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NON-SELECTIVE DECREASE OF COLLAGEN SYNTHESIS BY CULTURED FETAL LUNG FIBROBLASTS AFTER NON-LETHAL DOSES OF ETHANOL*

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Abstract—Addition of ethanol to cultured fetal lung fibroblasts resulted in decreases of both collagen and noncollagen protein syntheses. The inhibitory effect of ethanol on protein synthesis was dependent on the concentration of ethanol and the number of treatments with ethanol. Significant inhibition of collagen and noncollagen protein synthesis was observed 3 hr after a single treatment in 0.25% (v/v) ethanol. The maximum inhibitory effect of ethanol on protein synthesis was observed at 6 hr after drug addition. Inhibition of protein synthesis was observed when either proline or glycine was used as the precursor amino acid. An inhibition of alcohol dehydrogenase did not block the ethanol-mediated inhibition of protein synthesis. Ethanol, added to cell cultures throughout the log phase, inhibited cell growth during the late log and stationary phases. Ethanol inhibition of collagen and noncollagen protein synthesis was reversed when the cell cultures were washed and suspended in fresh media for 24 hr. These inhibitory effects of ethanol on macromolecular syntheses were not engendered by killing of cells. The viability of the cells, as indicated by trypan blue exclusion, was not affected significantly at the concentrations of ethanol used. The inhibitory effect of ethanol on protein synthesis also did not originate from drug-mediated inhibition of precursor amino acid uptake. Polysomes isolated from ethanol-treated fibroblasts incorporated proline into protein at a rate which was reduced commensurate with cellular protein synthesis. The resultant inhibition by ethanol of protein synthesis was not attributable to a direct effect of drug on polysomes. Treatment of fetal lung fibroblasts with ethanol also caused a marked inhibition of radioactive thymidine and uridine incorporation, indicating a reduction of both total cellular DNA and RNA synthesis. Accordingly, the decrease of protein synthesis resulted from inhibition of RNA synthesis. Furthermore, messenger RNA synthesis may have decreased since polysomes isolated from ethanol-treated fibroblasts synthesized less protein in the wheat germ cell-free system. Unlike other biochemical variables that were inhibited by ethanol treatment, the level of prolyl hydroxylase activity was elevated significantly. The elevated level of prolyl hydroxylase activity, however, was related neither to the rate of collagen polypeptide synthesis nor to the degree of proline hydroxylation of cellular collagen. The data suggest that the growth-retarding effects of nonlethal doses of ethanol on fetal development may result from inhibition of macromolecular synthesis in fetal fibroblasts.

The most widely abused drug in today's society is ethanol. Although the potential teratogenic effects of ethanol had been suspected, it was not until the work of Lemoine *et al.* [1] and Jones *et al.* [2] that a dysmorphic condition called 'fetal alcoholic syndrome', in which there is severe physical growth retardation was recognized. The effects of ethanol on the metabolic processes of fetal fibroblasts are of importance in light of the fetal growth retardation effect of ethanol. Fibroblasts synthesize collagen, which constitutes a major portion of the bulk weight of animals.

The effect of acute ethanol treatment on protein synthesis has been studied in a number of tissues and cells. Ethanol has been shown to inhibit both heme and protein syntheses in animals and human red blood cell precursors [3, 4]. Ethanol inhibition of rabbit reticulocyte protein synthesis occurs as a result of decreased heme synthesis involving inhibition of the rate-limiting enzyme δ-aminolevulinic acid synthetase [5]. Ethanol given *in vivo* has also been shown to reduce hepatic albumin synthesis [6]. This loss of albumin-synthesizing potential is associated with a reduction of hepatic ribonucleic acid and a disaggregation of the endoplasmic membrane-bound polysomes [7]. Ethanol *in vitro* depresses the incorporation of amino acids into protein in rat liver slices [8]. In still another study, hepatocytes exposed to ethanol had a reduced level of protein synthesis that did not result from a reduction of amino acid uptake [9]. Furthermore, the decrease in hepatic protein synthesis observed in ethanol-treated rats does not result from a reduction in free amino acid pools [10]. Acute ethanol administration also results in a decrease of protein synthesis in rat brain [11].

