. Denis Feierman, Mount Sinai School of Medicine) for 24 h and evaluated for collagen I expression by Western blot. The HSC/CYP3A4 co-culture produced a 2-fold increase in collagen I protein over the basal expression found in the HSC/C34 co-culture, whereas the HSC/E47 system produced a 8-fold increase in collagen over the basal HSC/C34 co-culture (Fig. 5). Thus, CYP2E1 was more effective than CYP3A4 in mediating paracrine stimulation of collagen type I protein levels in stellate cells.

Antioxidants Prevent the CYP2E1-mediated Increase in Collagen I Protein—Since there was an increase in H2O2 and lipid peroxidation in HSC co-cultured with the E47 cells, the inhibitory effect of antioxidants or a CYP2E1 inhibitor on collagen type I was assessed. Cells were plated and grown overnight; the next morning, the inserts and the incubation medium from the C34 or E47 cells were transferred onto the HSC and the co-cultures were immediately treated with either 1 mM concentration of the CYP2E1 inhibitor diallylsulfide, 2000 units of catalase, 25 μg vitamin E, or 2 mM glutathione ethyl ester or transfected with the empty vectors pZeo or pcDNA3 or with the expression vectors containing the cDNAs encoding for catalase (pZeo-CAT), GPX (pZeo-GPX), and Mn-SOD (pcDNA3-SOD). The co-cultures were maintained in the presence of these chemicals or transfection mix for 24 h, and cell lysates from the HSC were collected and analyzed by Western blot. Collagen I protein was almost 4-fold higher with the HSC/E47 than with the HSC/C34 co-culture (Fig. 6). Transfection with control plasmids had no effect on collagen levels in the HSC/E47 co-culture, validating the role of CYP2E1. Added catalase, which metabolizes H2O2, completely prevented the increase by CYP2E1-derived mediators on collagen I but decreased the basal expression of collagen I, indicating that endogenous H2O2 may have a parallel impact on endogenous collagen protein synthesis. Vitamin E, which prevents lipid peroxidation, had no effect on collagen levels in the C34 co-culture but completely prevented the increase in collagen I in the HSC/E47 system, suggesting that lipid peroxidation-de-
Derived products may be key mediators in the CYP2E1-dependent increase. Glutathione ethyl ester, a cell-permeable precursor of GSH, had no effect with the C34 co-culture but inhibited the increase by CYP2E1-derived diffusible mediators on collagen protein by 40% in the HSC/E47 system. Transfections with pZeo-CAT, pZeo-GPX, and pcDNA3-SOD completely blocked collagen protein levels in the E47 co-culture, suggesting a role for ROS in the increase produced by the E47 cell co-culture. Interestingly, these transfections blocked also the collagen expression in the C34 co-culture, suggesting that the basal pro-

**FIG. 2.** ROS production in the co-cultures. A, the concentration of ROS, mainly H₂O₂, in each cell line used in the co-cultures after 24 h of incubation was assayed by flow cytometry using the 2',7'-dichlorofluorescein diacetate method. Results are expressed as units of fluorescence and refer to mean ± S.E. (n = 3). ***, p < 0.005 compared with HSC and HSC/C34. B, the concentration of H₂O₂ in the incubation medium at 24 h was assayed by the FOX method. Controls included 2000 units of catalase, transfected catalase (pZeo-CAT), 5 μM H₂O₂, and the combination of transfected catalase and 5 μM H₂O₂. Results are expressed as μM H₂O₂ and refer to mean ± S.E. (n = 3). ***, p < 0.005 compared with HSC or HSC/C34. In all cases, the effects of catalase, transfected catalase, H₂O₂, and transfected catalase plus H₂O₂ were significantly different (p < 0.005 or p < 0.001) than the no addition control (first bar graph of each group).
duction of collagen type I protein is regulated, at least in part, by endogenous ROS. Taken together, these data suggest that CYP2E1 plays a role in the enhanced collagen I protein expression in the E47 co-culture by increasing production of ROS such as H$_2$O$_2$ and lipid peroxidation products. Western blot analysis as well as assays of the oxidation of $p$-nitrophenol showed that none of these treatments affected significantly the basal expression or activity of CYP2E1 except for diallylsulfide, which as expected, inhibited CYP2E1-dependent oxidation of $p$-nitrophenol.

