Intracellular Signaling Pathways Involved in Acetaldehyde-Induced Collagen and Fibronectin Gene Expression in Human Hepatic Stellate Cells

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Ethanol induces liver fibrosis by several means that include, among others, the direct fibrogenic action of acetaldehyde on hepatic stellate cells (HSC). However the mechanisms responsible for this effect are not well understood. In this communication we investigated signal transduction pathways triggered by acetaldehyde leading to upregulation of α2(I) collagen and fibronectin gene expression in human HSC. Run-on assays showed that acetaldehyde-enhanced transcription of these 2 genes as early as 2 hours, via *de novo* protein synthesis-independent and -dependent mechanisms. It also stimulated a time-dependent induction in phosphorylation of pp70S6K and extracellular-regulated kinase 1/2 (ERK1/2). These effects were completely prevented by calphostin C, a protein kinase C inhibitor. As expected, acetaldehyde-elicited ERK1/2 phosphorylation was inhibited by PD98059, a MEK inhibitor, but not by wortmannin, a PI3K inhibitor. On the other hand, both of these inhibitors partially inhibited phosphorylation of pp70S6K induced by acetaldehyde suggesting that its activation is ERK1/2- and PI3K-dependent. Acetaldehyde-elicited fibronectin and α2(I) collagen upregulation was inhibited by calphostin C. However, while PD98059, wortmannin and rapamycin (a pp70S6K inhibitor) completely abrogated α2(I) collagen upregulation, they had no effect on fibronectin expression. Overall, these data suggest that protein kinase C is an upstream component from which acetaldehyde signals are transduced to other pathways such as PI3K and ERK1/2. In addition, differential activation of these pathways is needed for the increase in fibronectin and α2(I) collagen gene expression induced by acetaldehyde in human HSC. (Hepatology 2001;33:1130-1140.)

Hepatic stellate cells (HSC) are the primary source of excess extracellular matrix proteins in liver fibrosis.1 In normal liver, HSC are localized in the space of Disse and store vitamin A in the form of retinyl esters.2 During the development of fibrosis, HSC undergo a process of activation acquiring a myofibroblast-like phenotype characterized by α-smooth muscle actin expression and increased proliferation and synthesis of extracellular matrix components, including type I collagen.3-4 In recent years several studies have been performed to elucidate some intracellular signaling pathways that underlie the fibrogenic behavior of HSC. For example, it has been shown that the extracellular signal-regulated kinase (ERK1/2) and the phosphatidylinositol 3-kinase (PI3K) play important roles in proliferation, chemotaxis, and collagen synthesis in HSC stimulated with different growth factors.5-9.

The development of liver fibrosis in alcoholics has been linked to the oxidation of ethanol to the highly reactive compound acetaldehyde.10 At concentrations that have been described in hepatic venous blood during alcohol consumption,11 acetaldehyde stimulates type I collagen synthesis and gene transcription in cultured rat and human HSC,12,13 in baboon liver myofibroblasts,14 and in human skin or liver fibroblasts.15 Unfortunately, the signaling mechanisms underlying this stimulatory effect are not well understood, although recent data have implicated a role for protein kinase C (PKC).16,17 These studies showed that acetaldehyde increases α1(I) and α2(I) collagen gene expression in rat HSC through Ca2+-independent PKC activation. On the other hand, in human HSC, characterization of the intracellular pathways involved in acetaldehyde-elicited type I collagen upregulation is still lacking.

In this communication, we examined the signal transduction pathways triggered by acetaldehyde in human HSC, and correlated this activation with α2(I) collagen and fibronectin mRNA upregulation and HSC proliferation. Our results show that acetaldehyde does not modify HSC proliferation, and upregulates α2(I) collagen gene transcription via a transduction pathway where PKC represents an upstream component from which acetaldehyde signals are transduced either to PI3K or ERK1/2 and then to pp70S6K, which is a downstream effector. On the other hand, a different intracellular pathway, branching downstream of PKC, is involved in increased fibronectin gene expression.