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The effect of chronic administration of ethanol on hepatic collagen metabolism has been studied extensively. Prolyl hydroxylase is elevated in the liver of rats and baboons after long-term feeding of ethanol [12]. The elevation of this key enzyme in collagen synthesis is associated with elevations of hepatic hydroxyproline content and an increased ability of tissue minces to form hydroxyproline [12]. Increased prolyl hydroxylase activity also has been demonstrated in liver samples of patients with alcoholic hepatitis [13]. Percutaneous liver biopsies from chronic alcoholic patients synthesize hydroxyproline present in proteins at increased rates [14, 15].

The effects of ethanol on collagen synthesis in connective tissues are limited. Incubation of embryonic chick tibia preparations with ethanol at low concentrations resulted in increased hydroxyproline in proteins, whereas at high concentrations, decreased hydroxyproline synthesis was observed [16]. Mouse 3T6 fibroblast cultures treated with ethanol had a reduction of hydroxyproline content in the cell layer [17]. This decrease in hydroxyproline content [17] could be related to either decreased collagen synthesis or increased collagen degradation. The concentrations of ethanol used in both of these latter studies are lethal blood levels in man.

The present study was undertaken to determine the effects of treatment with nonlethal doses of ethanol that produce moderate to advanced intoxication in humans on various measures of collagen synthesis in cultured fetal lung fibroblasts. Our data indicate that nonlethal concentrations of ethanol produced marked inhibition of collagen synthesis. At no concentration was a stimulation of collagen synthesis observed as described previously for calvaria minces [16]. The data further indicate that the reduced level of collagen synthesis was accompanied by a general decrease of protein synthesis. The decrease in protein synthesis in ethanol-treated cell cultures was not due to an inhibition of amino acid uptake or an alteration of amino acid precursor pools, since polysomes isolated from ethanol-treated cell cultures incorporated amino acids into protein in the wheat germ lysate system at a reduced rate. The decreased protein synthesis resulted from decreased cellular RNA synthesis, possibly including messenger RNA synthesis, because polysomes isolated from ethanol-treated cells synthesized decreased amounts of protein in a cell free system.

MATERIALS AND METHODS

Materials. Triton X-100, [2-^{3}H]glycine (15 Ci/mmole) and [5,6-^{3}H)uridine-5′-triphosphate (35 Ci/mmole) were obtained from the New England Nuclear Corp. (Boston, MA). [5-^{3}H)Proline (24 Ci/mmole) and [methyl-^{3}H]thymidine (50 Ci/mmole) were obtained from Amersham/Searle (Arlington Heights, IL). Sodium ascorbate was obtained from CalBiochem (Los Angeles, CA). Bio-Rad (Richmond, CA) supplied the Dowex 50 W-X8 (200–400 mesh). Form III collagenase was obtained from the Advance Biofactures Corp. (Lynbrook, NY). Fetal bovine serum, Eagle's minimum essential media with Earle's balanced salt solution, L-glutamine, trypsin, versene, sodium bicarbonate (7.5%, w/v), and gentamycin were obtained from Microbiological Associates (Bethesda, MD). Chlorotetracycline hydrochloride solution was obtained from the Grand Island Biological Co. (Grand Island, NY). Corning tissue culture flasks were obtained from Fischer (Boston, MA).

Cell culturing. IMR-90 fetal lung fibroblasts were obtained commercially from the Institute for Medical Research (Camden, NJ). SCOR IA fetal lung fibroblasts were collected as part of the SCOR program at the University of Vermont College of Medicine; this fibroblast cell line was grown initially from a lung biopsy specimen. These fibroblasts (SCOR IA) are a normal diploid cell line and have been characterized [18].

Fibroblasts were plated in 25 cm^2 flasks at a density of 1.4 to 1.8 $\times$ 10^5 cells. The cells were cultured in minimum medium supplemented with 10% (v/v) fetal calf serum and an antibiotic, either gentamycin (100 μg/ml) or chlortetracycline (100 μg/ml). The medium was changed on days 3 and 7 of growth. On days 8 and 9, the cells were supplemented with sodium ascorbate (2×10^{-4}M) and ethanol at the concentrations indicated; this concentration of ascorbate gave the maximum degree of collagen prolyl hydroxylation in control cultures. On day 10 of culture, the cells were supplied with ascorbate for 1 hr, followed by the addition of either radioactive proline, glycine, uridine or thymidine for the next 2 hr. The medium and cells were centrifuged at 1200 *g* for 20 min. The supernatant fraction was discarded and the cells were suspended in 0.9% (w/v) sodium chloride, 0.05 M Tris–HCl (pH 7.5) and washed twice.

