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Acetoacetate Induces CYP2E1 Protein and Suppresses CYP2E1 mRNA in Primary Cultured Rat Hepatocytes

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ABSTRACT

The ketone body acetoacetate (AA) in the absence of insulin or in the presence of diabetic insulin levels decreases CYP2E1 mRNA expression in a concentration- and time-dependent manner in primary cultured rat hepatocytes. AA activates p70 ribosomal S6 kinase (p70S6K) and protein kinase C (PKC) by ~2- to 2.5-fold, respectively, following 6-h treatment. The AA-mediated activation of p70S6K, but not PKC, was abolished by inhibition of PI 3-K with LY294002 [2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one] or wortmannin, in agreement with p70S6K being downstream of phosphatidylinositol 3-kinase (PI 3-K). Inhibition of PI 3-K, mTOR with rapamycin, or PKC with bisindolylmaleimide ameliorated the AA-mediated down-regulation of CYP2E1 mRNA expression. Neither the mitogen-activated protein kinase kinase inhibitor PD98059 (2′-amino-3′-methoxyflavone) nor the p38 mitogen-activated protein kinase inhibitor SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole] ameliorated the AA-mediated suppression of CYP2E1 mRNA expression. Heterogeneous nuclear RNA analysis revealed that AA suppressed CYP2E1 gene transcription by ~50% and that inhibition of PI 3-K and PKC diminished this AA-mediated effect on transcription. CYP2E1 mRNA half-life slightly increased from ~24 h in untreated hepatocytes to ~32 h in AA-treated cells. Interestingly, AA increased CYP2E1 protein levels by ~2- and 2.5-fold at 24 and 48 h, respectively. DL-β-Hydroxybutyrate was without effect. Polysomal distribution studies revealed that AA increased the proportion of RNA associated with the actively translated polysomal fractions versus the 40S to 60S untranslated fractions by ~40%. CYP2E1 protein half-life increased from ~8 h in untreated hepatocytes to ~24 in AA-treated cells. These data show that AA decreases CYP2E1 mRNA expression through inhibition of gene transcription while simultaneously elevating CYP2E1 protein levels through increased translation and decreased protein degradation.

Acetoacetate (AA) and DL-β-hydroxybutyrate (3HB) comprise two major ketone bodies, which are produced primarily in the liver. Ketone bodies are considered an emergency source of glucose (Casazza et al., 1984). In humans, ketone bodies (AA plus 3HB) with plasma concentrations higher than 1 mM are considered hyperketonemic. Ketone body concentrations may be elevated up to 10 mM in severe ketosis, relative to concentrations less than 0.5 mM under normal physiologic conditions (Jain et al., 2003). Hyperketonemia is a very common feature in diabetes mellitus.

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AA and 3HB effects on CYP2E1 expression remain controversial. An increase in CYP2E1 protein and/or activity has been associated with hyperketonemia (Shimojo, 1994); however, neither AA nor 3HB elevated CYP2E1 mRNA levels in the presence of insulin in primary cultured rat hepatocytes (Zangar and Novak, 1997) or in vivo (Barnett et al., 1992). In addition, we have reported that AA, but not 3HB, in the absence of insulin substantially decreased CYP2E1 mRNA levels in primary cultured rat hepatocytes compared with untreated hepatocytes (Woodcroft et al., 2002). The reason for these differences likely resides in the complexity of the regulatory signaling networks and the metabolic conditions in vivo and in vitro. Diabetes is a disease characterized by decreased levels of insulin, testosterone, thyroxine, and growth hormone, and increased levels of glucagon, glucose, lipids, and ketone bodies, all of which can be reversed over

ABBREVIATIONS: AA, lithium acetoacetate; 3HB, DL-β-hydroxybutyrate; PKC, protein kinase C; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PI 3-K, phosphatidylinositol 3-kinase; mTOR, mammalian target of rapamycin; p70S6K, p70 ribosomal S6 kinase; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; PD98059, 2′-amino-3′-methoxyflavone; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; DMSO, dimethyl sulfoxide; bp, base pair(s); hnRNA, heterogeneous nuclear RNA; PCR, polymerase chain reaction; TBS-T, Tris-buffered saline/Tween 20; PBS-T, 0.05% Tween 20 in phosphate-buffered saline; MEK, mitogen-activated protein kinase kinase; UT, untreated; HNF, hepatocyte-specific transcription factor; IL, interleukin.

time by insulin and all of which have been reported to alter CYP2E1 expression (for review, see Cheng and Morgan, 2001). Thus, it is extremely difficult to identify the singular effects of ketone bodies on CYP2E1 expression in diabetes in vivo. The regulation of CYP2E1 is also complex, involving transcriptional, post-transcriptional, translational, and post-translational events (for review, see Novak and Woodcroft, 2000), which also complicates our understanding of the regulatory mechanisms involved.

