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INDUCTION OF P4502E1 BY ACETONE IN ISOLATED RABBIT HEPATOCYTES

ROLE OF INCREASED PROTEIN AND mRNA SYNTHESIS

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Abstract—The molecular mechanism(s) underlying induction of the hepatic microsomal cytochrome P4502E1 (2E1) by xenobiotics (e.g. ethanol and acetone) is controversial. Proposed mechanisms include increased rates of enzyme synthesis due to elevated 2E1 mRNA levels, enhanced translation of pre-existing mRNA, or stabilization of 2E1 protein. To further assess which, if any, of these events predominates during the initial stages of 2E1 protein induction, we investigated the effects of acetone treatment on 2E1 content in cultured rabbit hepatocytes, an *in vitro* system that allows for precise control of the cellular mileau. Hepatocytes harvested from female rabbits and plated on plastic dishes with serum-supplemented medium were 90–100% viable for at least 48 hr in culture. Analysis of immunoreactive 2E1 content and aniline hydroxylase activity in microsomes isolated from hepatocytes cultured for up to 24 hr revealed that 2E1 expression was equal to that of microsomes from unplated cells and by 48 hr of culture, 2E1 levels decreased by only 35%. Moreover, microsomes isolated from cells exposed to 17 mM acetone for 24 hr exhibited a 53 and 62% increase in aniline hydroxylase activity and 2E1 content, respectively, compared to untreated cells. To explain these increases, the rate of 2E1 protein synthesis was determined in untreated cells or in cells treated with 17 mM acetone by first exposing hepatocytes to medium supplemented with ^{35}S-labeled methionine and cysteine ([^{35}S]Met/Cys) and subsequently assessing radiolabel incorporation into 2E1 protein. While no difference was found between untreated and acetone-treated cells in the incorporation of [^{35}S]Met/Cys into trichloroacetic acid-precipitable microsomal proteins, immunoaffinity purification of 2E1 revealed that incorporation of ^{35}S-labeled amino acids specifically into 2E1 was elevated by acetone to 200% of control values. Treatment of hepatocytes with the transcriptional inhibitor, α-amanitin, markedly inhibited this acetone-mediated increase in [^{35}S]Met/Cys incorporation into 2E1. Analysis of hepatocyte RNA revealed that acetone increased 2E1 mRNA to 130 and 160% of control levels at 6 and 24 hr, respectively, and that these increases were prevented by pretreatment with α-amanitin. Our results indicate that acetone increases 2E1 protein levels in cultured rabbit hepatocytes by stimulating its rate of *de novo* synthesis. Since this increase in 2E1 synthesis stems, at least in part, from the acetone-mediated enhancement of hepatocyte 2E1 mRNA content and is inhibitable by α-amanitin, transcriptional activation of the rabbit *CYP2E1* gene is apparently involved in the induction of 2E1 protein by acetone.

The cytochrome P450 system is a multigene family comprising numerous structurally related enzymes present in liver and extrahepatic tissues and is important in the metabolism of xenobiotics as well as endogenous compounds. One particular enzyme, cytochrome P4502E1 (2E1)‖, catalyzes the oxidation of drugs and environmental agents including ethanol, aniline, *p*-nitrophenol, *N*-nitrosodimethylamine (NDMA), acetaminophen, and carbon tetrachloride [1]. In addition to oxidizing these exogenous substrates, 2E1 also metabolizes the endogenous ketone acetone to acetol, which may then be converted to glucose [2]. Regulation of microsomal 2E1 enzyme levels is known to be affected by pathophysiological conditions including obesity [3], diabetes [4], and fasting [5], all of which produce an increase in the expression of this enzyme. Conversely, growth hormone, estrogen and testosterone appear to decrease 2E1 [6, 7]. Elevation of 2E1 also occurs following exposure to xenobiotics such as ethanol, acetone and imidazole [8]. Several investigations [9, 10] have indicated that the mechanism of 2E1 induction by fasting and diabetes is different from that involving xenobiotics. mRNA stabilization is reported to underlie 2E1 induction during conditions

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‖ Abbreviations: 2E1, cytochrome P4502E1; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IgG, immunoglobulin G; Met/Cys, methionine/cysteine; TCA, trichloroacetic acid; HEPES, (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]) sodium salt; PBS, phosphate-buffered saline; SSC, saline, sodium citrate buffer; NDMA, *N*-nitrosodimethylamine; and DTT, dithiothreitol.

of fasting and diabetes whereas protein stabilization and increased translational efficiency have both been reported to be involved in xenobiotic-promoted increases in 2E1 [10, 11]. Investigations concerning the mechanism of induction of this enzyme are particularly important because of its role in the bioactivation of xenobiotic agents such as the carcinogen NDMA [5] and the hepatotoxins halothane [12], enflurane [13], carbon tetrachloride [14], and acetaminophen [15].

In the present investigation, primary rabbit hepatocytes in culture were used to investigate the early phase of 2E1 induction by acetone. Primary cell culture provides an environment in which exposure to chemicals can be controlled more precisely than with intact animals and is thus more suited to mechanistic studies [16]. To evaluate P450 induction mechanisms, however, enzyme expression must be maintained in culture long enough for appreciable *de novo* protein synthesis to occur. This has been problematic with rat hepatocytes, where 2E1 enzyme levels rapidly decline upon culturing [17–19]. As a result, it has not been possible to observe with typical 2E1 inducers an increase in enzyme content in cultured rat hepatocytes over that of freshly-isolated cells [17–19]. Here, however, we show that 2E1 enzyme levels can be maintained in cultured rabbit hepatocytes for extended periods and that treatment with acetone, a typical 2E1 inducing agent, resulted in increased rates of *de novo* 2E1 protein synthesis as well as enhanced levels of 2E1 mRNA when compared to unplated cells.