Cell growth. The cells were plated as described previously. At the times indicated, the medium was suctioned off. The cells were washed with a versene–EDTA solution (1:5000). To detach the cells, 1 ml of a 0.25% (w/v) trypsin solution in calcium- and magnesium-free phosphate-buffered saline was applied to the monolayer for 5 min. The resuspended cells were removed and counted in a Coulter Counter.

Cellular collagen and noncollagen protein syntheses. Fibroblasts were grown to stationary phase. Two hours prior to collection either radioactive proline (100 μCi) or glycine was added to each flask. The cells were collected by scraping and were washed. The cells were suspended in 0.1 M NaCl, 50 mM Tris–HCl (pH 7.5). The sample was homogenized in a Polytron ST homogenizer (Brinkmann Co., Westbury, NY) for 45 sec and then placed in a boiling water bath for 10 min followed by homogenization for 30 sec. The sample was digested with collagenase [19] and the protein concentration was determined by the method of Lowry *et al.* [20], using bovine serum albumin as standard.

DNA and RNA syntheses. DNA and RNA syntheses were determined by radioactive thymidine and uridine incorporation, respectively. Cells were grown to stationary phase. Either tritium-labeled uridine (50 μCi/flask) or thymidine (100 μCi/flask) was added for 2 hr prior to the collection of cells. The cells in which uridine incorporation was determined were suspended in water and homogenized for 45 sec. An equal volume of trichloroacetic acid (10%, w/v) was added and the sample was allowed

to incubate on ice for 10 min. The sample was then centrifuged at 31,000 *g* for 20 min. The precipitate was washed with 20 ml of 5% (w/v) trichloroacetic acid, three times, and finally suspended in water. The sample was then homogenized for 45 sec and aliquots were used for either radiochemical analysis in 10 ml Aquasol-2 or protein determination.

After labeling with tritiated thymidine, the cells were collected, washed, suspended in H_2O and homogenized. After washing and centrifuging the trichloroacetic acid precipitated residue, the pellet was suspended in 6 N HCl and incubated at 90° for 20 min. The sample was then neutralized with NaOH and aliquots were assayed for radioactivity and protein concentration.

Cell viability. The cells from one flask were collected by trypsin treatment as described previously and suspended in serum-containing medium; an aliquot was added to a 0.4% (w/v) trypan blue solution. A total of at least 200 cells was counted with a hemocytometer and the percentage of viable cells was determined from the number of cells excluding trypan blue.

Cell culture preparation for the prolyl hydroxylase assay. Cells were grown to stationary phase and treated with ascorbate and ethanol as described above. After collecting the cells from flasks by scraping, they were suspended in 1 ml of a buffer containing 0.1% (w/v) Triton X-100 [19], and then homogenized and centrifuged at 20,000 *g* for 20 min. The supernatant fluid was assayed for prolyl hydroxylase activity as described [19]. Duplicate cultures were counted after trypsin release as described above.

Hydroxylproline formation. The cells were grown to stationary phase, treated with ethanol and ascorbate (see above), and then incubated with tritiated proline for 6 hr. The cells from six flasks were suspended in 6 ml of the Tris–NaCl buffer and homobenized for 45 sec, and then placed in a boiling water bath for 10 min and homogenized for an additional 30 sec. An aliquot was taken for collagenase digestion as described previously [19]. To the remaining homogenate an equal volume of 20% (w/v) trichloroacetic acid was added; the sample was incubated on ice for 15 min and then centrifuged at 31,000 *g* for 20 min. The pellet was resuspended in 25 ml of 5% (w/v) trichloroacetic acid and washed three times with trichloroacetic acid; the resulting pellet was suspended in 6 N HCl, hydrolyzed, decolorized and neutralized as described earlier [21]. The radioactive hydroxyproline was then isolated by Dowex chromatography [21].

Isolation of polysomes. IMR-90 fetal lung fibroblasts were grown to stationary phase and treated with ethanol and ascorbate; the cells were collected by scraping and washed as described above. The cells from six flasks were suspended in 15 ml of buffer [22]. The cell suspension was made to 0.5% (w/v) sodium deoxycholate and 0.2% (w/v) Triton X-100 and then was gently homogenized in a glass homogenizer with ten strokes by hand. The homogenate was centrifuged at 10,000 *g* for 30 min. The resulting supernatant fluid was layered over a 1.5 M sucrose cushion, and the polysomes were obtained as described previously [22] and translated in a wheat germ lysate prepared as described previously [22].

