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Early Response of $\alpha 2(I)$ Collagen to Acetaldehyde in Human Hepatic Stellate Cells is TGF- β Independent

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Abstract

Acetaldehyde is fibrogenic and induces the expression of type I collagen genes in hepatic stellate cells. Some of these acetaldehyde-dependent events are mediated by H₂O₂ and thus establish a direct connection between oxidative stress and collagen upregulation. We localized to the -378 to -183 region of the $\alpha 2(I)$ collagen (COL1A2) promoter an acetaldehyde-responsive element (AcRE) functional in human hepatic stellate cells (HHSCs) and investigated molecular mechanisms whereby acetaldehyde stimulates and modulates its transcriptional activity. Because the AcRE co-localized with a previously described transforming growth factor β (TGF- β)1-responsive element, and both acetaldehyde and this cytokine induce their effects through H₂O₂, we investigated whether all fibrogenic actions of acetaldehyde were mediated by this cytokine. Here we show that acetaldehyde-induced COL1A2 upregulation in HHSCs recognizes two distinct but overlapping early and late stages that last from 1 to 6 hours and from 6 to 24 hours, respectively. We present several lines of evidence to show that early acetaldehyde-mediated events are independent of TGF- β 1. These include significant time-course differences in the expression of COL1A2 and TGF- β 1 mRNAs and inability of neutralizing antibodies to TGF- β 1 to inhibit acetaldehyde-dependent collagen gene transcription and Smad 3 phosphorylation. We also show that although acetaldehyde-dependent upregulation of collagen was PI3K dependent, that of TGF- β 1 was PI3K independent. In conclusion, acetaldehyde-dependent mechanisms involved in COL1A2 upregulation are similar, but not identical, to those of TGF- β 1. We suggest that early acetaldehyde-dependent events induce the late expression of TGF- β 1 and create an H₂O₂-dependent autocrine loop that may sustain and amplify the fibrogenic response of this alcohol metabolite.

Abbreviations

HSC, hepatic stellate cell; Col1a1, $\alpha 1(I)$ collagen gene; ROS, reactive oxygen species; COL1A2, $\alpha 2(I)$ collagen; HHSC, human HSC; TGF, transforming growth factor; AcRE, acetaldehyde-responsive element; TbRE, TGF- β 1-responsive element

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A lcohol-induced liver fibrosis is multifactorial and involves local and systemic events. At the systemic level, multiple cytokines, including those involved in the acute phase response, play a role in the pathophysiology of the disease and contribute to the fibrogenic process inducing apoptosis and upregulating the expression of type I collagen and tissue inhibitor of metalloproteinase-1 mRNAs.^{1–6} At the local level, fibrogenic mechanisms are quite complex and involve various cell types, including hepatocytes, Kupffer cells, and hepatic stellate cells (HSCs).^{7–14} Whereas hepatocytes are the main cells involved in ethanol metabolism, Kupffer cells contribute with the production of cytokines, chemokines, and growth factors that play key roles in attracting inflammatory cells to injured sites and in activating and trans-differentiating HSCs to myofibroblasts, the main type I collagen-producing cells in the liver.^{11,12} Some of the molecular mechanisms whereby the various hepatic cell types contribute to liver fibrogenesis have been unraveled.^{11–14} However, important key events by which ethanol metabolized to acetaldehyde by hepatocytes activates production of type I collagen in HSCs remain to be fully elucidated. We have already shown that, in a functional co-culture system containing hepatocytes and HSCs, the former cells metabolize ethanol and the latter produce type I collagen.¹⁵ We and others have shown that acetaldehyde is fibrogenic *per se* and induces the expression of the $\alpha 1(I)$ collagen (*Colla1*) gene^{16–18} by a mechanism dependent on the accumulation of H₂O₂.¹⁸ We further established that H₂O₂ induces the activation, nuclear translocation, and DNA binding of members of the C/EBP β family of transcription factors to the –370 to –345 region of the *Colla1* gene.¹⁸ In addition, it has been shown that metabolites and reactive oxygen species (ROS) produced during ethanol metabolism by HepG2 cells or HSCs overexpressing CYP2E1 are fibrogenic.^{19–22} Taken together, these studies confirm the role of H₂O₂ and oxidative stress in liver fibrogenesis.

Although some publications have described general mechanisms of collagen production by HSCs and have unraveled some signal transduction pathways activated by acetaldehyde,^{23–28} little is known regarding to the molecular mechanisms whereby acetaldehyde upregulates the expression of the human $\alpha 2(I)$ collagen gene (*COL1A2*). Therefore, in this communication we describe molecular events triggered by acetaldehyde that result in *COL1A2* transcriptional activation. We show that acetaldehyde induces the rapid phosphorylation and nuclear translocation of Smads 3 to 4 via a TGF- $\beta 1$ -independent pathway and that protein complexes containing Sp1 and Smads 3 and 4 are essential for *COL1A2* upregulation by acetaldehyde in HHSCs.

Materials and Methods

Plasmids and Reagents

Transforming growth factor-beta-1 (TGF- $\beta 1$) was purchased from Roche Molecular Biochemicals (Indianapolis, IN). Catalase, cycloheximide, and mithramycin were purchased from Sigma Chemical Co. (St. Louis, MO). Wortmannin was purchased from Calbiochem (La Jolla, CA). Acetaldehyde was purchased from Fisher Scientific (Springfield, NJ). Details about the construction of the *COL1A2/LUC* chimeric plasmids, the *COL1A2* mutant constructs, MSp1 *COL1A2/LUC*, MCAGA *COL1A2/LUC*, and $\Delta 5ACOL1A2/LUC$, TKCAT plasmid, (AcRE)₃(TK)CAT plasmid and dominant negative Smad 2, Smad 3, and Sp1 plasmids have been published (<http://www.jbc.org/cgi/content/full/272/32/-B1829>).³⁰ The *COL1A2* and TGF- $\beta 1$ cDNAs have been described previously^{29,30} (<http://www.jbc.org/cgi/content/full/272/32/-B26>). The GAPDH and S14 ribosomal protein cDNAs were obtained from the ATCC (Rockville, MD). Oligonucleotides containing consensus recognition sequences for Sp1 and AP-1 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

HHSC Isolation and Culture

HHSCs were isolated from samples of human livers obtained during gastric bypass surgery for morbid obesity as previously described.²⁶ Informed consent in writing was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a prior approval by the institutional review committee. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hy-Clone Inc., Logan, UT) and antibiotics. Experiments were performed in triplicate using cells obtained from at least 3 different patients cultured for 2 to 6 passages.