**CYP2E1-derived Diffusible Mediators Increase the Rate of Synthesis of Intracellular Collagen Type I but Do Not Affect the Turnover of the Protein**—The results indicate that when HSC are co-cultured with E47 cells (or when conditioned medium from E47 cells is added to the HSC), collagen I protein, but not the mRNA level, increases in a time-dependent manner. This

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**FIG. 3. Lipid peroxidation production in the co-cultures.** A, the formation of lipid peroxidation products in each cell line used in the co-cultures after 24 h of incubation was assayed by the cis-parinaric acid method. Results are expressed as units of fluorescence and refer to mean ± S.E. ($n = 3$). ***, $p < 0.005$ compared with HSC or HSC/C34. B, lipid peroxidation products in the incubation medium at 24 h were determined by the TBARS method. Controls included 45 μM of arachidonic acid in the presence or absence of 25 μM vitamin E. Results are expressed as nM TBARS and refer to mean ± S.E. ($n = 3$). ***, $p < 0.005$ compared with HSC or HSC/C34. In all groups, the increase in TBARS produced by arachidonic acid and the prevention by vitamin E of this increase was significant ($p < 0.005$ or $p < 0.001$) compared with the nonaddition control.
effect could be due to regulation of collagen protein at different levels including 1) increased translational efficiency of the COL1A1 and/or COL1A2 mRNA or 2) decreased degradation of the protein. Although there were no differences in the expression of intra- and extracellular MMP-13 or in TIMP-1 or heat shock protein 47 (an important chaperone protein for type I collagen synthesis) (data not shown), direct analysis of collagen protein turnover by the co-cultures was performed. The HSC were labeled with [35S]methionine for 24 h and chased with unlabeled methionine for up to 12 h. Collagen I was immunoprecipitated, and the remaining incorporated label was quantified by SDS-PAGE and fluorography. The half-life of collagen and total protein was calculated from the semilogarithmic plot of counts incorporated per min versus time. At the 0-h time point, prior to the initiation of the chase, the cpm incorporated into collagen type I protein was 4-fold greater for the E47 co-culture, suggestive of an increase in collagen synthesis (evaluated below). Pulse-chase experiments revealed that the half-lives for collagen type I protein were 3.9 h for stellate cells cultured alone, 4.1 h for stellate cells co-incubated with C34 cells, and 4.5 h for stellate cells co-cultured with E47 cells (Fig. 7, A and B). The minor increase in the half-life of collagen protein in the E47 system is likely to make only a small contribution to the overall (about 4-fold) increase in collagen protein in this co-culture. These degradation experiments were carried out after first incubating the stellate cells or co-culture systems for 24 h with the EXPRE35S35S mix, followed by the chase (i.e. after steady state levels of collagen were synthesized). We also determined degradation of newly synthesized collagen, incubating the stellate cells or co-cultures with the EXPRE35S35S mix for 2 h and then chasing. Half-lives of newly synthesized collagen type I protein were 5.4 h for stellate cells cultured alone, 5.4 h for the C34 co-culture, and 5.7 for the E47 co-culture (data not shown). Rates of degradation of total HSC protein were similar for HSC cultured alone or with C34 or E47 cells (Fig. 7C).

The rate of collagen protein synthesis was assessed next by incubating with EXPRE35S35S mix for up to 12 h after a 24-h incubation of stellate cells with or without C34 or E47 cells, for comparative purposes with the rate of collagen degradation. In initial experiments on collagen synthesis, HSC were incubated with and without conditioned medium from confluent flasks of C34 or E47 cells in the presence or absence of different doses of cycloheximide (0, 25, 50, 100, and 150 μg/mL), an inhibitor of protein synthesis. When HSC were cultured with conditioned medium from the E47 cells, cycloheximide blocked the increase in collagen I protein (data not shown), suggesting that CYP2E1-metabolites increase collagen levels through a protein synthesis-dependent mechanism. Although these results indicate that de novo protein synthesis is required, they do not rule out the possibility that inhibition of synthesis of other proteins by cycloheximide could prevent the CYP2E1-dependent increase in collagen type I; to clarify this, a second approach was to study the rate of collagen I protein synthesis by cell labeling and immunoprecipitation of collagen I using an antibody that recognizes both the procollagen...
**CYP2E1-derived Oxidative Stress Induces Collagen I**

**HSC/C34**

![Image of HSC/C34]

**HSC/E47**

![Image of HSC/E47]

Fig. 6. Antioxidants prevent CYP2E1-mediated increase of collagen I protein. The HSC/C34 or HSC/E47 co-cultures were pretreated with 1 mM diallylsulfide (DAS), 2000 units of catalase, 25 μM vitamin E, 2 mM glutathione ethyl ester (GEE), or transfected with the plasmids containing the sequence encoding for catalase (pZeo-CAT), GPX (pZeo-GPX), and Mn-SOD (pcDNA3-SOD), starting at the time when both cell types were co-incubated. Controls included nontreated cells as well as transfections with the empty vectors pZeo and pcDNA3. Antioxidants were present in the incubation medium for 24 h, and HSC cell lysates were collected and analyzed for collagen I expression by Western blot. The signal was quantified by densitometric analysis, and results are expressed as relative units of collagen I expression, with the nontreated HSC co-cultured