Diabetes mellitus increases CYP2E1 at both the messenger RNA (mRNA) and protein levels (Dong et al., 1988). CYP2E1 is involved in biotransformation of several low molecular weight xenobiotics, including halogenated hydrocarbons, primary alcohols, and nitrosamines, as well as therapeutic agents and endogenous fatty acids and ketones (for review, see Novak and Woodcroft, 2000). CYP2E1 has been implicated in the generation of tissue damaging hydroxyl radicals in patients suffering from diabetes and liver disease (Lieber et al., 2004). Thus, elevated CYP2E1 levels may be a risk factor for xenobiotic metabolism, oxidative stress, and long-term hepatic injury. Diabetes mellitus is a risk factor for developing hepatocellular carcinoma (El-Serag et al., 2004).

We have previously reported a primary rat hepatocyte culture system that is responsive to xenobiotic-mediated increases in CYP2E1, 2B, 3A, and 4A mRNA and protein expression in a manner that parallels that in vivo (Zangar et al., 1995). We have reported, using this primary hepatocytes culture system, that AA, but not 3HB, decreases the CYP2E1 mRNA levels in the absence of any other hormones (Woodcroft et al., 2002).

Information regarding the mechanism(s) by which AA alters CYP2E1 levels is limited. We have previously reported that protein kinase C (PKC) signaling pathway is active in the AA-mediated activation of the extracellular signal-regulated kinase (ERK1/2) and the p38 mitogen-activated protein kinase (p38 MAPK) and in the production of cellular oxidative stress in primary cultured rat hepatocytes (Abdelmegeed et al., 2004). Jorquera and Tanguay (2001) reported that the phosphatidylinositol 3-kinase (PI 3-K) signaling pathway is active in the fumarylacetoacetate-mediated activation of ERK1/2 in Chinese hamster V79 cells and Hela cells. Fumarylacetoacetate is the precursor of AA.

The objective of the present study was to characterize the mechanisms and signaling pathway(s) involved in the AA-mediated effects on CYP2E1 mRNA and protein expression in primary cultured rat hepatocytes. We report that the AA-mediated decrease in CYP2E1 mRNA levels is the result of the inhibition of CYP2E1 gene transcription. We show that PI 3-K/Akt/mTOR/p70 ribosomal S6 kinase (p70S6K) and PKC are involved in the AA-mediated inhibition of CYP2E1 mRNA levels and gene transcription. In contrast, AA, but not 3HB, significantly increases microsomal CYP2E1 protein levels, which seems to occur through the concerted action of increased CYP2E1 translation and inhibition of protein degradation. Thus, AA effectively uncouples CYP2E1 gene transcription and CYP2E1 translation. A similar effect has been reported for ethanol (Ronis et al., 1993). These studies provide seminal information on the seemingly contradictory effects of AA on CYP2E1 gene transcription and protein expression and illustrate the complex mechanisms that regulate CYP2E1 gene expression and protein levels in response to ketone bodies.