Northern Blot Hybridizations and Run-on Transcription Assays

Confluent HHSCs were placed in a serum-free medium containing glutamine, nonessential amino acids, and antibiotics. Approximately 14 hours later, acetaldehyde was added at a final concentration of 200 $\mu\text{mol/L}$. For some experiments, cells were preincubated with either mithramycin (100 nmol/L), catalase (1,000 U/mL), wortmannin (100 nmol/L), or cycloheximide (30 $\mu\text{g/mL}$) for 30 minutes before acetaldehyde administration. Viability of the cells was estimated using the trypan blue exclusion test. In all cases, cellular viability was more than 90%. Total RNA was extracted at the times indicated in the figures and processed for Northern blot hybridizations according to standard protocols.¹⁸ Likewise, a standard protocol was used to determine rates of *COL1A2* and *TGF- β 1* transcription in control and acetaldehyde-treated cells using *GAPDH*, *S14*, and pBR322 DNA as controls.³¹ In some run-on transcription experiments, cells were pre-incubated for 2 hours before acetaldehyde administration, with a neutralizing antibody to TGF- β 1 (Promega Corp., Madison, WI) or an unrelated immunoglobulin G (Santa Cruz Biotechnology, Inc.), at a final concentration of 5 $\mu\text{g/mL}$. To determine the effectiveness of the anti-TGF- β 1 antibody, some HHSCs were also incubated with 2 ng/mL recombinant TGF- β 1 in the presence or absence of the corresponding neutralizing antibody, or an unrelated immunoglobulin G, following the protocol previously described. Three hours later, nuclei were isolated and used in run-on transcription assays as previously described. Relative intensity of the signals was determined by laser densitometric analysis of the radiographic films. Values are means of triplicate experiments \pm SD and were corrected for loading differences using *S14* as control.

Preparation of Nuclear Extracts, Footprinting Analysis, and Gel Mobility Shift Assays

Nuclear extracts were prepared from control and HHSCs treated with acetaldehyde for the times indicated in the figures as previously reported.²⁹ After incubating nuclear extracts with an end-labeled probe, gel mobility shift assays and footprinting analysis were carried out as previously described.²⁹ Binding conditions, as well as the sequences of the AcRE (-313 to -183) oligonucleotide and the oligonucleotide containing 3 copies in tandem of the *COL1A2* Smad binding site (-272 to -249) (*COL1A2* [CAGA]₃) used as either probes or unlabeled competitors, have been previously described.^{29,30} Identical amounts of nuclear proteins from each cell source were added to the binding reactions. Complexes formed were identified by autoradiography of the dried gels. For antibody interference assays, antibodies against Sp1 (PEP-2), Sp3 (D-20), Smads 2 (S-20), 3 (I-20) and 4 (C-20), c-fos (K-25), c-jun (D), and p50 (H-119), and p65 (C-20) nuclear factor kappaB (NF κ B) were purchased from Santa Cruz Biotechnology, Inc. These antibodies were used at a final concentration of 0.16 $\mu\text{g/mL}$. For competition studies, unlabeled oligonucleotides were added at a 100-fold molar excess.

Western Blot Analysis

Nuclear extracts (20 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on 10% gels and transferred onto a nitro-cellulose membrane. Membranes were probed with Smad 3 or TGF- β 1 goat antibodies at a final dilution of 1:1,000, followed by incubation with horseradish peroxidase-conjugated donkey anti-goat immunoglobulin G

(Santa Cruz Biotechnology) diluted 1:5,000. Proteins were detected with an enhanced chemiluminescence system (Renaissance; NEN Life Science Products, Boston, MA), as recommended by the manufacturer. Conditions used to assess the expression of FLAG epitope in cells stably transfected with the different DN proteins were described before using the M2 anti-Flag antibody from Sigma (<http://mcb.asm.org/cgi/content/full/20/3/-B16>).³¹ For detection of phosphorylated Smad 3, nuclear extracts were immunoprecipitated with 5 µg anti-phosphoserine antibody (Poly-Z-PS 1), (Zymed Laboratories, San Francisco, CA) and then subjected to Western blot analysis with Smad3 antibodies as described above.

Cell Transfections

Conditions for the preparation and transfection of plasmids into HHSCs by the calcium phosphate procedure have been detailed before (<http://www.jbc.org/cgi/content/full/275/50/-B27>).^{30,31} For the functional characterization of the acetaldehyde-responsive elements, HHSCs were treated with 10% glycerol for 90 seconds 16 hours after transfection and then placed in medium containing 0.1% fetal bovine serum. Two hours later, acetaldehyde was added at a 200 µmol/L final concentration. Twenty-four hours later, a second dose of acetaldehyde was added. Cells were procured 48 hours after the addition of the first dose of acetaldehyde, and used to determine luciferase or CAT assays as previously described.^{29,32} Transcriptional activity of each of the chimeric constructs was normalized against co-transfected pSv2CAT (when using luciferase vectors) or pSV40 LUC (when using CAT vectors). Transfections were performed multiple times and in duplicate. The statistical value of the data was evaluated by the Mann-Whitney *U* test. Stable transfectants were selected by culturing the cells for approximately 3 weeks in the presence of 400 µg/mL G418.³³ For some experiments, HHSCs were co-transfected with 7.0 µg of the -378 COL1A2LUC reporter construct and 2.5 µg of either one of the dominant negative Smad2, Smad3, or Sp1 vectors followed by treatment with acetaldehyde using the conditions described above.

Statistical Analysis

Values were expressed as mean \pm SD and are average from 3 to 6 values per experiment; experiments were repeated at least 3 times. Either Student's *t* test or the Mann-Whitney *U* test was used to evaluate the statistical differences between groups: a *P* value equal to or less than .05 was considered significant.