MATERIALS AND METHODS

Materials. Female New Zealand white rabbits were from Myrtle Laboratories (Thompson Station, TN). Collagenase (type IV), hydrocortisone hemisuccinate, aniline, insulin, sodium cholate, Lubrol PX, cycloheximide, α-amanitin, and (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]) sodium salt (HEPES) buffer were purchased from the Sigma Chemical Co. (St. Louis, MO). William's E medium, William's E medium deficient in methionine and cysteine, penicillin, streptomycin, and fungizone were from Gibco Laboratories (Grand Island, NY). Fetal bovine serum was purchased from Hyclone Laboratories Inc. (Logan, UT), and cyanogen bromide-activated Sepharose 4B was obtained from Pharmacia-LKB (Piscataway, NJ). Streptavidin-conjugated horseradish peroxidase and biotin-labeled goat anti-rabbit immunoglobulin G (IgG) were from Calbiochem Inc. (La Jolla, CA). ^{35}S-Labeled L-cysteine (15%) plus L-methionine (70%) (1110 Ci/mmol), also termed Tran ^{35}S-label, was purchased from ICN Biomedicals (Irvine, CA). Polystyrene tissue culture dishes (150 × 25 mm) were from Corning Glass Works (Corning, NY).

Hepatocyte isolation and culture. Hepatocytes were isolated from rabbits by a method adapted from Seglen [20]. Briefly, Hanks' balanced saline solution supplemented with 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA) was perfused through the portal vein at 30 mL/min. This was followed by William's E medium containing 0.05% collagenase. Single cells were filtered through cheesecloth and washed with medium containing 10% fetal bovine serum. Hepatocytes were then counted and viability was determined by trypan blue exclusion. The isolated cells were plated at a density of 10^7 cells/150 mm culture dish in 17 mL of William's E medium supplemented with 10 mM HEPES buffer, 5 mg/L hydrocortisone hemisuccinate, 24 IU/L insulin, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL fungizone and 7.5% fetal bovine serum. Hepatocytes were then incubated at 37° in a 5% CO_2/95% air humidified atmosphere. The original medium was replaced with fresh medium at 6 and 24 hr after plating. All treatments were begun 24 hr after the cells were initially plated. Thus, the designation "time 0" denotes cells that had been cultured for 24 hr, at which time the cells had attached to the polystyrene dishes. Cells were then exposed to various concentrations of acetone (8.5, 17 or 50 mM) for time periods ranging from 5 min to 48 hr; medium was replenished after 24 hr. Treatment with α-amanitin (5 μg/mL) or cycloheximide (10 μg/mL) was begun 30 min prior to acetone exposure. As determined by trypan blue exclusion, cells remained viable at both time points examined, 6 and 24 hr, in the presence of cycloheximide or α-amanitin. For the 2E1 radiolabeling studies, complete medium was replaced by medium deficient in both methionine and cysteine but containing tracer amounts (1.8 μCi/mL) of [^{35}S]Met/Cys; each plate contained 1×10^8 cpm. Hepatocytes were cultured continuously in the presence of these ^{35}S-labeled amino acids until harvested; medium was not changed once cells were exposed to the radiolabeled amino acids.

Following treatment, culture dishes were either rinsed with cold phosphate-buffered saline (PBS) and the hepatocytes harvested by scraping into 100 mM Tris–HCl, pH 7.4, containing 100 mM KCl and 1 mM EDTA or were disrupted with guanidinium thiocyanate/phenol/chloroform to isolate RNA [21]. Harvested cells were then frozen at −70° until preparation of microsomes. A portion of the harvested cells (0.5 mL, 5×10^7/mL) was lysed in 0.1 mM EDTA (pH 8.0), 10 mM Tris–HCl (pH 7.6), and 20 mM NaCl buffer, and the concentration of DNA determined fluorometrically [22]; standard curves were constructed with λ DNA.

Microsomal preparation. Hepatocytes were thawed and briefly sonicated to lyse cell membranes. This was followed by centrifugation at 10,000 *g* for 30 min at 4°. The supernatant was then centrifuged at 100,000 *g* for 90 min at 4° to harvest microsomes. The microsomal pellets were resuspended in 50 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol, 0.1 mM EDTA, and 0.1 mM dithiothreitol (DTT). Microsomal protein and P450 concentrations were determined as described by Bensadoun and Weinstein [23] and Omura and Sato [24], respectively.

Catalytic activity. Aniline hydroxylase activity was assessed by measuring the conversion of aniline to *p*-aminophenol as described by Mieyal *et al.* [25]. The reaction mixtures (0.5 mL) contained 100 mM potassium phosphate buffer (pH 7.4), 2 mM aniline, 1 mg microsomal protein and 1 mM NADPH.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblots. Microsomal proteins were initially resolved by SDS-PAGE [26], followed by electrophoretic transfer to nitrocellulose filters [27]. The filters were then immunochemically stained as described by Raucy *et al.* [15] using anti-hamster 2E1 IgG [3] as the primary antibody. 2E1 concentrations were determined using an Ultroscan XL scanning laser densitometer (Pharmacia-LKB Biotechnology). The linearity of immunochemical and Coomassie blue staining was assessed from standard curves constructed by applying 0.5 to 20 μg of hepatocyte microsomal protein to the immunoblots or gels. The staining intensity using 10 μg protein was within the linear portion of the standard curve and was thus utilized for all subsequent experiments.