MATERIALS AND METHODS

Materials. Culture media were from GIBCO (Grand Island, NY). Nycodenz was from Life Technologies (Milan, Italy). Calphostin C, PD98059, and rapamycin were from Calbiochem (La Jolla, CA). Bro-

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Abbreviations: HSC, human hepatic stellate cells; ERK1/2, extracellular signal-regulated kinase 1/2; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; SIFM, serum- and insulin-free Iscove’s medium; MEK, mitogen-activated protein kinase kinase; PLP, pyridoxal-5’-phosphate; PHMB, p-hydroxy-mercuribenzoate; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; BrdU, bromodeoxyuridine; ANOVA, analysis of variance.

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midoxyuridine was from Fluka (Flukachemie AG, Buchs, Switzerland). Nitrocellulose membranes (Hybond) were from Amersham (Milan, Italy). Pronase was from Boehringer Mannheim (Monza, Italy). Monoclonal antibromodeoxyuridine and peroxidase-conjugated rabbit antimouse immunoglobulins were from Dako (Glostrup, Denmark). Antibodies for pp70<sup>60k</sup> were from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents were from Sigma Chemical Co., Milan, Italy.

HSC Isolation and Culture. Human livers were obtained during gastric bypass surgery for morbid obesity. 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. HSC were isolated by the pronase-collagenase method as previously described. Small portions of each liver were routinely fixed in 4% phosphate-buffered formaldehyde and embedded in paraffin. Tissue sections (4 μm thick) were stained with hematoxylin-eosin for routine examination. Only HSC isolated from liver with minimal steatosis, without inflammation, were used for all experiments. Cells were cultured in Iscove’s modified Dulbecco’s Medium supplemented with 20% fetal bovine serum, 2 mmol/L glutamine, 0.1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, 0.6 U/mL insulin, and 1% antibiotic-antifungal solution. Primary cultures of HSC were grown to confluence, trypsinized (0.025% trypsin/0.5 mmol/L ethylenediaminetetraacetic acid), and subcultured in the same medium as described earlier. In this study, experiments were performed in triplicate using cells obtained from at least 3 different patients cultured for 1 to 3 passages.

Incubation of HSC with Acetaldehyde. Passaged activated HSC were seeded into 75-cm<sup>2</sup> scalable flasks until the monolayers were 75% to 80% confluent. The cell cultures were made quiescent by 24 hours, incubation in serum- and insulin-free Iscove’s medium (SIFM) and then incubated with 200 μmol/L of acetaldehyde for the indicated periods of time in the presence or absence of the different kinases or adduct formation inhibitors. These inhibitors have been previously tested in HSC, and their specificity at the concentrations used in the present study has been previously shown as: calphostin C (PKC-inhibitor), 1 μmol/L<sup>6,17,19</sup>; wortmannin and LY294002 (PI3K inhibitors), 100 nmol/L and 5 μmol/L respectively<sup>5-7,20-26</sup>; PD98059 (mitogen-activated protein kinase kinase [MEK] inhibitor), 50 μmol/L<sup>14,9,27-28</sup>; rapamycin (pp70<sup>60k</sup> inhibitor), 5 ng/mL<sup>29-32</sup>; pyridoxal-5’-phosphate (PLP) and p-hydroxy-mercuribenzoate (PHMB) (adduct protein adduct formation inhibitors), 10 μmol/L and 4 μmol/L respectively<sup>33,34</sup>. At these concentrations, all of these inhibitors completely prevented phosphorylation of their respective substrates, as shown in immunoprecipitation assays using p<sup>32</sup>-or-<sup>35</sup>S-orthophosphate-labeled proteins (data not shown). None of these compounds was toxic to HSC, as determined by the trypsin blue exclusion test (data not shown).

<sup>pp70<sup>60k</sup></sup> and ERK1/2 Activation Assays. Cells were scraped and homogenized in ice-cold buffer (pH 7.4) consisting of 50 mmol/L Tris pH 7.4, 150 mmol/L KCl, 1% Triton X-100, 1 mmol/L ethylenediaminetetraacetic acid, 5 mmol/L N-ethylmaleimide, and 0.2 mmol/L phenyl-methyl-sulfonyl-fluoride. Protein content was determined according to the Lowry method.<sup>35</sup> Homogenates were divided into aliquots and stored at −80°C for later use.