Results

Acetaldehyde induces COL1A2 expression in HHSCs by a transcriptional mechanism via a *de novo* protein synthesis-independent mechanism. Northern blot analysis of total RNA extracted from HHSCs treated with acetaldehyde for 6 hours revealed that α 2(I) collagen mRNA was induced 3.4 ± 0.4 fold compared with untreated cells ($P < .05$) (Fig. 1A). Time-course studies indicated that collagen mRNA upregulation is a relatively early event that starts approximately 3 hours after acetaldehyde administration and reaches its maximum approximately 16 to 24 hours later 4.6 ± 0.7 ($P < .05$) (Fig. 1B). Lack of inhibition by cycloheximide demonstrated that this early acetaldehyde-dependent increase in α 2(I) mRNA is independent of *de novo* protein synthesis (Fig. 1B). Although cycloheximide did not prevent acetaldehyde-mediated 2(I) collagen mRNA upregulation within the first 3 hours, further increase of COL1A2 gene expression is mostly dependent on *de novo* protein synthesis (Fig. 1B). Run-on transcription assays performed at 3 hours after acetaldehyde administration showed a 4.3 ± 0.3 -fold increase in transcription rates, demonstrating that the increase in COL1A2 gene expression in response to acetaldehyde is exerted at the transcriptional level (4.3 ± 0.3 vs. control untreated cells) ($P < .05$) (Fig. 1C).

The acetaldehyde-responsive element (AcRE) of the *COL1A2* gene is localized to the -378 to -183 promoter region and contains 2 distinct footprints.

To localize the region in the *COL1A2* promoter responsible for acetaldehyde-mediated transcriptional activation, transient transfections in HHSCs were performed using luciferase reporter vectors driven by progressive 5' deletions of the *COL1A2* upstream sequence. Cells transfected with a reporter vector driven by the 3,500-bp *COL1A2* promoter responded to acetaldehyde by augmenting luciferase activity 2.03 ± 0.1 -fold above control values ($P < .05$) (Fig. 2A). Similarly, cells transfected with a vector driven by the proximal 378-bp promoter responded to acetaldehyde by stimulating luciferase activity 2.47 ± 0.2 fold ($P < .05$) (Fig. 2A). By contrast, luciferase activity in cells transfected with a reporter vector driven by the 183-bp *COL1A2* promoter was not altered after acetaldehyde administration (Fig. 2A). These analyses therefore located the acetaldehyde responsive element (AcRE) somewhere within the -378 to -183 region of the *COL1A2* promoter. Footprinting analysis using the -378 to -183 sequence, and nuclear extracts prepared from control and acetaldehyde-treated HHSCs showed 2 distinct sites of DNA-protein interaction that were noticeably wider and more evident in acetaldehyde-treated than untreated cells (Fig. 2B). Interestingly, the 2 footprints are virtually identical to those previously reported in TGF- β -treated fibroblasts.²⁹ Further confirmation of the functional role of the footprinted region was obtained by showing that this sequence was capable of conferring acetaldehyde responsiveness to the otherwise unresponsive TK promoter (Fig. 2C).

The AcRE co-localizes with the previously described TGF- β 1-responsive element (*TbRE*) of *COL1A2*.²⁹ Because the *TbRE* contains Sp1 and Smad-binding sites, we determined whether these transcription factors are involved in the acetaldehyde-dependent upregulation of the *COL1A2* gene. HHSCs were therefore transfected with luciferase reporter vectors driven by the wild-type -378 promoter sequence or by -378 promoters containing mutations within the Sp1 (MSp1COL1A2/LUC) or the Smad (MCAGA COL1A2/LUC) binding sites (Fig. 2D). Whereas mutations in the Sp1-binding sites decreased substantially basal expression of the reporter vector and abrogated response to acetaldehyde, mutations of the Smad-binding site had only a minimal effect on basal gene expression but abrogated acetaldehyde responsiveness (Fig. 2D).

Sp1 and Smads 3 and 4 are required for acetaldehyde-mediated upregulation of *COL1A2* gene expression. The electrophoretic mobility shift assay was employed to study the possible role of Sp1 and members of the Smad family of transcription factors in acetaldehyde-induced *COL1A2* upregulation. To this end, we used nuclear extracts purified from control cells and from HHSCs treated with acetaldehyde from 1 to 24 hours. Consistent with the footprinting data, electrophoretic mobility shift assay showed increased binding to the -313 to -183 sequence after acetaldehyde treatment, whereas antibody interference assays demonstrated that complex 1 contained Sp1 but not AP-1 or NF κ B (Fig. 3A-B). The formation of Sp1-containing DNA complexes increased as early as 1 hour and remained elevated thereafter for at least 24 hours after acetaldehyde administration (Fig. 3A). The nature of complex 2 remains to be determined. However, as shown in Fig. 3A, binding of this complex was also altered by acetaldehyde treatment. However, the increment in complex 2 was found to be variable and not always reproducible (compare Figs. 3A and B). Increased Sp1 binding activity to the *COL1A2* promoter after acetaldehyde treatment was not unique; binding to an Sp1 high-affinity recognition sequence also increased (Fig. 3C). The essential role of Sp1 in acetaldehyde-mediated upregulating of *COL1A2* was further confirmed by experiments of interference with Sp1 DNA-binding activity. First, mitramycin at a concentration known to specifically inhibit Sp1 binding to DNA-abrogated acetaldehyde-stimulated α 2(I) collagen mRNA accumulation ($P < .05$) (Fig. 4A). Second, overexpression of a dominant negative form of Sp1 completely abrogated acetaldehyde-mediated up-regulation of the -378COL1A2 LUC vector (Fig. 4B).

Similar analyses established the role of Smad proteins in the acetaldehyde response. Electrophoretic mobility shift assays demonstrated binding of Smads 3 and 4 to the cognate site of the *COL1A2* promoter, which increased significantly after treatment (Fig. 5A). Time course studies revealed that the Smad 3–4 DNA complexes appear as early as 15 minutes after acetaldehyde administration and remain evident for at least 24 hours thereafter (Fig. 5B). Immunoblots of nuclear proteins from control and acetaldehyde-treated HHSCs demonstrated the presence of Smad 3 in both samples. However, phosphorylated Smad 3 was only present in acetaldehyde-treated cells, and this post-translational modification was not inhibited by cycloheximide (Fig. 5C). Co-transfection of HHSCs with the -378COL1A2LUC construct reporter and vector expressing a dominant negative isoform of Smad 3 documented abrogation of the acetaldehyde response (Fig. 6A). Furthermore, HHSCs stably transfected with the dominant negative Smad 3 expression vector displayed down-regulation of the endogenous *COL1A2* gene, in addition to blunting of acetaldehyde responsiveness (Fig. 6B). Similar findings were obtained with HHSCs transfected with a dominant negative Smad 4 construct (data not shown). In marked contrast, a dominant negative isoform of Smad 2, which is not part of the acetaldehyde-induced complex binding to the *COL1A2* promoter, had no effect on the activity of the -378 COL1A2LUC plasmid (Fig. 6A). We reasoned from previous study of the *coll1a1* that accumulation of H₂O₂ may be similarly responsible for stimulation of *COL1A2* expression in HH-SCs. Indeed, Northern blots showed that catalase prevents acetaldehyde-mediated upregulation of $\alpha 2(I)$ collagen mRNA accumulation (0.85 ± 0.17 vs. 3.49 ± 0.17 ; $P < .05$) (Fig. 7).