Determination of ^{35}S incorporation into microsomes and 2E1. Immediately following preparation of microsomes from hepatocytes labeled with [^{35}S]Met/Cys, an aliquot containing 100 μg protein was made 10% in trichloroacetic acid (TCA) and incubated for 20 min on ice. The mixture was further incubated at 90° for 15 min and centrifuged for 5 min at 1000 *g*. Following centrifugation, the protein pellet was washed three times with 5% TCA, and resuspended in 50 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol, 1 mM EDTA, 1 mM DTT, and 1% SDS. This solubilized protein was then mixed with 1.0 mL of ScintiVerse II (Fisher Scientific) incubated overnight at 25°, and subjected to scintillation counting.

Immunoaffinity purification of P4502E1. ^{35}S-Labeled 2E1 was isolated by an immunoaffinity purification procedure initially described by Wrighton *et al.* [28]. Briefly, 10 mg anti-hamster 2E1 IgG was coupled to 1 mL of CNBr-activated Sepharose 4B according to manufacturer's instructions. Hepatocyte microsomes were solubilized with 0.5% cholate plus 0.2% Lubrol PX, and incubated at 4° overnight with the antibody-coupled resin at a ratio of 15 mg microsomal protein/mL resin. Nonspecifically bound material was washed from the resin with four sequential wash buffers [28], and 2E1 was eluted from the resin with 100 mM glycine-HCl, pH 3.1. The yield was typically 0.03 mg 2E1 protein/mg microsomal protein (3%). 2E1 samples were dialyzed overnight against 100 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol, 0.1 mM EDTA and 0.1 mM DTT, and then subjected to SDS-PAGE. After fixation, polyacrylamide gels were soaked in En^{3}Hance (Dupont) and then exposed to X-Omat film for 3 days at −70° using intensifying screens.

Northern and slot blot analysis. Total hepatocyte RNA was size fractionated on denaturing formaldehyde gels and then transferred by vacuum to nitrocellulose filters. When slot blot analysis was performed, RNA (15 μg) was directly applied to the filters by vacuum. Filters were prehybridized for 20 min at 42° with a buffer containing 50% deionized formamide, 5× Denhardt's solution, 0.1% SDS, 5× SSC, and 250 μg/mL heat-denatured salmon sperm DNA (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0; 1× Denhardt's solution = 0.02% bovine serum albumin, 0.02% Ficoll and 0.02% polyvinylpyrrolidone). The DNA probes used, a human 2E1 cDNA and a human β-actin cDNA, were both labeled with [α-^{32}P]dATP (2.5×10^6 cpm/mL) by a random primer extension kit (Boehringer-Mannheim). The probes were then heat-denatured and incubated with the filters in the above buffer overnight at 42°. Filters were washed twice with 2× SSC buffer, 0.1% SDS for 10 min at 25°, followed by two washes with 0.1× SSC, 0.1% SDS for 10 min at 25°. The washed filters were then exposed to X-Omat film overnight at −70° and band densities were quantitated from the autoradiogram by laser densitometry. The 1.1 kb human 2E1 cDNA was obtained from Oxford Biomedical Laboratories, Inc. (Oxford, MI). The 1.8 kb β-actin cDNA was derived from Okayama-Berg plasmid vector pHFβ-1, which contains the full-length cDNA for human fibroblast cytoplasmic β-actin [29]. The β-actin cDNA insert, after excision from the vector with *Bam HI*, was amplified by the polymerase chain reaction [30] using 20-mer primers complementary to the 5′ and 3′ ends of the reported cDNA sequence [29].

Data analysis and statistics. Results are expressed as means ± SEM. Data were evaluated by linear

Table 1. Cytochrome P450 in microsomes from untreated hepatocytes

Time in culture* (hr)	Total P450† (nmol/mg)	Aniline hydroxylase† (nmol/min/mg protein)	P4502E1 content† (staining intensity, O.D./μg)
Unplated cells	0.92 ± 0.13	0.36 ± 0.03	0.65 ± 0.19
6	0.85 ± 0.20	0.38 ± 0.02	0.63 ± 0.11
12	0.87 ± 0.06	0.36 ± 0.05	0.64 ± 0.11
24	0.86 ± 0.14	0.32 ± 0.01	0.69 ± 0.06
48	0.57 ± 0.15‡	0.17 ± 0.09‡	0.41 ± 0.03‡

* Times in culture (6, 12, 24, and 48 hr) refer to times after the initial 24-hr stabilization period (time 0).

† Values denote the means ± SEM of three determinations made on microsomes from hepatocytes isolated from three different animals.

‡ Significantly different from unplated cells, $P < 0.05$.