Cell extracts (50 μg/lane) were boiled for 3 minutes in Laemmli sample buffer and separated by gel electrophoresis. Western blotting was performed according to Towbin.<sup>37</sup> Nonspecific binding sites were blocked by incubating nitrocellulose sheets for 1 hour in PBS containing 1% low fat dry milk. Nitrocellulose sheets were then incubated with antiyrosine phosphorylated ERK1 (or p44MAPK) and ERK2 (or p42MAPK) (1:10,000) antibody or with anti pp70<sup>60k</sup> (1:500) antibody overnight at room temperature, followed by goat antirabbit IgG conjugated with alkaline phosphatase (1:1,000, 2 hours at room temperature). Specific protein bands were detected colorimetrically using 7 mg nitroblue tetrazolium and 3.5 mg 5-bromo-4-chloro-3-indolyl phosphate as alkaline phosphatase substrates, in 20 mL of 10% diethanolamine buffer (pH 9.8).

The intensity of the bands was determined by scanning densitometry (Kodak Digital Sciences, Rochester, NY) and expressed as arbitrary units.

**Determination of HSC Proliferation.** HSC proliferation was measured by indirect immunoperoxidase staining of nuclei, which had incorporated bromodeoxyuridine (BrdU),<sup>3-6,30-38</sup> Briefly, HSC were incubated in SIFM for 24 hours. After this time, the medium was removed and cells were incubated in the same medium containing acetaldehyde (200 μmol/L) for an additional 24 hours. BrdU was added, at a final concentration of 5 × 10<sup>−5</sup> mol/L, during the last 4 hours of incubation.

Cells were counted using a computerized image analysis system connected to an Olympus microscope (Olympus Vanox AHB3, Olympus Optical Co. LTD, Tokyo, Japan). Data were expressed as labeling index (LI = % positive nuclei).

**RNA Extraction and Northern Blot Analysis.** Confluent cells were washed twice with PBS, and the medium was replaced for SIFM. Approximately 16 hours later, acetaldehyde was added in the presence or absence of the different kinase or adduct formation inhibitors, as described earlier. Twenty-four hours later, cells were harvested and used to obtain total RNA following the method described by Chomczynski and Sacchi.<sup>41</sup> Northern blot analysis was performed using 10 μg of total RNA and hybridization performed using [α-<sup>32</sup>P]-labeled cDNA probes for α2(I) collagen,<sup>42</sup> fibronectin,<sup>43</sup> and S14 ribosomal protein (ATCC, Rockville, MD), following conditions previously described.<sup>44</sup> After hybridization, membranes were washed 4 times with 2X sodium saline citrate containing 0.1% sodium dodecyl sulfate at 65°C. Relative intensity of the signals was determined by laser densitometric analysis of the radiographic film. Values are means of triplicate experiments ± SD and were corrected for loading differences using S14 as control.

**Run-on Transcription Assays.** Confluent cultures of HSC were placed in SIFM. Approximately 16 hours later, acetaldehyde was added in the presence or absence of the different kinase or adduct formation inhibitors at the concentrations described earlier. After 2 or 24 hours, cell nuclei were obtained and used to determine rates of α2(I) collagen, fibronectin, and S14 ribosomal protein, as previously described.<sup>35</sup> For some experiments, cells were preincubated with cycloheximide (30 μg/mL) for 2 hours before the addition of acetaldehyde. This concentration of cycloheximide blocks protein synthesis by 98% (data not shown).

**Acetaldehyde Determination.** Acetaldehyde concentration in the medium of cultured HSC was measured as previously described.<sup>46</sup> Two hours after the addition of 200 μmol/L acetaldehyde, the concentration of acetaldehyde in HSC culture media was 78 ± 14 μmol/L, and 52 ± 6 μmol/L after 24 hours. These values are within the range of concentrations previously reported.<sup>12,46</sup>

**Statistical Analysis.** Results are expressed as mean ± SD. Group means were compared by analysis of variance (ANOVA), followed by Student-Newman-Keuls test if the former was significant. A P value < .05 was considered statistically significant.