Early acetaldehyde-elicited upregulation of *COL1A2* is TGF- β 1 independent. The finding that the AcRE and the TbRE co-localize to the same promoter element and that Sp1 and Smads 3 and 4 are involved in both transcriptional responses raised the possibility of a functional relationship between these 2 signaling pathways. However, this possibility was not supported by experimental evidence. First, a neutralizing antibody to TGF- β 1 failed to prevent acetaldehyde-elicited phosphorylation of Smad 3 in HHSCs or acetaldehyde-dependent *COL1A2* upregulation (3.6 ± 0.36 vs. 3.03 ± 0.22) (Fig. 8A-B). Second, acetaldehyde treatment had virtually no effect on the expression of the TGF- β 1 gene in HHSCs at the point when *COL1A2* expression is already upregulated (Fig. 8C). Last, addition of an acetaldehyde inhibitor, wortmannin, had no effect on TGF- β stimulation of *COL1A2* expression in HHSCs (Fig. 8D) but completely abrogated the acetaldehyde-elicited response. Interestingly, and in agreement with data obtained by others, showing that acetaldehyde induced the late expression of TGF- β 1 mRNA and its receptor,^{16,28} we found that acetaldehyde upregulated the expression of this cytokine mRNA by 6 to 8 hours at a point in which *COL1A2* mRNA was already upregulated. As shown in Fig. 9A-B, changes in TGF- β 1 mRNA were accompanied by similar elevations in TGF- β 1 protein as determined by Western blot analysis.

Discussion

That acetaldehyde, the first metabolite of ethanol, is a fibrogenic factor in HSCs is well established.^{16,18,24–26} Thus, irrespective of whether ethanol is metabolized via alcohol dehydrogenase or CYP2E1, generated acetaldehyde triggers a signal transduction cascade that results in increased expression of type I collagen genes.¹⁷ Previous studies from our laboratory have localized an acetaldehyde-responsive element in the -370 to -344 of the *coll1a1* promoter and that this element co-localized with TGF- β 1- and TNF- α -responsive elements.^{18,32,33} Furthermore, we established that acetaldehyde upregulates *coll1a1* gene expression by a mechanism dependent on the generation of H₂O₂ and involving nuclear translocation and binding of members of the C/EBP β family of transcription factors. Type I collagen is a trimeric molecule product of 2 distinct genes that are coordinately expressed through apparently distinct transcriptional mechanisms. This study has further corroborated the diversity of the regulatory

mechanisms in type I collagen genes by demonstrating that responsiveness of COL1A2 to acetaldehyde is mediated by Sp1 and Smads 3 and 4 transcription factors.

Like the murine *colla1*, the AcRE of the human *COL1A2* gene co-localizes with the TbRE.²⁹ However, several lines of evidence exclude TGF- β involvement in acetaldehyde upregulation of COL1A2 at early points after acetaldehyde administration. First, *COL1A2* transcription is elevated in HHSCs within 1 hour after acetaldehyde administration, whereas that of TGF- β remains at background levels. Second, acetaldehyde-induced $\alpha 2(I)$ collagen mRNA accumulation does not require *de novo* protein synthesis, whereas that of TGF- $\beta 1$ does. Third, neutralizing antibodies to TGF- $\beta 1$ have no effect on acetaldehyde-mediated phosphorylation of Smad 3 and on transcriptional upregulation of the *COL1A2* gene. Fourth, wortmannin inhibits early acetaldehyde-induced accumulation of $\alpha 2(I)$ collagen mRNA, but has no effect on TGF- β -dependent *COL1A2* up-regulation. Although our findings did not address the formal possibility that acetaldehyde may activate pre-existing TGF- β and that this in turn may lead to autocrine stimulation of TGF- β expression, studies performed by others demonstrated that cytokine activation by acetaldehyde is a late event occurring at least 6 to 12 hours after acetaldehyde administration.²⁸ Moreover, his findings also showed that acetaldehyde induces the late expression of TGF- $\beta 1$ receptor type II (6 hours) and enhances the response of HSCs to TGF- β .²⁸ Altogether, these findings suggest that acetaldehyde exerts its fibrogenic actions by TGF- $\beta 1$ -independent and TGF- $\beta 1$ -dependent mechanisms. Because the late events are cytokine dependent, the findings strongly suggest that acetaldehyde starts the fibrogenic response and primes HSCs to further respond to this cytokine. Moreover, based on our previous findings showing that H₂O₂ is a mediator of acetaldehyde and TGF- $\beta 1$ -dependent responses, one could speculate that acetaldehyde, through this ROS, activates a TGF- $\beta 1$ -dependent autocrine loop.

Data from several laboratories have shown the critical nature of the proximal regions of the *COL1A1* and *COL1A2* promoters in regulating collagen gene expression.^{29-31,34-42} The region encompassing -183 to -378 of the *COL1A2* gene harbors TGF- β ,²⁹ TNF- α ,³⁰ acetaldehyde (this communication), and interferon-responsive elements.³⁶ These responsive elements bind members of the C/EBP family of transcription factors,³⁸ Sp1,^{29,31,43} (this communication), Smads 3 and 4,^{31,44,45} (this communication), Ets 1,³⁵ Y box-binding protein YB-1, and the co-activator p300.³⁶ Whereas Sp1, Smads 3 and 4, and p300 form DNA complexes that upregulate the transcription of the *COL1A2* gene, the others down-regulate gene expression. Ets 1 is acetylated after TGF- $\beta 1$ treatment; it associates with CBP/p300 protein complexes and shifts TGF- β response from collagen production to matrix degradation.³⁵ Conversely, YB-1 binds to the interferon responsive element and also interferes with the formation of Smad 3/p300 complexes.³⁶

Our studies demonstrate that acetaldehyde enhances binding of Sp1 and Smads 3 and 4 to the AcRE and that these transcription factors are essential for COL1A2 acetaldehyde responsiveness. These findings are in agreement with previous studies from our and other laboratories, demonstrating that Sp1 is essential for basal as well as TGF- $\beta 1$ -induced type I collagen gene expression.^{29,31,39,43-46} The molecular mechanisms whereby acetaldehyde activates Sp1 binding activity are yet to be determined, as is the potential role of Sp3. Smads 3 and 4 are essential for the acetaldehyde-dependent upregulation of the *COL1A2* gene because overexpression of their respective dominant negative isoforms completely abrogates acetaldehyde responsiveness. As shown in Fig. 8A, HHSC nuclei contain phosphorylated Smad 3 within 30 minutes after acetaldehyde administration.