Determination of total collagenolytic activity. Cells were grown to stationary phase and treated with ascorbic acid and ethanol as described previously. Six hours prior to collection, the culture medium was replaced with serum-free medium containing [5-^{3}H]proline (100 μCi/10 ml). At the end of this 6-hr period each cell culture was placed on ice for 20 min. The cells were harvested with the aid of a rubber policeman, recombined with the culture medium, and homogenized for 1 min using the polytron ST system. Each sample was split into two fractions; the first was dialyzed, the second was not dialyzed. Dialysis was performed four times against 400 ml of H_2O. An equal volume of 12 N HCl was then added to each fraction. The samples were hydrolyzed, and the radioactive hydroxyproline was isolated by Dowex chromatography as described previously [21].

RESULTS

The effect of ethanol treatment on cell growth of IMR-90 fetal lung fibroblasts is illustrated in Fig. 1. Ethanol treatment during the log growth phase caused a decrease of growth during late log and stationary phases. The ethanol concentrations that caused a diminution of growth were 0.1% (v/v) (Fig. 1A) and 0.25% (v/v) (Fig. 1B). In all subsequent experiments, cells were grown to stationary phase before the addition of ethanol.

The responses of collagen and noncollagen protein synthesis to various concentrations of ethanol are shown in Fig. 2. Statistically significant inhibition of collagen and noncollagen protein synthesis was observed at ethanol concentrations of 0.05, 0.10, 0.25 and 0.5% (v/v). Concentrations as high as 2% resulted in inhibition of protein synthesis. At no

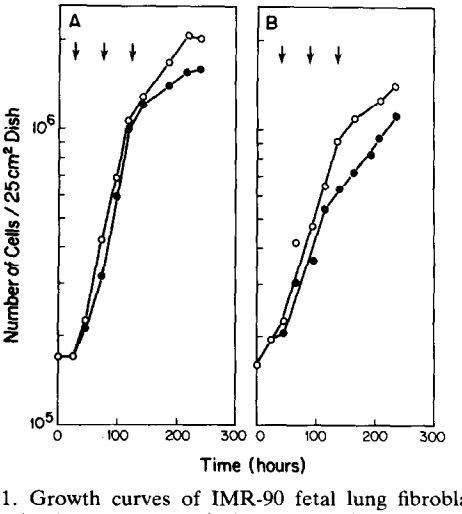

Fig. 1. Growth curves of IMR-90 fetal lung fibroblasts grown in the presence and absence of ethanol. IMR-90 fetal lung fibroblasts were plated at a density of 1.4 to 1.8×10^5 cells. After 2 days, ethanol (0.1%, v/v) (A) or (0.25%, v/v) (B) was added to the cultures (first arrow) and the cells were allowed to grow for another 2 days. The medium was then changed and the cells again received ethanol (second arrow). After another 2 days (third arrow) the cells were again treated with ethanol without changing the medium. Cell counts are the mean values of either duplicate or triplicate flasks. Key: control (-○-); and ethanol-treated (-●-).

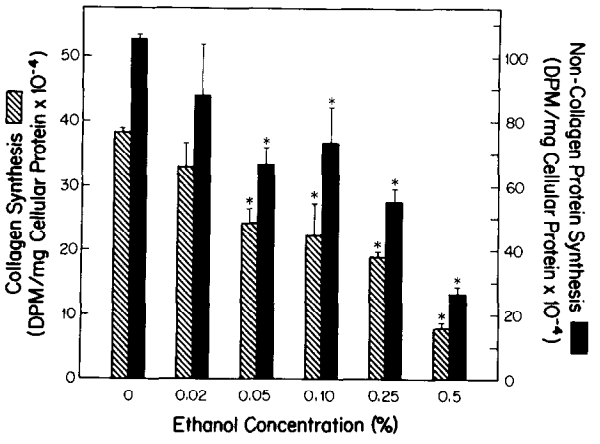

Fig. 2. Response of collagen and noncollagen protein synthesis in IMR-90 fetal lung fibroblasts to various concentrations of ethanol. IMR-90 fetal lung fibroblasts were plated in T-25 flasks at a density of 1.4 to 1.8×10^5 cells. The medium was changed on days 3 and 7 of growth. On days 8 and 9, the cells were treated with ethanol and ascorbic acid (2×10^{-4} M). The cell medium was not changed. On day 10, ascorbate was added. After 1 hr, [5-^{3}H]proline was added (100 μCi/10 ml medium). The cells were allowed to incorporate radioactive proline for 2 hr, and were then collected and proline incorporation into collagen and noncollagen protein was measured as described in the text. Collagen and noncollagen protein syntheses decreased 58 and 51 per cent, respectively, in 0.25% (v/v) ethanol when the data were expressed as the amount of proline incorporation into collagen and noncollagen protein per 10^6 cells. Each value is the mean ± S.E. of three cultures. An asterisk (*) indicates a significant difference from control at $P < 0.05$.