Materials and Methods

Materials. Modified Chee's medium and L-glutamine were purchased from Invitrogen (Carlsbad, CA). Insulin (Novolin R) was obtained form Novo-Nordisk (Princeton, NJ). Collagenase (type I) was obtained from Worthington Biochemicals (Freehold, NJ). Vitrogen (95–98% type I collagen, 2–5% type III collagen) was purchased from Cohesion Technologies (Santa Clara, CA). Horseradish peroxidase-conjugated rabbit anti-goat antibody was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Enhanced chemiluminescence reagents were obtained from Amersham Biosciences Corp. (Piscataway, NJ). Wortmannin, LY294002, rapamycin, bisindolylmaleimide [2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)-maleimide], PD98059, SB203580, and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Calbiochem (La Jolla, CA). Antibodies against phospho-p70 S6 kinase (Thr421/Ser424) (P-p70S6K), p70S6K, and phospho-PKC (pan) (betaII Ser660) (P-PKC) were obtained from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated goat anti-rabbit antibody and goat anti-mouse antibody were purchased from Bio-Rad Laboratories (Hercules, CA). AA, 3HB, and all other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Primary Rat Hepatocyte Culture. Hepatocytes were isolated from the livers of male Sprague-Dawley rats (200–300 g) using collagenase perfusion as described previously (Woodcroft and Novak, 1997, 1999). Briefly, hepatocytes were plated onto dishes covalently coated with vitrogen in modified Chee's medium fortified with 2 mM L-glutamine and 6.25 μg/ml transferrin, as described previously (Woodcroft and Novak, 1997, 1999), and supplemented with 0.1 μM dexamethasone and 1 μM insulin. Hepatocytes were plated at a density of 3×10^6 cells/60-mm dish for total RNA isolation and 10×10^6 cells/100-mm dish for microsome isolation, with three dishes per treatment. To examine the effects of ketone bodies on CYP2E1 gene expression without interference from insulin, which down-regulates CYP2E1 mRNA and protein levels (Woodcroft and Novak, 1997), the medium was replaced 4 h postplating with insulin-free medium for the duration of the experiment. Medium was changed every 24 h thereafter. Treatment with insulin and/or ketone bodies and kinase inhibitors was initiated 48 h after plating and continued for up to 48 h. Kinase inhibitors were dissolved in dimethyl sulfoxide (DMSO) and added 1.5 h before treatment with AA for 24 h. The final DMSO concentration in the medium ranged from 0.01 to 0.1%. DMSO concentrations up to 1% have no effect on CYP2E1 mRNA or on cell viability in primary cultured rat hepatocytes, relative to untreated hepatocytes. The RNA synthesis inhibitor actinomycin D (10 μg/ml) and the protein synthesis inhibitor cycloheximide (10 μg/ml) were added without replacement of medium 12 h after treatment of the hepatocytes with either medium alone (control) or AA. Treatment with AA was then continued following actinomycin D (0–10 h) and cycloheximide addition (0–12 h). The inhibitors were used at concentrations previously determined by our laboratory to be nontoxic based on the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide assay for mitochondrial succinate dehydrogenase activity (Woodcroft et al., 2002).

Northern Blot Analysis. Total RNA was isolated and Northern blot analysis was performed as described previously (Woodcroft and Novak, 1997, 1999). Northern blots were probed with CYP2E1 and cytoplasmic 7S cDNA probes for CYP2E1 mRNA and mRNA loading, respectively, as previously described (Woodcroft and Novak, 1997, 1999). Northern blot band densities were detected by enhanced chemiluminescence on Kodak X-OMAT film (Sigma-Aldrich) and quantified by densitometry using a Molecular Dynamics scanning laser densitometer and ImageQuant analysis program (Amersham Biosciences Corp.). CYP2E1 mRNA band densities were normalized for loading using the cytoplasmic 7S band densities. For CYP2E1 mRNA half-life calculation, first-order decay rate constants were derived and used to calculate half-life values CYP2E1 mRNA.

Generation of CYP2E1 Internal Standard for Heterogeneous Nuclear RNA Analysis. Heterogeneous nuclear RNA (hnRNA) analysis was performed as originally described previously (Elferink and Reiners, 1996) and adapted for CYP2E1 (Woodcroft et al., 2002). Genomic DNA (1 μg) was subjected to PCR on a PerkinElmer Cetus DNA Thermal Cycler using CYP2E1-specific forward and reverse primers (5′-CGGGATCCAGACACGGTCTATCTAAAGCA-3′ and 5′- CGGGATCCAGAGTGGGAAAATTAGCCCAC-3′, respectively) containing BamHI sites at the 5′ ends. An 887-bp fragment of CYP2E1 DNA corresponding to the last 61 bp of intron 1, all of exon 2 (coding region of CYP2E1 = amino acids 60–112), and the first 667 bp of intron 2 was isolated, BamHI-digested, ligated into BamHI-digested pBluescript KS+T7/A90, and transformed into JM109 cells. The resulting plasmid was digested with EcoRI and NcoI, which created a 75-bp internal deletion within intron 2. This construct was linearized with HindIII and in vitro transcribed.