Studies performed with mouse HSCs have localized an AcRE within a similar region of the *colla2* promoter. However, the transacting factor binding to the AcRE after acetaldehyde was identified as NF-1.⁴⁷ This nuclear transcription factor has been shown to play an important

role in TGF- β 1-mediated upregulation of the mouse *colla2*.⁴⁸ However, our studies have failed to demonstrate binding of NF1 to the COL1A2 AcRE, as antibody interference assays with NF1 antibodies had no effect on retarded bands induced by acetaldehyde (data not shown). These findings suggest that in addition to tissue-specific transcription factors there might be species-specific factors as well.

Previous work from our laboratory has established that several kinases, including PI3K and MAPK, are involved in acetaldehyde-dependent upregulation of COL1A2 mRNA.²⁶ However, we further need to establish which of these kinases, if any, are directly involved in phosphorylation of Smads or activation of Sp1 binding to DNA. Such studies will allow us to determine whether activation and phosphorylation of Smads 3–4 in acetaldehyde-treated cells is similar to that induced by TGF- β 1 and, therefore recognize a common mechanism.

We have already shown that acetaldehyde and TGF- β 1 induce accumulation of H₂O₂ in rat HSCs.^{18,32} In this communication, we further showed that H₂O₂ is involved in acetaldehyde-mediated upregulation of *COL1A2* in human HSCs. Therefore, this could be a common link in the fibrogenic cascade.¹⁸ Indeed, data in the literature suggest that H₂O₂ can induce the expression of TGF- β 1 in cultured hepatic stellate cells.⁴⁹ Accordingly, this could establish an important autocrine loop whereby acetaldehyde enhances the accumulation of H₂O₂ and this ROS upregulates expression of TGF- β 1 that will further generate more H₂O₂. This autocrine loop, which generates a strong oxidative stress response in HHSCs, could play a key role in perpetuation of liver fibrosis on discontinuation of the injurious stimuli. Therefore, agents that interfere with this autocrine loop directly or through modification of the protein kinases that activates it could represent a novel form of therapy for alcohol-induced liver fibrosis.

In summary, our findings demonstrate that acetaldehyde-induced fibrogenesis follows a complex signaling pathway that is independent of TGF- β 1 at early points but activates similar mediators used by the cytokine in upregulation collagen gene expression. In addition, this and previous work from our laboratory establishes the importance of oxidative stress in general and of H₂O₂ in particular in the fibrogenic mechanisms of acetaldehyde. Thus, antioxidants designed for blocking the production of H₂O₂ by HSCs and Kupffer cells could be of use in the treatment of liver fibrosis.

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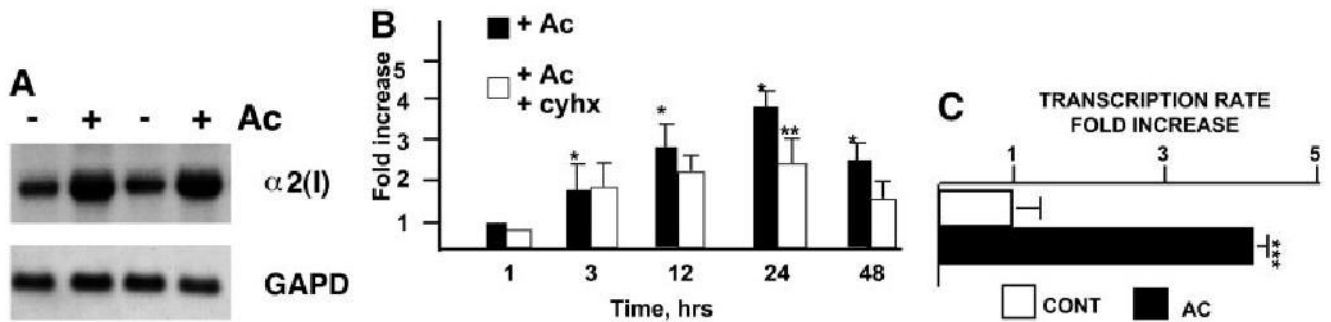
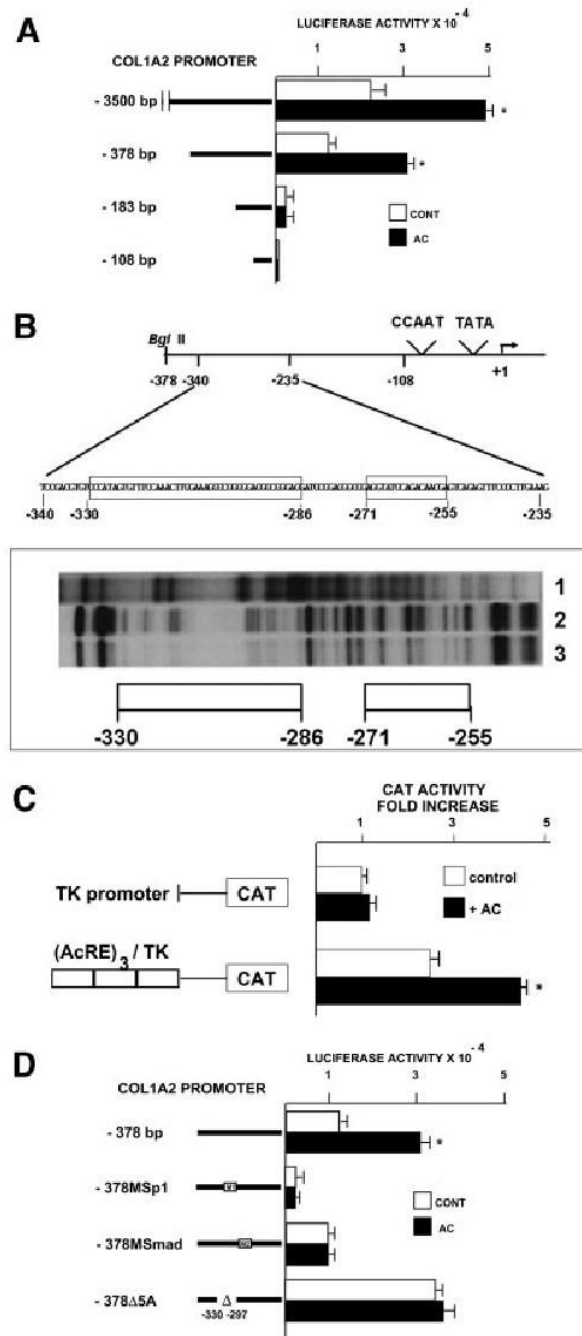