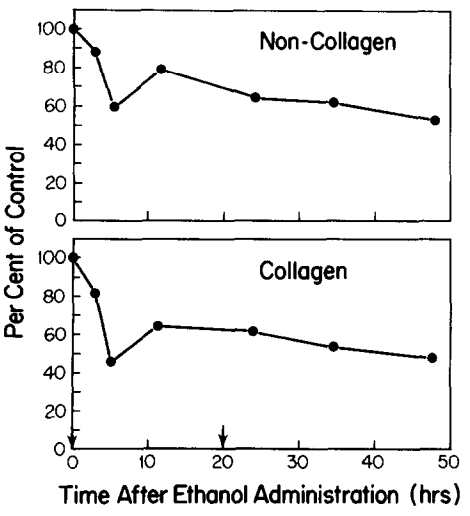

Fig. 3. Time response of ethanol inhibition of collagen and noncollagen protein synthesis. IMR-90 fetal lung fibroblasts were plated in T-25 flasks at a density of 1.4 to 1.8×10^5 cells. The medium was changed on days 3 and 7 of growth. On days 8, 9 and 10, ascorbic acid (2×10^{-4} M) was added to the cultures. Experimental cell cultures received 0.25% (v/v) ethanol at the times indicated by the arrows. Proline was added 2 hr before the harvesting of cells at the time periods indicated in the figure. The per cent-of-control values were determined from the mean values of data from at least three cultures.

concentration was a stimulation of collagen synthesis observed. The degree of inhibition of collagen synthesis was the same as that of noncollagen protein synthesis, thus indicating that ethanol caused a general inhibition of protein synthesis.

The temporal response of collagen and noncollagen protein synthesis to 0.25% ethanol is seen in Fig. 3. Significant inhibition of both collagen and noncollagen protein synthesis was observed as early as 3 hr after a single treatment with ethanol. The maximum inhibition of collagen and noncollagen protein synthesis was observed at 6 hr. The inhibition of protein synthesis was less at 12 hr than at 6 hr after a single treatment with ethanol. Addition of more ethanol at 20 hr resulted in a further depression of protein synthesis. The data in Figs. 2 and 3 indicate that the inhibitory effect of ethanol on collagen synthesis was not specific but was rather the result of a general inhibition of protein synthesis.

The ethanol-mediated inhibition of protein synthesis was also observed when tritium-labeled glycine was used as the precursor amino acid. The degree of inhibition of collagen and noncollagen protein synthesis was similar to that obtained with tritiated proline (data not shown).

The effect of ethanol on protein synthesis was also determined using another fetal lung fibroblast cell line (SCOR IA). Ethanol concentrations of 0.1 and 0.25% depressed both collagen and noncollagen protein syntheses to the same extent as that observed in IMR-90 fetal lung fibroblasts (data not shown).

Ethanol-mediated inhibition of collagen and noncollagen protein synthesis was reversible (Table 1). Cell cultures were treated for 2 days with 0.25% (v/v) ethanol. The medium was then withdrawn and the cells were replenished with fresh medium. After 24 hr, the level of collagen synthesis was equal to that of control cultures, while the level of noncollagen protein synthesis was elevated, compared to the control value. On days 2 and 3, collagen and noncollagen protein syntheses were equal to control values.

The inhibition of collagen and noncollagen protein synthesis did not result from an ethanol-mediated killing of cells. At concentrations of 0.1, 0.25 and 0.5% (v/v) ethanol, the cells were 95–97 per cent viable, as determined by trypan blue exclusion (data not shown).

Pyrazole, a potent inhibitor of alcohol dehydrogenase [23–25], did not block the inhibition of protein synthesis by ethanol (Table 2). Lower doses of pyrazole also had no effect, whereas higher doses of pyrazole inhibited protein synthesis in these cells.