α1 and α2. Synthesis of collagen type I protein increased as a function of time of incubation with the 35S pulse with all three systems; however, the rate of collagen synthesis was highest with the E47 co-culture at all time points studied (Fig. 8, A and B). At 12 h, there was a 4-fold increase in the rate of collagen synthesis in the HSC/E47 co-culture compared with HSC cultured alone or in the presence of the C34 cells (Fig. 8, A and B). Cycloheximide blocked collagen synthesis in all systems at 2 h. Total protein synthesis by HSC was not altered by co-culturing with E47 cells (Fig. 8C). These results suggest that increased collagen synthesis and not changes in collagen degradation are associated with increased collagen protein in the presence of CYP2E1-mediated oxidative stress. Since COL1A1 or COL1A2 mRNA levels were not changed in the HSC/E47 co-culture, the increase in collagen protein synthesis by this co-culture appears to reflect an increase in translation of collagen mRNA by the CYP2E1-derived diffusible mediators. Hepatocytes from Pyrazole-treated Rats Increase Collagen I Protein Expression in Freshly Isolated HSC—To verify the results obtained in the T6-HSC and the HepG2 cell lines to primary HSC and intact hepatocytes, freshly isolated HSC were co-incubated with primary hepatocytes from either control or pyrazole-treated rats. Pyrazole induces CYP2E1 protein expression about 3-fold in hepatocytes and stabilizes the protein against degradation (22). Collagen I protein levels in HSC cultured alone or with hepatocytes isolated from saline control rats were undetectable at 2 days, whereas HSC co-cultured with hepatocytes from pyrazole-treated rats showed an induction in collagen type I protein levels (Fig. 9A). This increase was prevented by added catalase and vitamin E, indicating that ROS were involved. Importantly, the increase in collagen I protein was decreased by the CYP2E1 inhibitors diallylsulfide and phenyl isothiocyanate (Fig. 9A). Validation that these compounds inactivate CYP2E1 is shown by the 5-fold lower content and catalytic activity of CYP2E1 in pyrazole hepatocytes treated with diallylsulfide (DAS) and phenyl isothiocyanate (PICT) (Fig. 9B). Evidence for the increase in CYP2E1 content by the pyrazole treatment at the beginning and after 2 days in culture is shown by the 3-fold higher level and catalytic activity of CYP2E1 in pyrazole versus saline hepatocytes (Fig. 9B, right, Western blots and activity data). These levels of CYP2E1 in the pyrazole hepatocytes are about 7-fold higher than the levels of CYP2E1 in cell extracts of the E47 cells.

**DISCUSSION**

Although the development of liver fibrosis is a multicellular process involving paracrine signaling between resident liver cells and nonresident (immigrated) inflammatory cells, the central event in the initiation of liver fibrogenesis relates to the activation of stellate cells in the area of tissue necrosis and inflammation in which ROS play an important role. Direct stimulation of HSC proliferation and collagen synthesis by products generated from hepatocytes may contribute to iron- and alcohol-related fibrosis. Production of prooxidant agents or lipid peroxidation products in parenchymal cells can initiate a pathway leading to an increase in collagen production by stellate cells (3, 4). Results with the co-culture model indicate that CYP2E1-derived ROS can increase collagen protein synthesis by HSC.

To characterize the intercellular communication between the hepatocyte and the stellate cell and to better understand the mechanisms by which oxidative stress and other mediator molecules perpetuate the fibrogenic response in stellate cells, a co-culture model containing HSC with a HepG2 cell line that overexpresses cytochrome P450 2E1 (E47 cells) or a control HepG2 cell line (C34 cells) was used. This system, with constant generation of ROS, may be more reflective of the physiological conditions in the liver and 1) allows identification of diffusible mediators responsible for modulating collagen production, 2) permits evaluation of the role of ROS in extracellular matrix production, and 3) may help to explore molecular mechanisms underlying the increase of collagen type I protein.

The antioxidant defense of each cell type in both co-cultures was first characterized; no changes in the activities of any of the antioxidant enzymes or in GSH levels in the HSC were found. Except for glutathione transferase, the activities of the other enzymes in the HSC were much lower than those found in HepG2 cells, indicating a low antioxidant defense in HSC. There were increases in both intra- and extracellular H2O2 and in the intra- and extracellular lipid peroxidation end products in HSC co-incubated with the E47 cells. These observations of elevated H2O2 and lipid peroxidation levels in the HSC/E47 co-culture suggest that ROS produced by CYP2E1 metabolism in the HepG2 cells may diffuse and enter the HSC, with a subsequent modulation of HSC function such as increased collagen type I protein.