**RESULTS**

Effect of Acetaldehyde on pp70<sup>60k</sup> and ERK1/2 Activation. Western blot analysis experiments were performed to evaluate the effect of acetaldehyde on pp70<sup>60k</sup> and ERK1/2 activation. Because pp70<sup>60k</sup> is downstream of PI3-K, its activation is usually an indirect measure of PI3-K activation.<sup>3,23,24</sup> Stimulation of pp70<sup>60k</sup> activity is accompanied by its increased phosphorylation on serine and threonine residues, resulting in reduced mobility of the protein on SDS-PAGE.<sup>32</sup> On the other hand, the effect of acetaldehyde on ERK1/2 phosphorylation was determined using an antibody that specifically recognizes the active tyrosine-phosphorylated form of ERK1 and ERK2.<sup>37</sup>

As shown in Fig. 1 (A and B), acetaldehyde induces significant pp70<sup>60k</sup> phosphorylation, as determined by the noticeable decrease in the mobility pattern of the 70-kd and 85-kd
protein triplets, as compared with control, untreated samples. This change in gel mobility pattern becomes evident 60 minutes postacetaldehyde treatment, with values returning to normal after 6 hours. Likewise, as shown in Fig. 1 (A and C) acetaldehyde induces a significant increase in ERK1/2 after 10 minutes of incubation that declined at 60 minutes and then returned down to control levels at 360 minutes.

**Effect of Adduct Formation Inhibitors on pp70S6K and ERK1/2 Activation.** We and others have shown that formation of acetaldehyde-protein adducts accounts, at least in part, for the stimulatory effect of acetaldehyde on HSC. In view of these data, we tested the effect of 2 different adduct formation inhibitors, namely PHMB and PLP, on acetaldehyde-induced pp70S6K and ERK1/2 activation. As shown in Fig. 2 (A-C) treatment of HSC with PHMB completely abrogates acetaldehyde-induced pp70S6K and ERK1/2 phosphorylation, thus suggesting that activation of these pathways by acetaldehyde may be mediated, at least in part, by formation of adducts with proteins. Similar results were obtained when using PLP (Fig. 2B and C).

**Effect of Different Intracellular Pathway Inhibitors on Acetaldehyde-Induced pp70S6K and ERK1/2 Activation.** It has been previously shown that pp70S6K and ERK1/2 are important elements in signal transduction pathways, which can be differentially regulated by upstream components. To study more specifically signal transduction pathways triggered by acetaldehyde in human HSC leading to enhanced extracellular matrix gene expression, the effect of various kinase inhibitors on pp70S6K and ERK1/2 activation, and on α2(1) collagen and fibronectin gene expression was evaluated. Because work from other laboratories has previously shown that acetaldehyde activates PKC in rat HSC, we first tested the effect of the PKC inhibitor calphostin C. As shown in Fig. 3 (A-C), calphostin C completely abolishes acetaldehyde-induced pp70S6K and ERK1/2 phosphorylation.

Because pp70S6K activation is, at least in part, PI3K-dependent in PDGF-stimulated HSC, we next tested the effect of wortmannin and LY294002, 2 different PI3K inhibitors, in HSC incubated with 200 μmol/L acetaldehyde. As shown in Fig. 4 (A and B), wortmannin reduces acetaldehyde-induced pp70S6K phosphorylation. However, the intensity of the phosphorylated bands in these samples is still significantly higher than that observed in control, untreated cells. On the other hand, as shown in Fig. 4 (A and B), this inhibitor does not reduce ERK1/2 activation induced by acetaldehyde. Similar results were obtained when using a structurally unrelated PI3K inhibitor LY294002 (Fig. 4B and C).

Overall, the results of the experiments presented earlier suggest that, in this experimental system, enzymes other than PI3K could be a plausible mediator. To further explore this possibility, the effect of the pp70S6K inhibitor rapamycin was tested. As shown in Fig. 6 (A and B), rapamycin significantly inhibits pp70S6K phosphorylation in control and acetaldehyde-treated HSC. In contrast to this result, and as shown in Fig. 6 (A and C), incubation with rapamy-
cycin fails to prevent the increase in ERK1/2 phosphorylation induced by acetaldehyde, thus confirming that the pp70S6K represents a downstream component in this signaling pathway.