The effect of ethanol treatment on prolyl hydroxylase of IMR-90 fetal lung fibroblasts was also examined. Although collagen synthesis was decreased, prolyl hydroxylase activity per cell was elevated by 71 per cent (Table 3). Accordingly, the level of prolyl hydroxylase activity following ethanol treatment of fetal lung fibroblasts was not associated with an increase of collagen synthesis. Since prolyl hydroxylase activity was elevated after ethanol treatment, we determined the degree of proline hydroxylation of cellular collagen (Table 4). Ethanol treatment inhibited the formation of hydroxyproline and collagen polypeptide synthesis to the same extent.

Table 1. Reversibility of the ethanol-mediated decrease in collagen and noncollagen protein synthesis in IMR-90 fetal lung fibroblasts*

Days of culture	Treatment	Collagen (dpm/mg protein $\times 10^{-4}$)	Noncollagen
10	Control	32.2 ± 1.6 (3)	121.9 ± 9.7
	Ethanol	14.4 ± 1.4† (3)	63.4 ± 0.9†
11	Control	30.8 ± 2.0 (4)	101.8 ± 5.9
	Ethanol: washed	32.3 ± 2.0 (4)	137.1 ± 10.9†
12	Control	32.0 ± 2.8 (4)	78.1 ± 3.9
	Ethanol: washed	23.9 ± 2.9 (4)	92.9 ± 6.0
13	Control	29.3 ± 4.9 (4)	80.0 ± 8.7
	Ethanol	28.8 ± 1.2 (4)	81.8 ± 3.3

* IMR-90 fetal lung fibroblasts were plated in T-25 flasks at a density of 1.4 to 1.8×10^5 cells. The cells were grown to stationary phase and treated with ascorbate and ethanol (0.25%, v/v) as described in the legend of Fig. 2. Two hours before collection on day 10 of cell culture, the cells were given radioactive proline (100 μCi). One hour before administration of radioactive proline, the cells were treated with ascorbate (2×10^{-4} M). On day 10 the remaining ethanol cultures were replenished with fresh medium after being washed twice with serum-free media. Proline incorporation into collagen and noncollagen protein was determined by the collagenase digestion assay. Each value is the mean ± S.E. The values in parentheses are the numbers of samples.

† Significantly different from control at $P \leq 0.05$.

Table 2. Lack of effect of pyrazole on the ethanol-mediated inhibition of protein synthesis in IMR-90 fetal lung fibroblasts*

Treatment	Proline incorporation (dpm/mg protein $\times 10^{-5}$)
No addition	13.3 ± 0.2 (3)
Ethanol	7.5 ± 0.2† (4)
Pyrazole (2 mM)	14.7 ± 2.0 (5)
Ethanol + pyrazole (2 mM)	5.1 ± 0.8† (3)

* Cell cultures were plated in T-25 flasks at a density of 1.4 to 1.8×10^5 cells. The cells were grown to stationary phase and treated with ascorbic acid (2×10^{-4} M) and ethanol (0.25%) on days 8 and 9 of growth. The medium was not changed during this treatment period. On day 10 the cells were treated with ascorbate and [5-^{3}H]proline was added.

† Significantly different at $P \leq 0.05$.

Table 3. Effect of ethanol treatment on prolyl hydroxylase activity of IMR-90 fetal lung fibroblasts*

Treatment	Prolyl hydroxylase activity (dpm of [^{3}H]water formed/10^6 cells $\times 10^{-3}$)
Control	5.9 ± 0.5 (3)
Ethanol	10.1 ± 0.9† (4)

*IMR-90 fetal lung fibroblasts were plated in T-25 flasks at a density of 1.4 to 1.8×10^5 cells. The cells were grown to stationary phase and treated with ascorbate and ethanol (0.25%, v/v) as described in the legend of Fig. 2. On day 10 of cell culture prolyl hydroxylase activity was determined by the tritium release assay. Each value is the mean ± S.E. The values in parentheses are the numbers of samples.

† Significantly different from control $P \leq 0.05$.

Table 4. Lack of effect of ethanol treatment on the degree of hydroxylation of proline residues in cellular collagen*

Treatment	Hydroxyproline formed (A) (dpm/mg protein $\times 10^{-4}$)	Collagen (B) (dpm/mg protein $\times 10^{-4}$)	A/B
Control	69.0 ± 4.3 (3)	86.5 ± 9.6	0.79 ± 0.07
Ethanol	39.0 ± 1.8† (3)	51.9 ± 3.9†	0.75 ± 0.02

* IMR-90 fetal lung fibroblasts were plated in T-25 flasks at a density of 1.4 to 1.8×10^5 cells, grown to stationary phase, and treated with ascorbate and ethanol (0.25%, v/v) as described in the legend of Fig. 2. Six hours before collection on day 10 of cell culture, the cells were treated with radioactive proline (100 μCi/10 ml). Hydroxyproline formation and radioactive collagen were determined. Each value is the mean ± S.E. The values in parentheses are the number of samples.