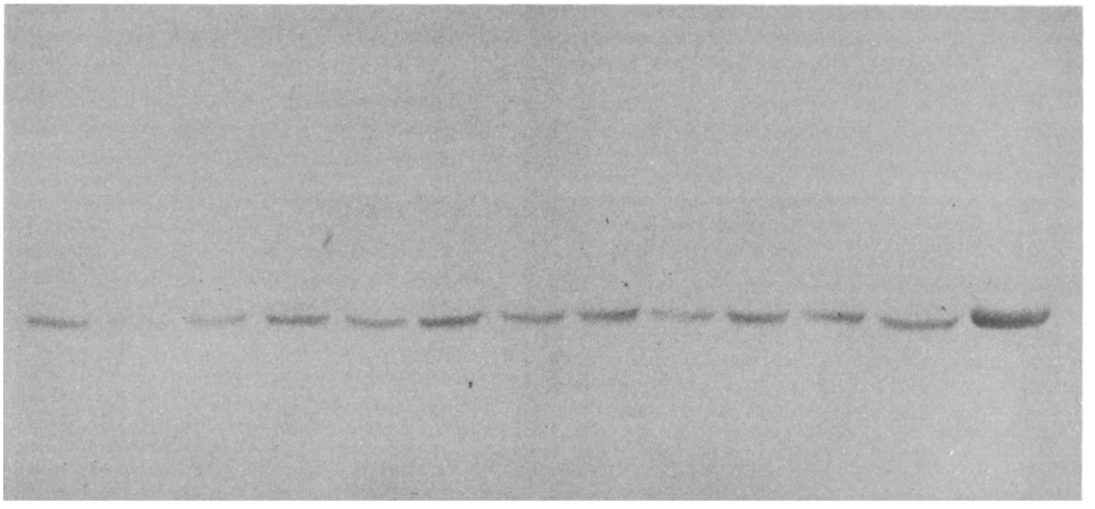

Fig. 1. Effects of acetone on 2E1 content in cultured rabbit hepatocytes. Hepatocyte microsomal proteins (10 μg) were first separated by SDS-PAGE and transferred to a nitrocellulose filter; the filter was immunochemically stained with anti-hamster 2E1 as described in Materials and Methods. Lanes 1 and 2 contain microsomes from unplated cells (10 and 5 μg, respectively); lanes 3, 5, 7, 9, and 11 are microsomes (10 μg) from untreated cells which were harvested at 1, 3, 6, 12, and 24 hr, respectively. Lanes 4, 6, 8, 10, and 12 are microsomes from 17 mM acetone-treated cells harvested at 1, 3, 6, 12, and 24 hr after exposure. Lane 13 contains microsomes (10 μg) from rabbit liver.

regression and correlation or by Student's *t*-test. Differences were attributed to treatment rather than change variation when $P < 0.05$.

RESULTS

Cultured rabbit hepatocytes were utilized to examine 2E1 induction at early time points after acetone treatment. Rabbit livers perfused with collagenase typically produced yields of 5×10^9 cells with > 90% viability. DNA content was determined at various time points (time 0, 6, 12, 24, and 48 hr) as an indicator of cell integrity. The content of cellular DNA was maintained throughout the time points examined until 48 hr, at which time it was 80% of that at time 0. Analysis of P450 content in rabbit hepatocyte microsomes revealed that total P450 concentrations were maintained in culture at a level similar to that of cells freshly isolated from liver (unplated) for at least 24 hr, and by 48 hr aggregate P450 and aniline hydroxylase activity were decreased to 62 and 47%, respectively, of that observed in unplated hepatocytes (Table 1). It is important to note here that all comparisons to unplated cells were made after hepatocytes had been initially cultured for 24 hr (see Materials and Methods). Immunoblot analysis of rabbit hepatocyte microsomes revealed levels of 2E1 identical to that of unplated cells for 24 hr of culturing; by 48 hr, immunoreactive protein content decreased by only 37% relative to that in freshly isolated cells (Table 1 and Fig. 1).

Microsomes from hepatocytes treated with acetone for various time periods were assessed for changes in 2E1 content and aniline hydroxylase activity. Microsomal aniline hydroxylase activity was used as a probe of 2E1 metabolic activity because treatment with acetone has been shown to specifically elevate only the 2E1-promoted component of this activity in rabbit liver microsomes [2, 31]. In preliminary concentration–response studies, hepatocytes were exposed to three different concentrations of acetone, namely 8.5, 17, and 50 mM. Increases in microsomal 2E1 content and aniline hydroxylase activity elicited by 8.5 mM acetone were always less (10–20%) than those elicited by 17 mM acetone, whereas 50 mM acetone resulted in a 20–30% decrease in enzyme content and activity relative to untreated cells, possibly due to cellular toxicity. Therefore, we chose 17 mM acetone as the optimal dose for subsequent studies. Immunoblot analysis of microsomes from hepatocytes treated with 17 mM acetone revealed a time-dependent increase in microsomal 2E1 compared to unplated cells and cells at time 0 (Figs. 1 and 2). Under the same conditions, aniline hydroxylase activity reached a maximum of 53% at 24 hr above unplated control and time 0 hepatocyte microsomes (Fig. 2).

To determine whether the acetone-mediated increase in immunoreactive 2E1 content was due to enhanced *de novo* synthesis of 2E1 protein, the enzyme was immunoaffinity purified from rabbit

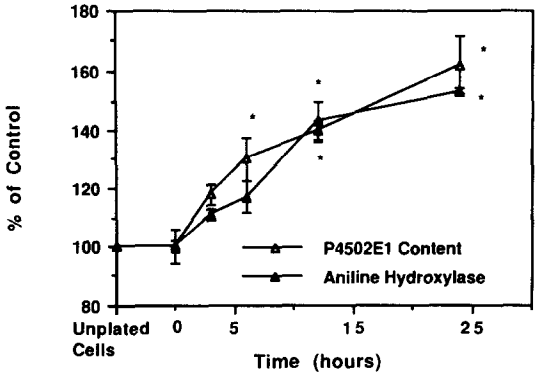

Fig. 2. Effect of acetone treatment on aniline hydroxylase activity and 2E1 content in cultured rabbit hepatocytes. Aniline hydroxylase activity was determined in microsomes from hepatocytes incubated with 17 mM acetone for times indicated in the figure. P4502E1 content in microsomes from cells treated with acetone was assessed by densitometric scanning of immunoblots similar to that shown in Fig. 1. Values for both aniline hydroxylase activity and P4502E1 content are expressed as percent of control values, which were determined in microsomes from cells maintained in the absence of acetone at the corresponding time points, at time 0, and in unplated cells. Control values of aniline hydroxylase activity at times 0, 3, 6, 12, and 24 hr were 0.37 ± 0.031, 0.36 ± 0.03, 0.38 ± 0.02, 0.36 ± 0.05, and 0.32 ± 0.01 nmol/min/mg microsomal protein, respectively. Control values for 2E1 content were 0.65 ± 0.19, 0.57 ± 0.07, 0.63 ± 0.11, 0.64 ± 0.11, and 0.69 ± 0.06 O.D./μg, at the respective time points. Each value represents the mean $\pm$ SEM of three determinations made on hepatocytes prepared from three separate rabbits. Asterisks indicate significant differences between untreated and acetone treated cells at the individual time points ($P < 0.05$).