In the absence of an inflammatory response, CCl4 treatment
Fig. 7. CYP2E1-derived diffusible mediators do not alter collagen I protein degradation. A, the rate of collagen degradation was studied in HSC cultured alone or with C34 or E47 cells after pulsing with [35S]methionine for 24 h, followed by chasing for 0, 1, 2, 4, 8, and 12 h. Immunoprecipitation with anti-collagen I antibody, washing, and fluorography was carried out as described under “Experimental Procedures.” Collagen levels were determined from PhosphorImager analysis of fluorographs shown in A, whereas total protein degradation was determined from the decline in trichloroacetic acid-precipitable counts. B and C show the rate of collagen and total protein degradation, respectively.
FIG. 8. CYP2E1-derived diffusible mediators stimulate collagen I protein synthesis. A, the rate of collagen synthesis was determined at 0, 2, 4, 8, and 12 h by labeling with $[^{35}S]$methionine and immunoprecipitation as described under “Experimental Procedures.” A set of experiments in which cells were incubated with 40 μM cycloheximide was included at 2 h. B and C show the rate of collagen and total protein synthesis, as determined by PhosphorImager analysis of fluorographs of immunoprecipitated collagen or by counting cell pellets after trichloroacetic acid precipitation.
oxidation of 

**FIG. 9. Hepatocytes from pyrazole-treated rats induce collagen I protein in primary HSC.** Livers from control rats were perfused with bacterial collagenase and Pronase to isolate primary HSC, which were seeded and grown in 10% fetal bovine serum-MEM. Following overnight incubation, the cells were washed, and fresh medium was added. Rats were injected twice intraperitoneally with either saline or pyrazole, and 24 h after the last injection the livers were perfused with bacterial collagenase to isolate the hepatocytes, which were immediately seeded on the inserts and placed on top of the HSC. Some HSC were also cultured alone as controls. Where indicated, cells were treated with 2000 units of catalase, 25 μM vitamin E, 5 mM diallylsulfide (DAS), or 10 μM phenyl isothiocyanate (PICT). Either cell lysate from the HSC or microsomes from the hepatocytes were collected at 48 h of co-culture. A. Western blot for collagen type I shows that the pyrazole-treated hepatocytes induce collagen I protein in primary HSC at 48 h. No detectable collagen expression was observed in HSC cultured alone or co-incubated with control hepatocytes. B. Western blot depicting the expression of CYP2E1 in pyrazole-treated hepatocytes treated with or without diallylsulfide or phenyl isothiocyanate (first three lanes). CYP2E1 protein levels in pyrazole-treated and control rats at 0 and 48 h in culture are shown on the right. Results are expressed as relative densitometric units. CYP2E1 activity is expressed below the blots as pmol/min/mg of protein of p-nitrocatechol formed from the oxidation of p-nitrophenol.

of hepatocyte/stellate cell co-cultures increases lipid peroxidation and collagen gene expression in stellate cells (29). We investigated whether the co-incubation of HSC with the C34 or E47 cells could increase collagen expression in HSC. An increase in collagen production was detected in the HSC co-incubated with the E47 cells, which was prevented by catalase and vitamin E, as well as by transfectected catalase, GPX, and Mn-SOD, validating the participation of ROS in this effect. Moreover, similar results were observed in freshly isolated HSC co-incubated with hepatocytes from pyrazole-treated rats with high CYP2E1 induction. The increase in collagen protein by the HSC/E47 co-culture was not associated with an increase in COL1A1 or COL1A2 mRNA levels. Previously, a HSC line that overexpresses CYP2E1 was established; COL1A2 mRNA levels were elevated as compared with empty vector-transfected HSC. The increase in COL1A2 mRNA was blocked by antioxidants and was associated with increased transcription of the COL1A2 gene (15, 16). In contrast, in the co-culture model, increases in collagen protein were not associated with elevated collagen mRNA. This may reflect 1) different effects of intracellularly generated ROS versus extracellularly diffusible ROS, 2) differences in the levels of ROS in the HSC containing overexpressed CYP2E1 versus those diffusing from the HepG2 cells through the insert to the HSC, and 3) possible paracrine effects by other factors in the co-culture model, not present in the HSC-CYP2E1 model.