Analysis of hnRNA. Deoxyribonuclease-treated total RNA (5 μg) and the CYP2E1 internal standard (750,000 copies) were reverse transcribed using an intron 2-specific CYP2E1 primer (5′-TCACCTAAAGCCTGGTGCGTT-3′) and amplified in the presence of [^{32}P]-dATP using CYP2E1-specific forward and reverse primers (5′-GGGGACATTCCTGTGTTCCAG-3′ and 5′-CAAGTAGAGTGCCAGGCAAGG-3′, respectively). The PCR products were separated on a 10% nondenaturing polyacrylamide gel with Tris-base/boric acid/EDTA running buffer for 6 h at 30 mA and visualized by exposure of the dried gel to X-ray film for 2 h. Band densities were determined using a Molecular Dynamics scanning laser densitometer and ImageQuant analysis program. Relative levels of CYP2E1 hnRNA band densities were normalized to the corresponding internal standard band densities. The internal standard is nearly identical to CYP2E1 hnRNA transcript. This similarity is of particular importance to ensure that both the internal standard RNA and CYP2E1 hnRNA transcripts are copied with equal efficiency during the reverse transcription reaction and the exponential phase of PCR (Elferink and Reiners, 1996). Thus, CYP2E1 hnRNA calculations are more meaningful after normalization to the internal standard, since it obviates errors that may arise due to differences in efficiencies in the reverse transcription reaction or PCR. The use of the internal standard for normalization of data are also helpful in comparison between different samples and different experiments.

Immunoblot Analysis. To determine the phosphorylation state of p70S6K and PKC, cell lysates were prepared by scraping the cells directly into 500 μl of Laemmli sample buffer (62.5 mM Tris-HCL, pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.1% bromphenol blue). Cell lysates (10–15 μl) were separated by SDS-polyacrylamide gel electrophoresis on 10% gels and electrophoretically transferred to nitrocellulose membrane (Bio-Rad). Membranes were blocked for 1 h in 5% milk powder in Tris-HCL buffered saline containing 0.05% Tween 20 (TBS-T). Membranes were then probed with phospho-specific antibodies (diluted 1:500 in 5% bovine serum albumin in TBS-T) overnight at 4°C, followed by incubation with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (diluted 1:2000 in 5% milk powder in TBS-T) or goat anti-mouse horseradish peroxidase-conjugated secondary antibody for GAPDH (diluted 1:20,000 in 5% milk powder in TBS-T). Proteins were detected by enhanced chemiluminescence on Kodak X-OMAT film (Sigma-Aldrich) and quantified by densitometry with a Molecular Dynamics scanning laser densitometer and ImageQuant analysis program (Amersham Biosciences Corp.). Following probing with phospho-specific antibodies, blots were stripped and reprobed for total levels of the respective kinases or GAPDH, and relative levels of phosphorylated kinases were normalized to the total levels of the respective kinase or GAPDH.

For CYP2E1 protein detection, microsomes were prepared from cultured rat hepatocytes as described previously (Woodcroft and Novak, 1997). Protein concentrations were determined using the bicinchoninic acid protein assay (Sigma-Aldrich). For immunoblot analysis, microsomal protein (20 μg/lane) was separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and blocked for 3 h in 5% milk powder in PBS-T (0.05% Tween 20 in phosphate-buffered saline). For immunodetection, blots were incubated overnight at 4°C with CYP2E1 antibody (diluted 1:1000 in 0.1% bovine serum albumin in PBS-T). The preparation of CYP2E1 antibody was described previously (Kim et al., 1991). This was followed by incubation of the membrane with rabbit anti-goat secondary antibody conjugated to horseradish peroxidase (diluted 1:5000 in 5% milk powder in PBS-T) for 3 h at room temperature. Proteins were detected by enhanced chemiluminescence and quantified by scanning laser densitometry as described above.