† Significantly different at P ± 0.05.

Table 5. Protein synthesis by polysomes isolated from ethanol-treated IMR-90 fetal lung fibroblasts in the wheat germ lysate system*

Treatment	Proline incorporation (dpm/A_{260} unit of polysome $\times 10^{-4}$)
Control	142.0 ± 9.0 (3)
Ethanol	80.2 ± 3.3† (3)

*IMR-90 fetal lung fibroblasts were plated in T-25 flasks at a density of 1.4 to 1.8 $\times 10^5$ cells, grown to stationary phase, and treated with ascorbate and ethanol (0.25%, v/v) as described in the legend of Fig. 2. On day 10 of cell culture the polysomes were isolated and translated in the wheat germ lysate system. Each value is the mean ± S.E. The values in parentheses are the numbers of samples.

† Significantly different at $P \leq 0.05$.

Table 6. Lack of a direct effect of ethanol on the ability of polysomes to synthesize protein in the cell free system*

Ethanol conc (%)	Proline incorporation (dpm/A_{260} unit of polysome $\times 10^{-4}$)
0.0	107.4
0.01	111.9
0.02	108.5
0.03	108.8
0.05	112.5
0.08	105.7
0.1	112.6
0.13	107.7
0.15	113.9
0.25	112.4
0.50	102.1

* Polysomes were isolated as described in the text. Polysomes were incubated for 5 min at room temperature with the ethanol concentrations indicated above. The remaining components of the *in vitro* protein synthetic system were added and the samples were incubated for an additional 60 min. Each value is the mean of duplicate samples. The polysomes used in these experiments were isolated from cells different than those in Table 4, which accounts for the difference in incorporation rate per A_{260}.

However, ethanol did not increase the degree of proline hydroxylation of cellular collagen.

The reduced level of protein synthesis observed in ethanol-treated cell cultures could have resulted from a decrease of precursor amino acid uptake.

Table 8. Lack of effect of ethanol on total collagenolytic activity of IMR-90 fibroblasts*

Treatment	Per cent dialyzable hydroxyproline
Control	82.3 ± 3.8 (3)
Ethanol	82.7 ± 3.3 (3)

* IMR-90 fetal lung fibroblasts were plated in T-25 flasks at a density of 1.4 to 1.8 $\times 10^5$ cells and grown to stationary phase. The cells were treated with ascorbate and ethanol as described in the legend of Fig. 2. Six hours prior to collection, the culture medium was replaced with serum-free medium containing [5-^{3}H]proline (100 μCi/10 ml). The amount of proteinaceous radioactive hydroxyproline was determined for both dialyzed and undialyzed samples. The per cent dialyzable hydroxyproline was calculated by subtracting the amount of radioactive hydroxyproline in the dialyzed fraction from that in the undialyzed fraction, dividing this number by the radioactive hydroxyproline in the undialyzed fraction, and multiplying by 100. Each value is the mean ± S.E. The values in parentheses are the numbers of samples assayed.

However, when polysomes were isolated from control and ethanol-treated cell cultures and translated in the wheat germ lysate system, the degree of inhibition of protein synthesis was similar to that observed for cellular protein synthesis (Table 5). Incubation of control polysomes with ethanol *in vitro* did not result in a loss of ability of these polysomes to synthesize protein in the cell free system (Table 6).

Ethanol treatment of IMR-90 fetal lung fibroblasts also caused a marked inhibition of incorporation of thymidine into DNA and of uridine into RNA (Table 7). Uridine incorporation was depressed by 44 per cent, while thymidine incorporation was reduced by 33 per cent. Because polysomes isolated from ethanol-treated cells synthesized reduced amounts of protein in the cell free system, an ethanol-mediated decrease in messenger RNA synthesis may be the cause of the inhibition of cellular protein synthesis.

Although ethanol inhibited collagen synthesis, collagen degradation was not altered (Table 8). Accordingly, the ethanol-mediated inhibition of collagen accumulation by IMR-90 fibroblasts must have resulted from an alteration of collagen synthesis and not of degradation.