Fig. 1. Acetaldehyde stimulates COL1A2 gene expression at the transcriptional level by protein synthesis–dependent and –independent mechanisms. (A) Northern blot analysis of total RNA extracted from HHSCs cultured in the presence or absence of acetaldehyde (AC) for 6 hours; the identity of the probes is indicated on the right side of the autoradiograph. (B) Time-course analysis COL1A2 mRNA expression in AC-treated cells cultured in the absence or presence of cycloheximide (cyhx). (C) Run-on transcription assays of the endogenous *COL1A2* gene in HHSCs treated with AC for 3 hours. * $P < .05$ when compared with control, untreated HSCs. ** $P < .05$ when compared with AC-treated cells. *** $P < .05$ when compared with control values.

**Fig. 2.**

The acetaldehyde-responsive element of the *COL1A2* gene in HHSCs is located between nucleotides -378 and -183 . (A) Deletion analysis in control and acetaldehyde (AC)-treated HHSCs transfected with luciferase reporter constructs driven by different lengths of the *COL1A2* promoter. The length of each of the constructs used is shown on the left side of the figure. $*P < .05$ vs control without AC. (B) Footprinting analysis of the -378 to the -183 *COL1A2* promoter region performed with nuclear extracts from control (lane 2) and acetaldehyde-treated HHSCs (lane 3) for 3 hours. As a negative control, DNA incubated without nuclear extracts was used (lane 1). (C) CAT activity in control and acetaldehyde-treated HHSCs (AC) transfected with a reporter CAT vector driven by the TK promoter alone or with

a vector containing 3 copies in tandem of the acetaldehyde-responsive element (AcRE) upstream of the TK promoter. * $P < .05$ vs. cells transfected with the (AcRE)₃ TKCAT vector without AC. (D) Mutation analysis in control and acetaldehyde-treated HHSCs transfected with luciferase reporter constructs driven by mutated versions of the -378 COL1A2LUC vector. For these experiments, the following sequences of the -378COL1A2LUC were mutated: -301 to -302, -292 to -291 and -275 to 274, sequences corresponding to the 3 COL1A2 Sp1 binding sites (M_{Sp1} COL1A2/LUC) or the -272 to -249 sequence corresponding to the SMAD binding site (MCAGA COL1A2/LUC). In addition, a construct in which the -330 to -297 area was deleted (Δ 5A) was also used. * $P < .05$ vs. control cells without AC.

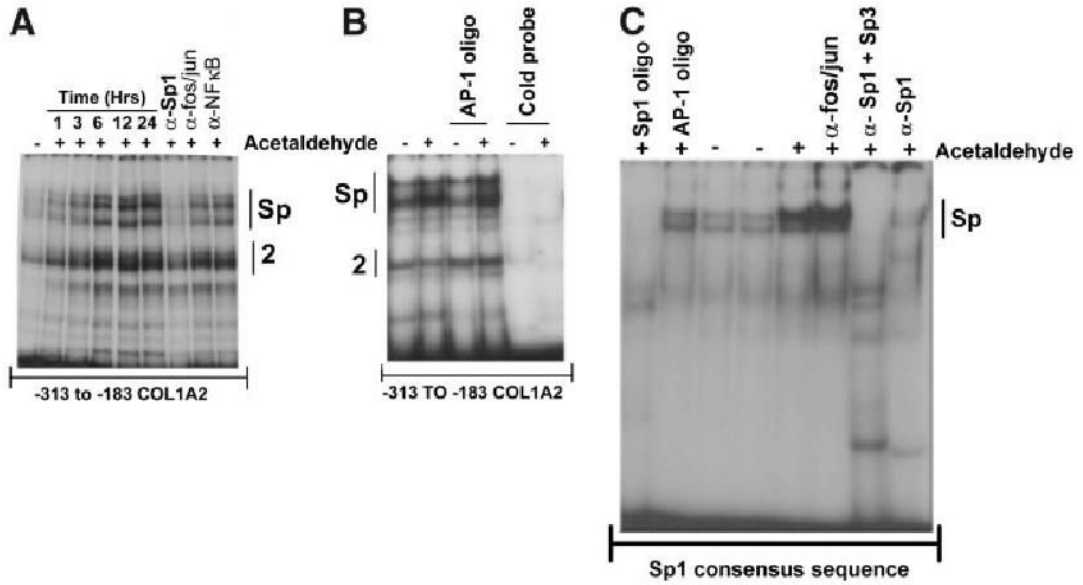


Fig. 3.

Acetaldehyde enhances binding of a Sp1-containing complex to the -313 to -183 region of the *COL1A2* gene in HHSCs and to a Sp1 high-recognition sequence. (A) Electrophoretic mobility shift assays of nuclear extracts from control (-) and acetaldehyde-treated (+) HHSCs for 1, 3, 6, 12, and 24 hours using the -313 to -183 *COL1A2* region. As shown in this panel, acetaldehyde significantly enhances protein binding to this region. Antibody interference assays demonstrated that the upper complex contains Sp1, but not AP-1 (fos/jun) or NFκB. The identity of the lower complex (labeled “complex 2” remains unknown). (B) The complexes formed after acetaldehyde treatment (3 hours) are specific, as they can be competed by unlabeled -313 to -183 *COL1A2* sequence (“cold probe”) but not by an AP-1 high-affinity recognition oligonucleotide. (C) Increased binding of nuclear extracts of HSCs treated for 24 hours with AC to an Sp1 high-recognition oligonucleotide. The complex is specific, as it can be competed with excess (100-fold molar) unlabeled Sp1 consensus sequence, but not AP-1, and is recognized by antibodies to Sp1 and Sp3 (α-Sp1 + α-Sp3) but not AP-1 (anti-fos/jun). Panel C also shows that whereas the combination of α-Sp1 + α-Sp3 antibodies completely eliminates (or supershifts) the DNA-protein complexes obtained with acetaldehyde, the α-Sp1 antibody was less efficient and did not eliminate the complex completely.