Figure 2. Effect of adduct formation inhibitors on acetaldehyde-induced phosphorylation of pp70^S6K and ERK1/2. Cells were cultured for 24 hours in serum- and insulin-free medium and then incubated in the same medium with or without acetaldehyde in the presence or absence of the adduct formation inhibitors p-hydroxy-mercuribenzoate (PHMB) and pyridoxal-5'-phosphate (PLP) at the concentrations indicated in the Materials and Methods section. (A) Representative Western blot. Cell lysates were obtained as described in Materials and Methods. Proteins (50 μg/lane) were separated by SDS-PAGE, transferred to nitrocellulose, and then incubated with specific antibodies for pp70^S6K (upper blot) and phosphorylated ERK1/2 (lower blot). Molecular weight markers are represented on the left of each panel. The arrow shows the position of phosphorylated pp70^S6K. (B) Histograms summarize the results of triplicate experiments for pp70^S6K, with control assigned a value of 1 and refers to mean ± SD. *(P < .05 vs. all other groups. PHMB, p-hydroxy-mercuribenzoate; PLP, pyridoxal-5'-phosphate; Ace, acetaldehyde.

Figure 3. Effect of protein kinase C on acetaldehyde-induced phosphorylation of pp70^S6K and ERK1/2. Cells were cultured for 24 hours in serum- and insulin-free medium and then incubated in the same medium with or without acetaldehyde in the presence or absence of calphostin C at the concentrations indicated in the Materials and Methods section. (A) Representative Western blot. Cell lysates were obtained as described in Materials and Methods. Proteins (50 μg/lane) were separated by SDS-PAGE, transferred to nitrocellulose, and then incubated with specific antibodies for pp70^S6K (upper blot) and phosphorylated ERK1/2 (lower blot). Molecular weight markers are represented on the left of each panel. The arrow shows the position of phosphorylated pp70^S6K. (B) Histograms summarize the results of triplicate experiments for pp70^S6K, with control assigned a value of 1 and refers to mean ± SD. *(P < .05 vs. all other groups. Calp, calphostin C; Ace, acetaldehyde.
Effect of Adduct Formation and Intracellular Pathway Inhibitors on Acetaldehyde-Induced α2(I) Collagen and Fibronectin Gene Expression, and Cell Proliferation in Human HSC. One of the main events occurring during alcoholic liver injury in vivo is the activation of HSC, with a concomitant increase in their proliferation rate and in their capacity to synthesize extracellular matrix components. In view of these, we investigated the effect of acetaldehyde on α2(I) collagen and fibronectin mRNA expression in human HSC and attempted to link these changes with the acetaldehyde-triggered signal transduction pathways identified earlier.

As shown in Fig. 7 (A-C), α2(I) collagen and fibronectin mRNA expression is induced by 2.5 ± 0.3 and 3.0 ± 0.6, respectively, in HSC incubated with 200 μmol/L acetaldehyde for 24 hours. Time-course studies revealed that this effect is observed as early as 4 to 6 hours postacetaldehyde administration (data not shown). Run-on transcription assays performed with nuclei obtained from control cells, and from cells treated with acetaldehyde, showed that the acetaldehyde effect is exerted at the transcriptional level (Fig. 8A and B). As shown in this Fig. 8, acetaldehyde stimulates 2.2 ± 0.4 and 4.4 ± 0.3 α2(I) collagen and fibronectin gene transcription, respectively, 2 hours postacetaldehyde administration, and 4.3 ± 1.2 and 5.7 ± 1.4, respectively, after 24 hours. Addition of cycloheximide (30 μg/mL) did not abolish the acetaldehyde effect at early time points (Fig. 8A). However, after 24 hours, it completely blocked the acetaldehyde effect (Fig. 8B). These results suggest that acetaldehyde stimulates α2(I) collagen and fibronectin gene transcription through a protein synthesis-independent and -dependent mechanism. In contrast to these results, transforming growth factor-β1 (TGF-β1) stimulated α2(I) collagen and fibronectin gene expression in HSC only via a de novo protein synthesis-independent mechanism (data not shown).

We have previously shown that formation of acetaldehyde adducts with the carboxyl-terminal propeptide of type I procollagen may account, at least in part, for this effect.48,50 In agreement with this, incubation of HSC with the inhibitor of adduct formation PHMB completely abolished acetaldehyde-induced α2(I) collagen and fibronectin mRNA upregulation (Fig. 7A-C). The effect of PHMB is exerted at the transcriptional level, as shown by run-on transcription assays (Fig. 9A and B). Similar results were obtained when using PLP, another inhibitor of adduct formation (data not shown).