The prevention by antioxidants of the increase in collagen protein indicates that ROS play an important role in the CYP2E1-dependent up-regulation. However, it is not clear whether ROS are directly responsible for the increased collagen type I protein synthesis or if they act to modulate other proteins or signaling pathways, which subsequently result in an increase in collagen synthesis. Since the only major difference between the E47 and C34 cells is the expression of CYP2E1, the differences in ROS production and collagen expression between the HSC/C34 and HSC/E47 co-cultures is probably due to CYP2E1. This was validated by showing that a CYP2E1 inhibitor, diallylsulfide, prevented the increase in collagen by the HSC/E47 co-culture. A different cytochrome P450, CYP3A4 could not mimic the stimulatory effects found with the CYP2E1-expressing cells. Pyrazole hepatocytes with high levels of CYP2E1 were more effective than saline hepatocytes in elevating collagen I protein levels. Specificity for CYP2E1, however, requires evaluation of other P450 enzymes (e.g. CYP4A10 and CYP4A14, which were recently found to be potent generators of ROS in CYP2E1 knockout mice (30)).

Since the mechanism by which ROS increased collagen protein expression in HSC co-cultured with E47 cells did not involve changes at the mRNA level, the stability of collagen type I protein was studied. Results indicated that although the rate of collagen type I protein degradation is important for extracellular matrix remodeling in this co-culture model, only slight differences in the half-life of the collagen type I protein (as well as in the rate of total HSC protein degradation) were observed; therefore, collagen accumulation, as a consequence of decreased turnover, accounts minimally for the differences in collagen protein observed in the E47 co-culture. These results are in agreement with the fact that no differences were observed in the expression of MMP-13 or TIMP-1, proteins that regulate degradation of both normal and fibrotic liver matrix. Since it is believed that collagen degradation lags behind collagen synthesis, it follows that degradation is a posttranslational rather than a co-translational process, and the fact that degradation and secretion are kinetically distinguishable suggests that these processes may occur in parallel pathways (31).

To explain why collagen protein was elevated in the HSC/E47 co-culture in the absence of major changes in collagen mRNA or turnover, experiments were carried out to determine whether these differences were due to a more efficient translation of the collagen type I mRNAs. The rate of collagen type
I synthesis was similar between HSC alone or cultured with the C34 cells, but a 4-fold increase in synthesis was found in HSC cultured with the E47 cells. This extent of increase in synthesis is similar to the increases in collagen levels observed by Western blot analysis (Fig. 4). Some specificity for the increase in collagen synthesis is apparent from the lack of effect on several other HSC proteins, such as catalase, MMP-13, and TIMP-1, and the lack of effect of the HSC/E47 co-culture on total protein synthesis by HSC. These results suggest that increased translation of collagen mRNA was mainly responsible for the increase in collagen protein produced by the E47 co-culture. This is, however, to our knowledge, the first evidence that increased translation of collagen mRNA in HSC may occur by a ROS-dependent mechanism. There are several reports in the literature about possible mechanisms of regulation of translation of collagen mRNA. De Crombrugghe and Schmidt (32) found a sequence of about 50 nucleotides surrounding the translation initiation codon remarkably conserved in the a1(I) and a2(I) procollagen mRNAs; this sequence plays a role in determining the level of expression of these genes by modulating translational efficiency. Kirk et al. (33) have shown that there are pauses in the translation of procollagen α-chains in intact cells. The association of completely elongated but only partially modified procollagen chains with the polysome complex facilitates the carboxyl-terminal interactions that lead to triple helix formation. Veis and Kirk (34) observed that polysome-bound nascent procollagen contains chymotrypsin, chymotrypsin plus trypsin, and pepsin-resistant α-chain size components. Rossi and de Crombrugghe (35) speculated that translational efficiency of both α1(I) and α2(I) collagen mRNA could be modulated by influencing the equilibrium between monomer and dimer. Guta et al. (36) have shown that while synthesis of pro-a1(I) and pro-a2(I) collagen chains proceeds in parallel within the same endoplasmic reticulum compartments, their elongation rates are not coordinated. Whether oxidative stress directly or indirectly could affect the formation of the translational initiation codon for collagen or export of the peptides into the endoplasmic reticulum, the elongation rates, or the folding and formation of the triple helix, still remains to be elucidated.

It is of interest that while changes in collagen protein by ROS-dependent mechanisms can be associated with transcriptional activation of the collagen genes, other possibilities, including stability of collagen mRNA (37, 38) and increased translational efficiency of collagen mRNA may be important for further evaluation. The co-culture models described in this report may be useful in furthering our understanding of intercellular communication between hepatocytes and stellate cells and the impact of CYP2E1-derived ROS on HSC function, activation, and collagen metabolism.

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