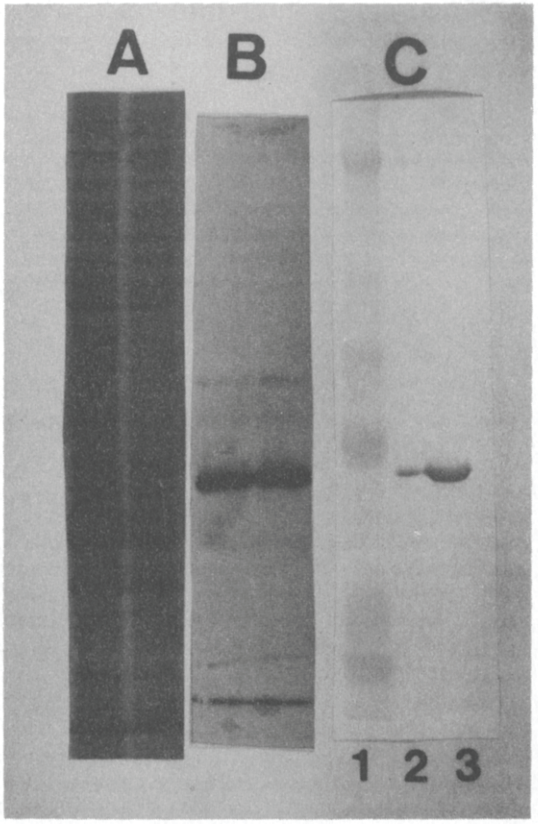

Fig. 3. [^{35}S]Met/Cys incorporation into total microsomal protein and 2E1 in cultured rabbit hepatocytes. Panel A: Autoradiograph of [^{35}S]Met/Cys incorporation into hepatocyte microsomes (10 μg microsomal protein/lane); lane 1 (left), untreated hepatocyte microsomes; lane 2 (right), microsomes from acetone-treated hepatocytes. Panel B: Autoradiograph of [^{35}S]Met/Cys radiolabeled immunoaffinity-purified P4502E1 (2 μg protein/ lane) from hepatocytes derived from two separate rabbits after exposure to acetone for 12 hr. Panel C: Immunoblot of purified 2E1. Lane 1, pre-stained protein standards with molecular weights of 180,000, 116,000, 84,000, 58,000, 40,000, and 27,000; lane 2, immunoaffinity-purified hepatocyte microsomal P4502E1 (2 μg); and lane 3, liver microsomes (10 μg) from an acetone-treated rabbit.

hepatocyte microsomes that had been radiolabeled with [^{35}S]Met/Cys. Autoradiographic analysis of hepatocyte microsomal proteins resolved by SDS-PAGE shows the radiolabeling of proteins with molecular weights ranging from 12,000 to 250,000 (Fig. 3A). Figure 4A demonstrates that incorporation of radiolabeled Met/Cys into microsomes was linear with time ($r^2 = 0.99$) and was not affected by exposure to acetone.

^{35}S-Labeled rabbit hepatocyte microsomes, upon solubilization, were then subjected to immunoaffinity chromatography on a Sepharose 4B column to which anti-2E1 IgG had been linked. Following extensive washing of the column, 2E1 protein was eluted under acidic conditions, after which the purified enzyme was subjected to SDS-PAGE and fluorography. As illustrated in Fig. 3B, affinity purification yielded one predominant ^{35}S-labeled protein. Immunoblot analysis with anti-2E1 IgG (Fig. 3C) showed that this protein co-migrated with the major acetone-inducible protein in rabbit liver microsomes, namely 2E1. In a separate experiment, the 2E1 species isolated by immunoaffinity chromatography from rabbit hepatocytes was found to possess the same molecular weight (50,000) as purified rabbit liver 2E1 (data not shown) [32].

Rates of [^{35}S]Met/Cys incorporation into 2E1 were linear over 24 hr in both untreated ($r^2 = 0.97$) and acetone-treated cells ($r^2 = 0.96$) (Fig. 4B). However, [^{35}S]Met/Cys incorporation rates were 2-fold higher in acetone-treated hepatocytes than in control cells as assessed from the slopes of the regression lines. Exposure of hepatocytes to cycloheximide, a protein synthesis inhibitor, for 6 hr resulted in a marked decrease (> 85%) in [^{35}S]Met/Cys incorporation into 2E1 from both untreated and acetone-treated cells, indicating that protein synthesis was occurring (data not shown).