Different intracellular pathways stimulated in HSC by acetaldehyde could selectively stimulate cell DNA synthesis and/or gene expression. Thus, we evaluated the effect of the different kinase inhibitors on steady state levels of α2(I) collagen and fibronectin mRNA (Fig. 7A-C) and gene transcription (Fig. 9A and B). As shown in Fig. 7 (A and B), the PKC inhibitor calphostin C significantly decreases to control levels the acetaldehyde-elicited response of both mRNAs. As shown in Fig. 9 (A and B), this effect is exerted at the transcriptional level. Acetaldehyde-induced α2(I) collagen gene expression is also inhibited by the PI3K inhibitor wortmannin, by the MEK antagonist PD98059 and by the pp70<sup>60k</sup> inhibitor rapamycin (Fig. 7A and B). The effect of these compounds is also observed at the level of gene transcription (Fig. 9A). In contrast to these results, addition of these inhibitors has no effect on acetaldehyde-elicited fibronectin mRNA upregulation or enhanced gene transcription (Fig. 7A and C, Fig. 9B). Although ERK1/2 and/or pp70<sup>60k</sup> have been shown to be differentially involved in cell proliferation,3-7,23,24 acetaldehyde failed to induce any increase in the number of S-phase nuclei com-
DISCUSSION

The mechanisms whereby excess alcohol consumption results in hepatic fibrogenesis are not entirely understood. Data in the literature suggest that ethanol-induced liver fibrosis is a complex and multifactorial process that involves different liver cell types and diverse pathological mechanisms. Work from various laboratories has established that acetaldehyde, ethanol’s first metabolite, is fibrogenic and induces the expression of various extracellular matrix components in HSC. However, the molecular mechanisms whereby acetaldehyde exerts its fibrogenic actions remain to be fully elucidated.

In this article, we show that acetaldehyde induces collagen and fibronectin gene expression at the transcriptional level, and that this induction is observed as early as 2 hours post-acetaldehyde administration. These results are consistent with data previously published using rat HSC and human fibroblasts. We also show that acetaldehyde-elicited collagen and fibronectin gene upregulation is biphasic. Whereas at early time points (2 hours postacetaldehyde administration) it does not require de novo protein synthesis, at late time points (24 hours) it is protein synthesis-dependent. These results are in partial agreement with those previously reported by Casini et al., who showed that cycloheximide completely blocks acetaldehyde-induced upregulation of collagen gene transcription occurring after 6 hours. One possible explanation for this protein-synthesis dependency at late time points may be related to the production of TGF-β1 by HSC. As previously shown, acetaldehyde stimulates steady state levels of TGF-β1 mRNA in HSC. Thus, it is conceivable that the increased expression of type I collagen and fibronectin observed at late time points is due, at least in part, to TGF-β1 produced via an autocrine loop by HSC in response to acetaldehyde. This possibility is currently being explored in our laboratory.

Because changes in phosphorylation/dephosphorylation may account, at least in part, for the early changes observed in collagen and fibronectin gene expression elicited by acetaldehyde, in this study we focused our attention at identifying some signal transduction pathways triggered by acetaldehyde, leading to enhanced extracellular matrix expression. We selected to investigate specifically the role of PKC, PI3K, ERK1/2, and pp70S6K because it has been established that these kinases are involved in TGF-β1- and/or IGF-1-mediated type I collagen gene upregulation in several cell types including HSC, fibroblasts, mesangial cells, and osteoblasts. This to this end, we took advantage of specific inhibitors of intracellular kinases, namely calphostin C, wortmannin, LY294002, PD98059, and rapamycin, which at the concentrations used in this study, have been shown to effectively and selectively inhibit their cognate kinases in HSC.

Studies performed with calphostin C, a specific inhibitor of PKC, revealed that acetaldehyde-mediated collagen and fibronectin mRNA upregulation is a PKC-dependent event. This event is exerted at the transcriptional level, as shown by run-on transcription assays. Our data suggest that PKC plays a key role in activating downstream components, namely ERK1/2 and pp70S6K, which ultimately stimulate extracellular matrix gene expression.
Our studies also revealed that acetaldehyde stimulates α2(I) collagen and fibronectin gene expression through different signal transduction pathways. The former involves an ERK1/2-, PI3K-, and pp70S6K-dependent pathway, because wortmannin and LY294002, 2 PI3K inhibitors, PD98059, a MEK inhibitor, and rapamycin, a pp70S6K inhibitor, completely abolished acetaldehyde-mediated α2(I) collagen mRNA expression and gene transcription. On the other hand, a different intracellular pathway branching downstream of PKC is involved in increased fibronectin gene expression. This conclusion is based on the lack of effect of the different inhibitors on acetaldehyde-induced fibronectin mRNA and gene transcription.