Polysomal Distribution Analysis. Polyribosome distribution of CYP2E1 mRNA in rat hepatocytes was performed as previously described (Johannes and Sarnow, 1998) with some modification. Cells were plated at a density of 10×10^6 cells/100 mm-dish, with four dishes per treatment. To harvest, cells were placed on ice, washed twice with ice-cold phosphate-buffered saline containing 0.1 mg/ml cycloheximide, and lysed directly on the plate following the addition of 400 μl of polysome extraction buffer (15 mM Tris-CL, pH 7.4, 15 mM $MgCl_2$, 0.3 M NaCl, 1% Triton X-100, 0.1 mg/ml cycloheximide, and 1 mg/ml heparin). All of the following procedures were performed on ice. Extracts for each treatment were combined into one Eppendorf tube. The samples were kept on ice for 10 min, mixed occasionally, and centrifuged at 10,000 rpm for 10 min to remove nuclei and debris. One milliliter of the supernatant was then placed onto 11 ml of a 10 to 60% sucrose gradient composed of extraction buffer with no Triton X-100. Samples were centrifuged at 36,000 rpm for 2 h in a Beckman SW40 Ti rotor (Beckman Coulter, Inc., Fullerton, CA) at 4°C. Gradients were collected as 0.8-ml fractions, using an ISCO fraction collection system (ISCO, Lincoln, NE), into disposable 15-ml conical tubes at 0.375 ml/min-flow rate and collection at ~144-s intervals. Fractions were adjusted to 2 ml with diethyl pyrocarbonate-treated H_2O, and 3 ml of 8 M guanidine HCl was added to the samples, which were vortexed for 2 min. A volume of 5 ml of 100% ethanol was then added to the samples, and the fractions were stored at −80°C overnight. The fractions were centrifuged at 12,000 rpm to for 25 min in a Beckman 25R Allegra centrifuge with a TA 10-250 rotor at 4°C. Pellets were washed with 70% ethanol and resuspended in 40 μl of Tris-Base/EDTA, pH 7.5. A volume of 156 μl of 3 M NaOAc and 1.39 ml of 100% ethanol was added to the samples, which were stored at −80°C overnight followed by recentrifugation. Pellets were washed with 70% ethanol and resuspended in 100 μl of Tris-Base/EDTA, pH 7.5. Equal volumes of fractions 3 through 14 were subjected to Northern blot analysis, and band densities were determined using a Molecular Dynamics scanning laser densitometer and the ImageQuant analysis program.

Statistical Analysis. Significant differences between groups were determined by analysis of variance followed by a multiple comparison test ($P < 0.05$). Statistical analysis was performed on duplicate or triplicate cell lysates from a single hepatocyte preparation. Reproducibility of results was confirmed in two to four separate hepatocyte preparations.

Results

We have previously reported that ketone bodies had no effect on CYP2E1 mRNA levels in the presence of 1 μM insulin in the medium for the duration of experiment (Zangar and Novak, 1997). We have also showed that the ketone body AA, but not 3HB, decreased CYP2E1 mRNA significantly following 24-h treatment in the absence of 1 μM insulin, compared with the untreated rat hepatocytes (Woodcroft et al., 2002). To address whether the variation in results between the two studies was mainly due to the presence of insulin, we examined the effects of AA on CYP2E1 mRNA

levels in the presence and absence of various concentrations of insulin (0–1 μM) (Fig. 1). Insulin decreased CYP2E1 mRNA significantly with maximal inhibition of ~80% monitored at 10 nM and 1 μM insulin (Fig. 1). AA (5 mM) in the absence of insulin significantly inhibited CYP2E1 mRNA levels by an ~50% relative to untreated hepatocytes (Fig. 1). In the presence of diabetic levels of insulin (0.1 nM), AA also inhibited CYP2E1 mRNA by an additional ~50%, compared with the corresponding 0.1 nM insulin-treated hepatocytes and by an ~75% relative to hepatocytes controls in the absence of insulin (Fig. 1). At higher concentrations of insulin (10 nM and 1 μM), AA was without any significant effect on CYP2E1 mRNA, compared with the corresponding insulin-treated hepatocytes (Fig. 1). Thus, AA inhibits CYP2E1 mRNA in the absence of insulin or in the presence of diabetic concentrations of insulin, whereas it exhibits no effects in the presence of higher concentrations of insulin. Because the basal level of CYP2E1 mRNA expression was markedly decreased (~50%) in the presence of 0.1 nM insulin, we investigated the effects of ketone bodies on CYP2E1 mRNA and protein levels in the absence of insulin in all subsequent experiments.