RNA isolated from control rabbit hepatocyte cultures was subjected to Northern blot analysis with either a 2E1 cDNA probe or a β-actin cDNA probe

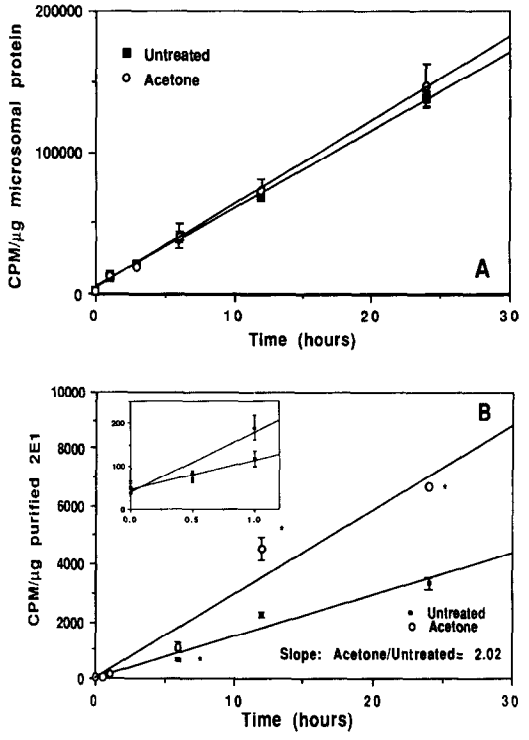

Fig. 4. Effect of acetone on [^{35}S]Met/Cys incorporation into microsomes and purified 2E1 in cultured rabbit hepatocytes. Panel A: Incorporation of radiolabeled amino acids into hepatocyte microsomes was determined for the indicated times and expressed as cpm/μg microsomal protein. Linear regression analysis revealed $r^2 = 0.99$ for microsomes from untreated cells and $r^2 = 0.99$ for microsomes from acetone-treated cells. Each value is the mean ± SEM of determinations for three separate animals. The values comprising each determination for an individual animal represent a pool of eight culture dishes. Significant differences were not observed in [^{35}S]Met/Cys incorporation between microsomes isolated from untreated and acetone-treated hepatocytes ($P > 0.05$). Panel B: Incorporation of radiolabeled Met/Cys into 2E1. The data are expressed as cpm/μg of purified 2E1. Each value is the mean ± SEM of three separate rabbits as described above for panel A. The asterisks at 6, 12, and 24 hr of acetone exposure indicate significant differences ($P < 0.05$) in the rate of [^{35}S]Met/Cys incorporation into 2E1 between acetone and untreated hepatocytes. Linearity was assessed by regression analysis of ^{35}S incorporation into 2E1 isolated from control ($r^2 = 0.97$) and acetone-treated ($r^2 = 0.96$) cells. The slopes of the individual lines were 145 for untreated and 294 for 2E1 from acetone-treated cells. The inset represents the earlier time points (time 0, 0.5, and 1 hr). Significant differences in the rate of [^{35}S]Met/Cys incorporation into 2E1 between acetone and untreated hepatocytes were not observed at the early time points ($P > 0.05$).

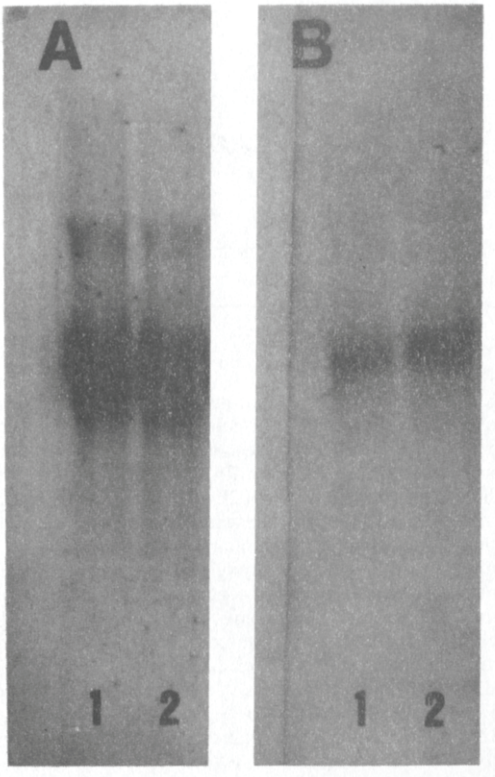

Fig. 5. Northern blot analysis of 2E1 mRNA and β-actin mRNA in cultured rabbit hepatocytes. Total RNA (10 μg) isolated from untreated hepatocytes from two separate rabbits was subjected to electrophoresis in agarose gels containing formaldehyde, followed by vacuum transfer to nitrocellulose filters. The filters were then hybridized with either a β-actin cDNA probe (panel A) or a 2E1 cDNA probe (panel B) as described in Materials and Methods; autoradiography was used to visualize β-actin mRNA/cDNA and 2E1 mRNA/cDNA hybrids. Lanes 1 and 2 contain RNA from two separate hepatocyte preparations.

(Fig. 5). Autoradiography showed a single, compact hybridization signal in the case of either probe, indicating that the RNA was not degraded. The autoradiogram also indicated that the 2E1 cDNA did not appear to hybridize with 2E2 mRNA, which exhibits a greater size than 2E1 mRNA [33]. RNA slot blot analysis revealed that acetone treatment of cultures for 6 and 24 hr significantly enhanced 2E1 mRNA content by 30 and 60%, respectively, relative to control levels (Fig. 6). This acetone-mediated increase in hepatocyte 2E1 mRNA was abolished by prior treatment of the cells with the RNA polymerase inhibitor α-amanitin (Fig. 6). Moreover, exposure of cells to α-amanitin prior to acetone treatment also prevented the increase in [^{35}S]Met/Cys incorporation into immunoaffinity purified 2E1. The nearly 2-fold enhancement of [^{35}S]Met/Cys incorporation into newly-synthesized 2E1 protein (from 650 ± 60 to 1090 ± 150 cpm/μg) noted after 6 hr of acetone exposure was abolished (440 ± 120 cpm/μg) in cells treated with acetone together with α-amanitin. However, addition of this inhibitor had essentially no effect on the rates of [^{35}S]Met/Cys incorporation into newly synthesized 2E1 in cells treated with α-amanitin alone (580 ± 60 cpm/μg).