The role of ERK1/2- and PI3K-dependent signal transduction pathways in upregulating type I collagen gene expression in HSC and other cell types, has been previously documented. ERK1/2 belongs to the mitogen-activated protein kinases (MAPK) group, which controls fundamental cellular processes such as proliferation, differentiation, apoptosis, and protein synthesis. Activation of different kinases of this group has been observed in several models of liver injury. Administration of CCl4 induces c-jun N-terminal kinase (JNK) and p38MAPK activities in whole liver. Likewise, Marra et al., have shown ERK activation in HSC during acute liver damage. In vitro, several fibrogenic growth factors, such as IGF-1 and TGF-β1 have been shown to exert their activities, at least in part, through stimulation of ERK1/2 and/or PI3K phosphorylation. Our present data are also in agreement with several reports showing that some biological and environmental stimuli up-regulate collagen gene expression via ERK. In vitro, several fibrogenic growth factors, such as IGF-1 and TGF-β1 have been shown to exert their activities, at least in part, through stimulation of ERK1/2 and/or PI3K phosphorylation. Our present data are also in agreement with several reports showing that some biological and environmental stimuli up-regulate collagen gene expression via ERK.8,58-60 More recently, it has been shown using rat HSC that acetaldehyde activates JNK1/2, a known downstream element of the ERK pathway.62

Although ERK1/2- and PI3K activation has been shown to play a role in the modulation of cell proliferation, in this study we found no increase in the number of S-phase nuclei in HSC incubated with acetaldehyde, in spite of an increase in ERK1/2 and PI3K phosphorylation. Although we have no explanation for this apparent discrepancy, it is noteworthy to mention that proliferation of HSC is not always associated with ERK 1/2 activation. Indeed, we have previously shown that HSC proliferation stimulated by insulin is ERK1/2-independent. Interestingly, in smooth muscle cells, ERK activation is associated with a negative signal for cell proliferation in cyclooxygenase-2 (COX-2)-expressing cells through the production of prostaglandin E2 (PGE2). Because COX-2 is constitutively expressed in HSC and acetaldehyde or ethanol (through its transformation in acetaldehyde) induces PGE2 production in HSC, it is conceivable that in human HSC, activation of ERK by acetaldehyde leads to the production of prostaglandins, which, in turn, inhibit cell proliferation. This possibility, although feasible, needs to be tested experimentally.

Our results also establish an important role for the pp70S6K in acetaldehyde-mediated α2(I) collagen upregulation. This kinase is a growth factor-regulated serine/threonine kinase that is known to phosphorylate the 40S ribosomal protein S6 in vitro, and is usually a downstream component of PI3K. Unfortunately, the molecular mechanisms whereby phosphorylation of pp70S6K leads to upregulation of type I collagen gene transcription are unknown. However, in other cellular systems, this kinase is induced by H2O2, a reac-
As already indicated, our findings showed that PKC is involved in acetaldehyde-elicited α2(I) collagen gene upregulation. Thus, it was important to establish whether induction of pp70<sup>S6K</sup> activation was PKC-dependent. Indeed, our findings revealed that calphostin C completely prevented acetaldehyde-dependent activation of pp70<sup>S6K</sup>, thus suggesting that PKC is upstream of this kinase. The mechanisms whereby PKC induces its activation follows at least 2 distinct pathways. One appears to be mediated by ERK1/2, and the other by PI3K. This conclusion is based on the fact that wortmannin, a PI3K inhibitor, prevented acetaldehyde-mediated induction of the α2(I) collagen gene, and also inhibited by approximately 50% activation of pp70<sup>S6K</sup>. Likewise, PD98059, a specific inhibitor of MEK, also inhibited by 50% activation of pp70<sup>S6K</sup> and completely abolished acetaldehyde-mediated α2(I) collagen upregulation. Thus, the pp70<sup>S6K</sup> appears to represent a common element to which both PI3K and the MEK/ERK pathway converge. This convergence has been shown in other experimental systems.