The concentration-dependent effects of AA on CYP2E1 mRNA expression in primary cultured rat hepatocytes were examined at 24 h, using concentrations of AA (2–5 mM) representative of those present in the plasma of diabetic patients. AA at 2 to 3 mM resulted in an ~30% decrease in CYP2E1 mRNA levels, whereas an ~50% decrease in CYP2E1 mRNA levels was monitored at 4 to 5 mM AA, relative to untreated hepatocytes (data not shown). Because 5 mM AA achieved the most significant suppression of CYP2E1 mRNA expression, we used this concentration for all subsequent experiments. The time course of AA effects on CYP2E1 mRNA expression was examined (Fig. 2). AA was without effect on CYP2E1 mRNA expression following treatment for 3 h, whereas there was an ~14, 34, 53, and 84% inhibition of CYP2E1 mRNA expression following treatment with AA for 6, 12, 24, and 48 h, respectively, relative to untreated hepatocytes (Fig. 2). These data show that AA, at diabetic insulin concentrations, suppressed CYP2E1 mRNA levels in a concentration-and time-dependent manner. Because the results following 24-h treatment with AA were significantly different, we used this time period for all subsequent experiments.

We next investigated the signaling pathway(s) that may be involved in mediating the AA suppression of CYP2E1 mRNA levels. Our laboratory has shown that the PI 3-K signaling pathway mediates the insulin-dependent decrease in CYP2E1 mRNA levels (Woodcroft et al., 2002). In addition, the PI 3-K signaling pathway has been implicated in ERK1/2 phosphorylation by fumarylacetoacetate in Chinese hamster V79 cells (Jorquera and Tanguay, 2001). The PI 3-K signaling pathway proceeds through the Akt/mTOR and the p70S6K (Asnaghi et al., 2004). Thus, we investigated whether PI 3-K plays a role in the AA-mediated decrease in CYP2E1 mRNA levels (Fig. 3A). Primary cultured rat hepatocytes were treated with the PI 3-K inhibitors, LY294002 (10 or 20 μM), or wortmannin (100 or 500 nM) 1.5 h before AA treatment (Fig. 3A). LY294002 ameliorated the decrease in CYP2E1 mRNA levels resulting from treatment with AA

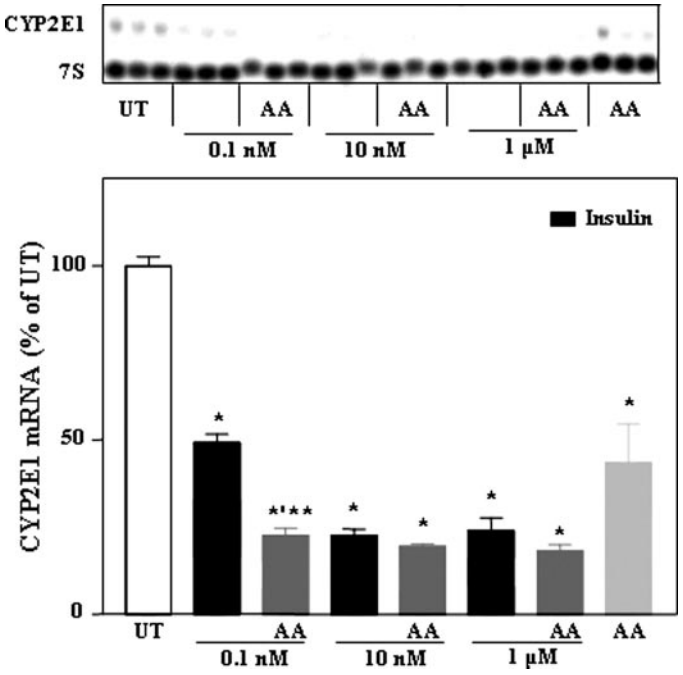

Fig. 1. The effects of AA on CYP2E1 mRNA expression in the absence or presence of insulin. Hepatocytes were treated with insulin (0–1 μM) in the absence or presence of 5 mM AA for 24 h. Untreated hepatocytes (UT) were cultured in the absence of insulin or AA. CYP2E1 mRNA levels were monitored by Northern blot analysis, and band density was normalized to 7S RNA. CYP2E1 mRNA levels are plotted as a percentage of the CYP2E1 mRNA levels monitored in untreated hepatocytes. Data are means ± S.E.M. of Northern blot band densities of three preparations of total RNA. *, significantly different from UT; **, significantly different from corresponding hepatocytes treated with insulin only ($P < 0.05$).