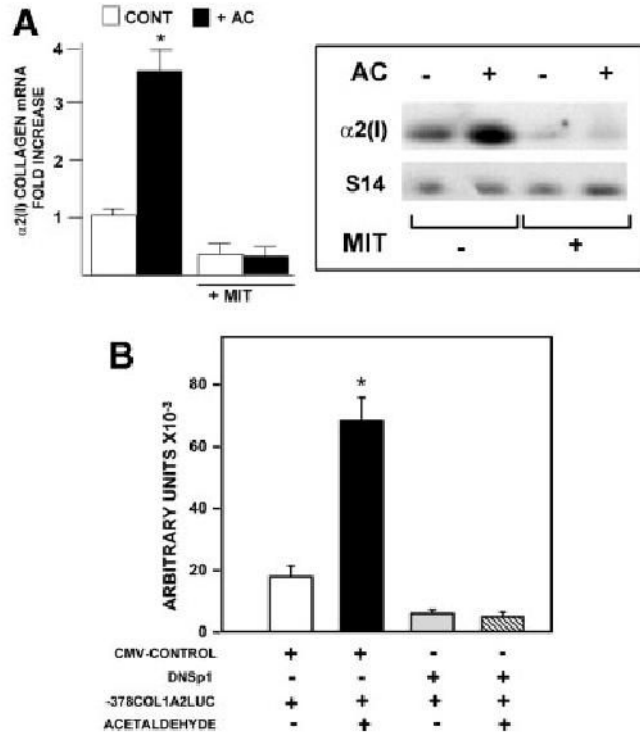


Fig. 4. Sp1 is essential for the acetaldehyde-dependent upregulation of the COL1A2 gene in HHSCs. (A) Northern blot analysis of $\alpha 2(I)$ collagen mRNA performed with total RNA extracted from control and acetaldehyde (AC)-treated HHSCs cultured in the presence or absence of 100 nmol/L mithramycin (MIT) for 6 hours. The histogram on the left summarizes the results of quadruplicate experiments. The right panel depicts a representative Northern blot. Differences in loading were corrected with the signal generated with an S14 mRNA probe. * $P < .05$ versus control cells without AC. (B) Effect of acetaldehyde on HHSCs transiently co-transfected with the -378 COL1A2LUC reporter construct and a dominant negative Sp1 expression plasmid (DNSp1). Activity is expressed as arbitrary units of luciferase activity. As a control for these experiments, some HHSCs were co-transfected with the -378 COL1A2LUC and an empty CMV expression vector (CMV-CONTROL). * $P < .05$ versus control cells without AC.

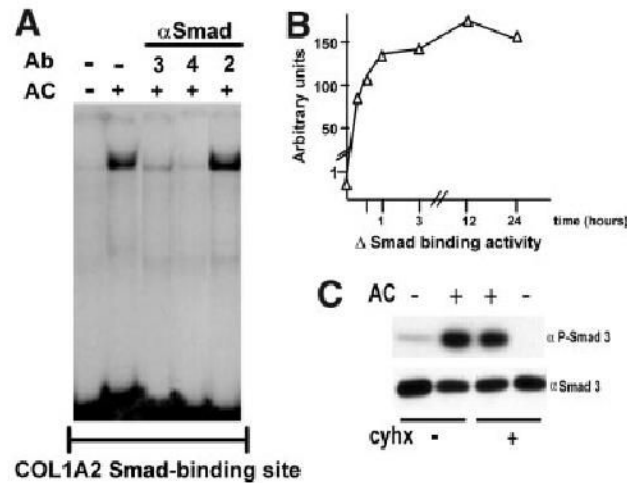
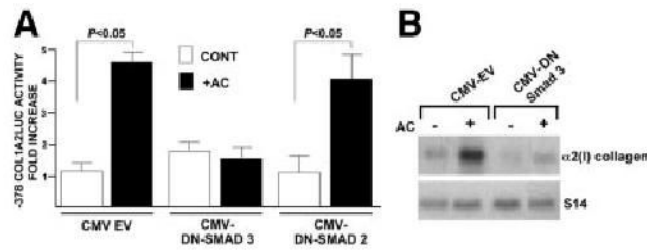


Fig. 5. Acetaldehyde enhances phosphorylation of Smad 3 and binding of a Smad3/4-containing complex to the -272 to -249 COL1A2 region by a protein synthesis-independent mechanism. (A) Electrophoretic mobility shift assay performed with nuclear extracts from control and acetaldehyde (AC)-treated HHSCs for 6 hours, using an oligonucleotide containing 3 copies in tandem of the COL1A2 Smad binding site (CAGA)₃ as probe. Antibody interference assays with antibodies (Ab) to either Smads 2, 3, or 4 revealed that the complex contained Smads 3/4 but not Smad 2. (B) Time-course analysis of the effect of acetaldehyde on the binding of the Smad 3/4-containing complex to the (CAGA)₃ oligonucleotide. The graph was constructed with values obtained after densitometric analysis of bands generated in duplicate electrophoretic mobility shift assays performed at the indicated times and expressed as arbitrary units. (C) Western blot analysis of nuclear extracts obtained from control and acetaldehyde (AC)-treated HHSCs for 6 hours in the presence or absence of cycloheximide (cyhx). Samples were probed with an anti-Smad3 antibodies directly (α Smad3) or after immunoprecipitation with an anti-phosphoserine antibody (α P-Smad3).

**Fig. 6.**

Smad 3 but not Smad 2 is essential for acetaldehyde-dependent COL1A2 gene upregulation in HHSCs. (A) Effect of acetaldehyde (AC) on luciferase activity of HHSCs transiently co-transfected with the -378 COL1A2LUC reporter vector and either a control empty CMV vector, or dominant negative Smad3 (DN-SMAD3) or Smad2 (DN-SMAD2) expression vectors. Values are expressed as fold increase above controls. (B) Northern blot analysis of total RNA extracted from HHSCs harboring stably integrated copies of an empty CMV vector or an expression vector encoding DN-Smad3 cultured in the presence or absence of AC. The identity of the probes is indicated on the right side of the autoradiography.