As mentioned earlier, the effect of these inhibitors was relatively specific, because, except for calphostin C, they had no effect on acetaldehyde-elicited fibronectin mRNA and gene transcription. Moreover, the effect of all of these compounds was not the result of toxic effects on the cells because in all experiments HSC viability was shown to be at least 90% (data not shown). On the other hand, it is important to emphasize that although the use of chemical inhibitors to study cell sig-

**Fig. 7.** Effect of kinase and adduct formation inhibitors on acetaldehyde-induced upregulation of α2(I) collagen and fibronectin mRNAs. Northern blot analysis was performed with 10 μg of total RNA extracted from control, untreated cells, and from cells treated with acetaldehyde in the presence or absence of p-hydroxy-mercuribenzoate (PHMB), calphostin C, wortmannin, PD98059 or rapamycin at the concentrations indicated in Materials and Methods. (A) Representative Northern blot. α2(I), α2(I) collagen; FN, fibronectin; S14, S14 ribosomal protein. (B) Effect of kinase and adduct formation inhibitors on acetaldehyde-induced upregulation of α2(I) collagen mRNA. Histograms summarize the results of triplicate experiments, and are expressed as relative α2(I) collagen mRNA expression with control assigned a value of 1 and refers to mean ± SD. (C) Effect of kinase and adduct formation inhibitors on acetaldehyde-induced fibronectin mRNA upregulation. Histograms summarize the results of triplicate experiments, and are expressed as relative fibronectin mRNA expression with control assigned a value of 1 and refer to mean ± SD. All values were corrected for loading differences after hybridization with a S14 ribosomal protein cDNA.*P < .05 vs. all other groups. Continued □, acetaldehyde ▪.

**Fig. 8.** Effect of cycloheximide on acetaldehyde-mediated α2(I) collagen and fibronectin gene transcription after 2 hours (A) or 24 hours (B). Run-on assays were performed using nuclei isolated from HSC treated with 200 μmol/L acetaldehyde in the presence or absence of cycloheximide for either 2 or 24 hours. Results are expressed as relative α2(I) collagen or fibronectin transcription rates with controls assigned a value of 1 and refer to mean ± SD. All values were corrected for loading differences using a S14 ribosomal protein cDNA. *P < .05 vs. all other groups. □ Continued; ▪ acetaldehyde.
naling has proved very useful, there are some caveats which need not to be overlooked, particularly regarding their specificity when used at high concentrations. In this regard, the use of dominant negative mutants and/or antisense technologies may prove useful to further elucidate signal transduction pathways leading to increased extracellular matrix production by acetaldehyde.

Overall, our data show that although induction of α2(I) collagen and fibronectin gene expression by acetaldehyde is PKC-dependent, downstream pathways diverge and involve different kinases. Although we did not investigate further which kinases downstream of PKC lead to upregulation of fibronectin gene expression, we established that PI3K and ERK1/2 are involved in stimulating α2(I) collagen gene expression, and that both of these kinases exert their effects via activation of pp70S6K. Whether other kinases, in addition to PI3K and ERK1/2, are involved in stimulating procollagen type I and fibronectin gene transcription in cultured rat fat-storing cells, remains to be investigated.

The mechanisms whereby acetaldehyde induce all of these activities are presently unknown. Indeed, it would be interesting to determine how a small molecule such as acetaldehyde can trigger activation of intracellular signal transduction cascades. We show that PHMB and PLP, 2 inhibitors of adduct formation, prevent acetaldehyde-induced ERK1/2 and pp70S6K activation, as well as α2(I) collagen and fibronectin gene upregulation. It is important, however, to emphasize that these data need to be taken with caution because PHMB and PLP could have other biological activities in addition to their properties as inhibitors of adduct formation. Nevertheless, they are consistent with our previous findings showing that formation of acetaldehyde-protein adducts accounts, at least in part, for the fibrogenic properties of acetaldehyde in HSC. Thus, conceivably, acetaldehyde could induce activation of PKC and/or ERK1/2 and pp70S6K through formation of protein adducts. This hypothesis, albeit provocative, needs to be further tested.

In summary, our study represents an identification of differential signal transduction pathways triggered by acetaldehyde leading to α2(I) collagen and fibronectin gene upregulation in human HSC. A better understanding of these pathways may allow in the early future the development of more rational therapies to counteract the devastating effects of alcohol-induced liver fibrosis.

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