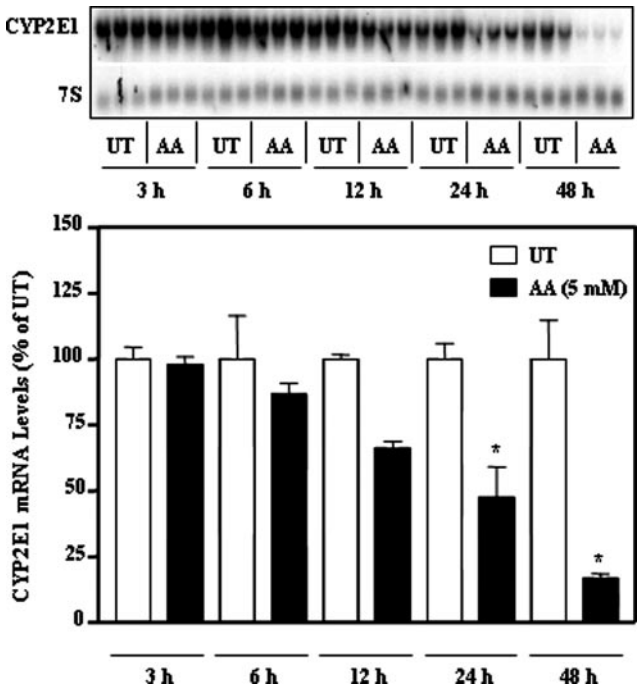

Fig. 2. Time-dependent effects of AA on CYP2E1 mRNA expression. Hepatocytes were treated with AA (5 mM) for various time periods (3–48 h). Untreated hepatocytes (UT) were cultured in the absence of AA. CYP2E1 mRNA levels were monitored by Northern blot analysis, and band density was normalized to 7S RNA. CYP2E1 mRNA levels are plotted as a percentage of the CYP2E1 mRNA levels monitored in untreated hepatocytes. Data are means ± S.E.M. of Northern blot band densities of three preparations of total RNA. *, significantly different from UT ($P < 0.05$).