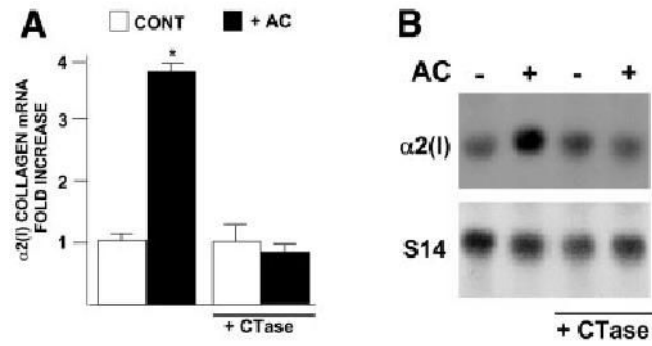


Fig. 7.

Catalase abrogates acetaldehyde-induced $\alpha 2(I)$ collagen mRNA expression in HHSCs.

Northern blot analysis of $\alpha 2(I)$ collagen mRNA performed with total RNA extracted from control and acetaldehyde (AC)-treated HHSCs cultured in the presence or absence of catalase (1,000 U/mL) for 24 hours. (A) The histogram summarizes the results of quadruplicate experiments. (B) Representative Northern blot. Differences in loading were corrected with the signal generated with an S14 mRNA probe. * $P < .05$ versus control cells without AC.

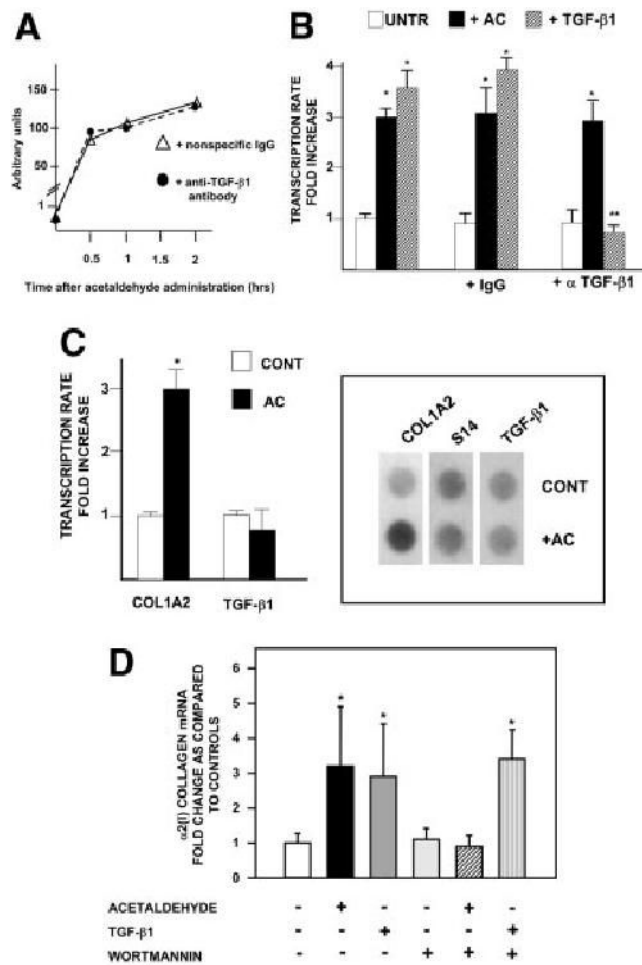


Fig. 8. TGF-β1 is not involved in early acetaldehyde-dependent up-regulation of the *COL1A2* gene. (A) Effect of neutralizing antibodies to TGF-β1 on acetaldehyde-induced phosphorylation of Smad3. HHSCs were incubated with acetaldehyde in the presence of a nonspecific immunoglobulin G (triangles) or an anti-TGF-β1 neutralizing antibody (circles). At the indicated times, nuclear extracts were obtained for Western blot analysis of phosphorylated Smad 3 as described in Materials and Methods. The graph was constructed with values obtained after densitometric analysis of bands generated in duplicate Western blots performed at the indicated times and expressed as arbitrary units. (B) Run-on transcription assays of control and acetaldehyde-treated HHSCs for 3 hours in the presence or absence of a nonspecific immunoglobulin G or a neutralizing antibody to TGF-β1 (α TGF-β1). As a control for these experiments, some HHSCs were treated with 2 ng/mL of TGF-β1 in the absence or presence of the neutralizing antibody to the cytokine. * $P < .05$ versus control untreated cells. ** $P < .05$ when compared with TGF-β1-treated cells in the absence of the neutralizing antibody. (C) Run-on transcription assays of *COL1A2* and *TGF-β1* gene expression performed with nuclear extracts from control and acetaldehyde (AC)-treated HHSCs for 1 hour. The histogram on the left summarizes the results of triplicate experiments. The right panel depicts a representative run-on assay. Differences in loading were corrected with the signal generated with an S14 probe. * $P < .05$ versus control cells without AC. (D) Northern blot analysis of HHSCs treated with either acetaldehyde (200 μmol/L) or TGF-β1 (2 ng/mL) in the absence or presence of 100 nmol/L wortmannin. For these experiments, HHSCs were pretreated for 3 hours with

wortmannin followed by 12 hours with acetaldehyde or TGF- β 1. The histogram summarizes results of triplicate experiments, and the results are expressed as fold-increase above control untreated HHSCs. * $P < .05$ versus control cells without AC, TGF- β 1, or wortmannin.

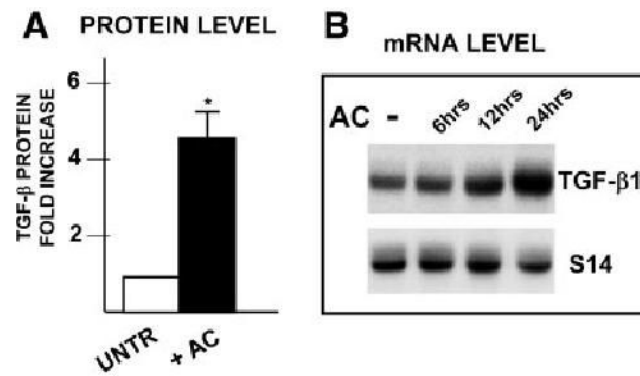


Fig. 9. Acetaldehyde induces late expression of $\alpha 2(I)$ collagen mRNA by a TGF- $\beta 1$ -dependent mechanism. (A) Western blot analysis of TGF- $\beta 1$ expression in control and acetaldehyde-treated HHSCs for 24 hours. The histogram summarizes triplicate experiments, and the results are expressed as fold increase above untreated controls. * $P < .05$ versus control cells without AC. (B) Time-course analysis of TGF $\beta 1$ mRNA expression in HHSCs treated with 200 $\mu\text{mol/L}$ acetaldehyde. The identity of the probes is indicated on the right side of the autoradiograph.