DISCUSSION

Using primary cultures of rabbit hepatocytes, we

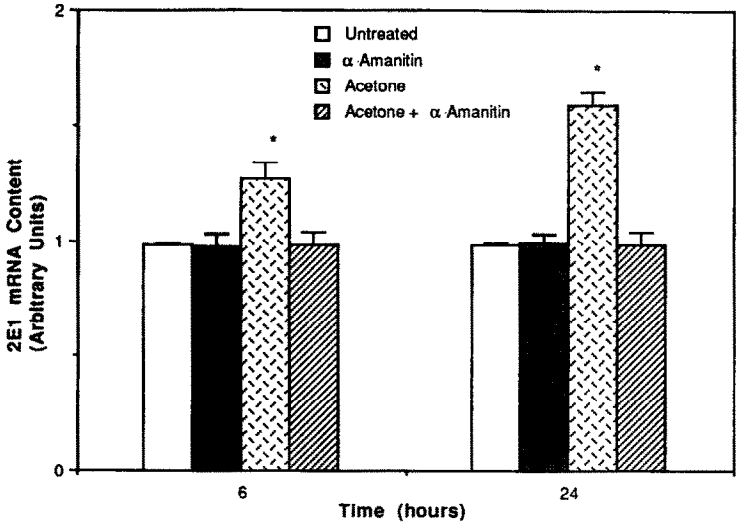

Fig. 6. Effect of α-amanitin on 2E1 mRNA levels in control and acetone-treated rabbit hepatocytes. Hepatocytes were cultured for 6 or 24 hr in the presence of the agents indicated. Total RNA was isolated from cells pooled from four individual dishes and 15 μg was directly applied to nitrocellulose filters using a slot-blotting device. After hybridization with a 2E1 cDNA (see Materials and Methods), autoradiography was used to visualize 2E1 mRNA/cDNA hybrids. Scanning densitometry was then employed to measure the intensity of 2E1 mRNA/cDNA hybridization signals. Each value is the mean ± SEM of three determinations made on hepatocytes prepared from three separate rabbits. All values for 2E1 mRNA have been normalized to β-actin mRNA. The asterisks indicate a significant difference ($P < 0.05$) from untreated cells.

have demonstrated for the first time that acetone increases the synthesis not only of 2E1 protein but also its encoding mRNA. We have also shown that cultured rabbit hepatocytes constitute an *in vitro* experimental system permitting the evaluation of 2E1 regulatory mechanisms under conditions of P450 enzyme maintenance. Isolated rabbit hepatocytes were kept in culture for up to 24 hr, during which time aggregate P450 concentrations as well as immunoreactive 2E1 content remained at levels approximating those found in unplated cells. Under these culture conditions, treatment of hepatocytes with acetone was found to elicit a time-dependent increase in both 2E1 content and one of its inherent activities, namely aniline hydroxylation, compared to untreated cells. By assessing the incorporation of radiolabeled Met/Cys into 2E1, this elevation of the enzyme by acetone was shown to correlate with an increase in its rate of *de novo* synthesis, a phenomenon selective for 2E1 since acetone failed to elevate rates of amino acid incorporation into total microsomal protein. The inhibition of newly synthesized 2E1 (^{35}S-labeled enzyme) by α-amanitin provided additional evidence that acetone promoted induction by protein synthesis. This study also revealed that the increase in hepatocyte 2E1 levels in response to acetone was accompanied by a corresponding increase in 2E1 mRNA transcripts, indicating that the latter accounts, at least in part, for the former. Importantly, treatment of cultures with the RNA polymerase II inhibitor, α-amanitin, prevented the acetone-mediated increase in 2E1 transcripts, implying that acetone can influence 2E1 protein expression in rabbit hepatocytes at the level of *CYP2E1* gene transcription.

In this study, we used rabbit hepatocytes as a model system for examining the mechanisms underlying 2E1 protein induction by acetone. Rabbit hepatocytes were found to maintain 2E1 protein at levels equivalent to unplated cells, and those at time 0, for 24 hr (Table 1 and Fig. 1); only a 35% decrease in cellular 2E1 content was noted after 48 hr in culture. Such 2E1 maintenance was achieved by culturing the cells directly on plastic in a serum-supplemented medium. It is important to note here that 2E1 levels were actually maintained for 48 hr since all measurements were made after cells had been allowed to stabilize in culture for 24 hr. In contrast, 2E1 expression in rat hepatocytes decreases markedly within the first 24 hr in culture [17, 18], regardless of whether hepatocytes are cultured as described here or using more exotic culture conditions (e.g. culturing on Matrigel in a chemically-defined serum-free medium). The reason for this superior maintenance of 2E1 in cultured rabbit hepatocytes is not entirely clear at present. However, Morel *et al.* [34] have shown that human hepatocytes maintain 2E1 levels greater than 75% of those found in unplated cells for up to 60 hr in culture, suggesting that the species from which hepatocytes are derived is an important determinant of whether P450 enzymes can be maintained in culture.