Table 7. Inhibitory effect of ethanol on thymidine and uridine incorporation by IMR-90 fetal lung fibroblasts*

Treatment	[^{3}H]Thymidine incorporation (dpm/mg protein $\times 10^{-5}$)	[^{3}H]Uridine incorporation (dpm/mg protein $\times 10^{-4}$)
Control	30.2 ± 3.5 (3)	69.5 ± 5.4
Ethanol	20.2 ± 0.8† (3)	38.9 ± 5.4†

* IMR-90 fetal lung fibroblasts were plated in T-25 flasks at a density of 1.4 to 1.8 $\times 10^5$ cells. The cells were grown to stationary phase, and treated with ascorbate and ethanol (0.25%, v/v) as described in the legend of Fig. 2. On day 10 of cell culture, [^{3}H]thymidine or [^{3}H]uridine was added for 2 hr before the cells were collected. Nucleotide incorporation was determined as described in the text. Each value is the mean ± S.E. The values in parentheses are the number of samples.

† Significantly different from control at $P \leq 0.05$.

DISCUSSION

In the present study, the addition of nonlethal concentrations of ethanol to the culture medium resulted in a marked inhibition of protein synthesis by fetal lung fibroblasts. These results may be related to the known growth-retarding effect of maternal alcohol ingestion in the 'fetal alcoholic syndrome' [1, 2]. The inhibition of fetal fibroblast protein synthesis is a likely molecular basis for the growth-retarding effect of ethanol in fetuses, since collagen is synthesized predominantly by fibroblasts and this protein accounts for a significant amount of the bulk weight of animals. One cannot exclude, however, nutritional, genetic and immunologic mechanisms that were not investigated in this cell culture model.

Chronically administered ethanol induces fibrosis and an elevation of hepatic protein synthesis [11], in particular an increase of collagen synthesis [12, 14, 15]. In contrast, acute ethanol treatment results in a depression of hepatic protein synthesis [8, 9, 11, 26]. One study reported that addition of ethanol to liver biopsy specimens from patients with alcoholic hepatitis or active cirrhosis caused an increase in the incorporation of proline and hydroxyproline by collagen [14]. Another study demonstrated a selective increase of hydroxyproline formation after the addition of ethanol to calvaria minces. Both of these experiments were proposed as models of the ethanol stimulation of collagen synthesis that occurs during ethanol-induced hepatic fibrosis. In the present study, however, an inhibition of collagen synthesis was observed after addition of ethanol to IMR-90 or SCOR IA fetal lung fibroblasts. Furthermore, the effect on collagen synthesis of ethanol was not selective, since noncollagen protein synthesis was decreased to the same extent as collagen synthesis.

The ethanol-mediated decrease of collagen and noncollagen protein syntheses in fetal lung fibroblasts was not not due to either inhibition of precursor amino acid uptake or decreased cell viability. The degree of inhibition of protein synthesis was similar in whole cells and polysome preparations. The ethanol-mediated inhibition of protein synthesis also was not due to cell killing. Although the viability of liver cells is markedly decreased by 0.25% and higher concentrations of ethanol [27], this dramatic decrease of cell viability was not observed in IMR-90 fetal lung fibroblasts.

Ethanol inhibition of protein synthesis in brain and liver is associated with a decrease of RNA synthesis [26, 28]. Ethanol inhibition of protein synthesis by fetal lung fibroblasts may be mediated by a decrease in RNA synthesis and, specifically, messenger RNA synthesis, since polysomes isolated from ethanol-treated fibroblasts synthesized less protein than did polysomes isolated from control cells in a cell free system. The diminished ability of polysomes isolated from ethanol-treated fibroblasts to synthesize protein in the cell free system was not due to a direct effect of ethanol on polysomes, as indicated in Table 6.

The data presented in this report also are important because ethanol has been used as a vehicle to study the effects of glucocorticoids on protein synthesis in isolated fibroblasts. Concentrations of ethanol of 0.05–1% (v/v) have been used to administer glucocorticoids to cell cultures [29–37]. The present study demonstrates that treatment of IMR-90 or SCOR 1A fetal lung fibroblasts with these concentrations of ethanol results in a marked decrease in both collagen and noncollagen protein synthesis. Accordingly, ethanol should not be used as a drug vehicle in cell culture, since this alcohol may mask the effects of the drug on collagen and noncollagen protein syntheses.

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