Rabbit hepatocytes, unlike those from rat, also exhibited a bona fide time-dependent induction of 2E1 enzyme content and activity upon acetone exposure (Figs. 2 and 4B). 2E1 levels in acetone-

treated cells were not only higher than in the corresponding untreated cells but were also higher than in unplated cells and those at time 0. By contrast, 2E1 levels in rat hepatocytes after treatment with other 2E1 inducing chemicals (e.g. ethanol, imidazole, pyrazole, and dimethylsulfoxide) are increased only with respect to untreated cells cultured for the same time period [17]. Since enzyme content in "induced" rat hepatocytes was still significantly lower than in freshly isolated cells, others have concluded that ethanol and similar agents induce 2E1 by stabilizing the enzyme against proteolytic degradation rather than by increasing its synthesis [17, 19]. With regards to acetone itself, Song *et al.* [35] have reported that 2E1 induction *in vivo* by this compound also involves protein stabilization. However, these workers examined the acetone-mediated induction process under conditions where the enzyme was maximally induced (after acetone treatment for 10 days), a state that may differ markedly from the acute phase of 2E1 induction. In our own study, which was aimed at assessing the initial 2E1 induction phase, we found increased rates of 2E1 synthesis in rabbit hepatocytes during the first 24 hr of acetone treatment (Fig. 4B). Had acetone treatment stabilized 2E1 protein against degradation during this time period, the curves presented in Fig. 4B would have been reversed, with untreated cells exhibiting the greater rate of [^{35}S]-Met/Cys incorporation into 2E1. Kim *et al.* [11] and Kim and Novak [36] have also reported that increased rates of enzyme synthesis underlie the enhanced hepatic 2E1 levels found in rats treated acutely with pyridine. Since Kim and co-workers could find no increase in liver 2E1 mRNA, this increase in 2E1 synthesis rates was attributed to enhanced translational efficiency of pre-existing 2E1 message. However, the results presented here suggest that acetone enhances rates of 2E1 synthesis by a different mechanism, namely activation of *CYP2E1* gene transcription. In the absence of acetone, α-amanitin had little effect on the rates of [^{35}S]Met/Cys incorporation into 2E1 protein, suggesting that in the basal state translation of pre-existing 2E1 mRNA is sufficient to maintain levels of the protein for at least 6 hr. In the presence of acetone, however, α-amanitin decreased the enhanced rates of radiolabel incorporation into 2E1 to values similar to those in untreated cells, indicating that transcriptional activation is involved in 2E1 induction by acetone.

It is necessary to emphasize that the 2-fold elevation of ^{35}S-labeled 2E1 protein from acetone-treated cells described here represents newly synthesized enzyme exclusively and not enzyme present before treatment. Consistent with these results, we also found a similar increase in 2E1 by immunoblot analysis, a less sensitive technique that detects total 2E1 protein. If protein stabilization had occurred simultaneously, then the increase noted in 2E1 protein in acetone treated cells would have been greater. Consistent with our results, *in vivo* treatment of rabbits with ethanol for 12 or 24 hr results in only a 61% increase in hepatic 2E1 [37]. In contrast prolonged treatment (7 days) results in a much greater increase, suggesting that synthesis as well as stabilization may underlie 2E1 induction in animals treated chronically with 2E1 inducers [37, 38]. With regard to diabetic or fasted rats, 50–200% elevations in 2E1 have been described, a phenomenon believed to be caused by an increase in circulating ketone bodies (i.e. acetone) [39]. Therefore, the 2-fold increase in rabbit hepatocyte 2E1 elicited by acetone treatment is directly comparable with values obtained in animals acutely treated with 2E1-type inducers *in vivo* or in fasted or diabetic rats.

It should be pointed out that our study is the first to describe the elevation of hepatocyte 2E1 mRNA *in vitro* in response to treatment with a 2E1 chemical inducing agent. At two time points, 6 and 24 hr, acetone was found to clearly increase 2E1 mRNA content in cultured rabbit hepatocytes (Fig. 6). With the exception of two previous studies [40, 41], the majority of reports have indicated that xenobiotics (including acetone) which induce liver 2E1 enzyme levels in rats as well as rabbits have either no effect or actually decrease 2E1 message levels [1, 17, 35, 37]. Although Kubota *et al.* [40] and Diehl *et al.* [41] found that ethanol treatment elevates hepatic 2E1 mRNA levels in hamsters and rats, respectively, the underlying mechanism was not examined. One possible mechanism for the increase in 2E1 message noted in these previous investigations as well as in the present study involves mRNA stabilization by ethanol and acetone. However, our results indicate that transcriptional activation of the *CYP2E1* structural gene plays a more important role in the acetone-mediated enhancement of 2E1 transcripts in cultured rabbit hepatocytes. Indeed, we found that prior exposure of acetone-treated hepatocytes to α-amanitin, a potent inhibitor of gene transcription, completely blocked the increase of both 2E1 mRNA and newly synthesized protein. Transcriptional regulation of *CYP2E1* gene expression, which is a novel finding with regards to a 2E1 chemical inducing agent, has been reported previously to play an important role in the ontogenic regulation of this P450 enzyme [42].

In conclusion, rabbit hepatocyte 2E1 levels were maintained in culture at concentrations similar to that of unplated cells for 24 hr. During this time, treatment of hepatocytes with acetone elevated 2E1 protein concentrations above that observed at either time 0 or in unplated cells. Induction of 2E1 by acetone resulted from an increase in the rate of protein synthesis. Moreover, this increase in 2E1 protein was accompanied by an elevation in its corresponding mRNA. By treating hepatocytes with the transcriptional inhibitor, α-amanitin, we demonstrated for the first time that acetone-mediated increases in 2E1 mRNA and protein were due, at least in part, to transcriptional activation of the *CYP2E1* gene.

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