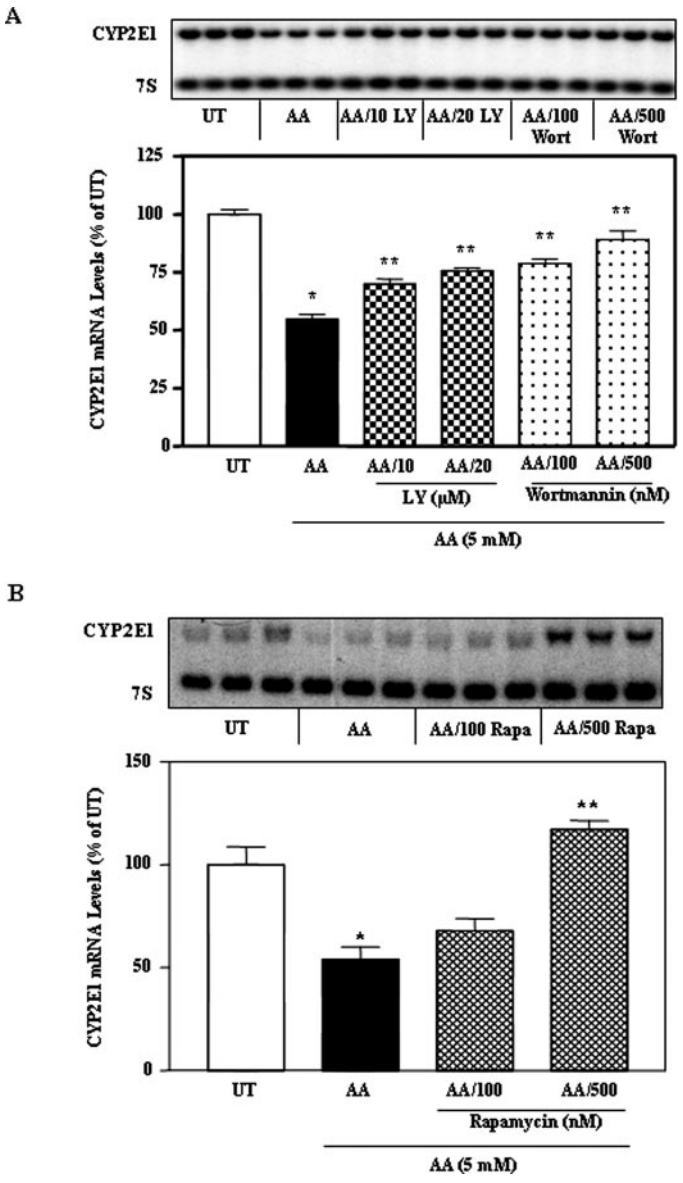

Fig. 3. The effects of the PI 3-K inhibitors, LY294002 and wortmannin, and the mTOR inhibitor rapamycin on the AA-mediated decrease in CYP2E1 mRNA levels. Hepatocytes were treated with the PI 3-K inhibitors LY294002 (LY) (10–20 μM) or wortmannin (Wort) (100–500 nM) (A) or with the mTOR inhibitor rapamycin (Rapa) (100–500 nM) (B) for 1.5 h before treatment with 5 mM AA for 24 h. Untreated hepatocytes (UT) were cultured in the absence of AA, PI 3-kinase inhibitors, and mTOR inhibitor. CYP2E1 mRNA levels were monitored by Northern blot analysis, and band density was normalized to 7S RNA. CYP2E1 mRNA levels are plotted as a percentage of the CYP2E1 mRNA levels monitored in untreated hepatocytes. Data are means ± S.E.M. of Northern blot band densities of three preparations of total RNA. *, significantly different from UT; **, significantly different from hepatocytes treated with AA only ($P < 0.05$).

(Fig. 3A). The inhibition of the AA-mediated decrease in CYP2E1 expression in cells pretreated with LY294002 was dose-dependent with an ~1.3- or ~1.4-fold increase in CYP2E1 mRNA monitored relative to AA-treated cells, in response to 10 or 20 μM LY294002, respectively (Fig. 3A). LY294002 alone was without significant effect on CYP2E1 mRNA levels (not shown). Pretreatment of cells with wortmannin also revealed a dose-dependent amelioration of the AA-mediated decrease in CYP2E1 mRNA expression (Fig. 3A). Wortmannin at 100 or 500 nM produced an ~1.5- or ~1.8-fold increase, respectively, in CYP2E1 mRNA levels relative to AA-treated cells (Fig. 3A). Wortmannin alone (100 nM) was without significant effect on CYP2E1 mRNA levels, whereas 500 nM wortmannin slightly increased CYP2E1 mRNA levels ~1.3-fold (not shown). These data suggest that the PI 3-K signaling is involved in the AA-mediated suppression of CYP2E1 expression. To determine whether the downstream Akt/mTOR target plays a role in AA-mediated suppression of CYP2E1 mRNA expression, primary cultured rat hepatocytes were treated with the mTOR inhibitor rapamycin (500 nM) prior to AA treatment (Fig. 3B). Rapamycin inhibited the AA-mediated decrease in CYP2E1 mRNA levels by an ~124 and 200% at 100 and 500 nM, respectively (Fig. 3B). The inhibition of the AA-mediated decrease in CYP2E1 expression in cells pretreated with rapamycin was dose-dependent, although the maximal effect on CYP2E1 mRNA occurred with 500 nM rapamycin, with CYP2E1 mRNA achieving levels comparable with those present in untreated cells (Fig. 3B). Rapamycin alone was without any significant effect on CYP2E1 mRNA expression (not shown). Together, these data suggest that the PI 3-K/Akt/mTOR/p70S6K signaling pathway plays a significant role in mediating the inhibitory effects of AA on CYP2E1 mRNA expression.

The p70S6K is a well established downstream effector of PI 3-K signaling pathway (Asnaghi et al., 2004) and is a good indicator of PI 3-K signaling activation. To verify that AA activates the PI 3-K/Akt/mTOR/p70S6K signaling pathway, we examined the effect of AA on p70S6K phosphorylation and the effects of both of the PI 3-K inhibitors, LY294002 (20 μM), and wortmannin (500 nM), and the mTOR inhibitor rapamycin (500 nM) on the AA-mediated activation of p70S6K following 6-h treatment (Fig. 4, A and B). As shown in Fig. 4, A and B, AA increased p70S6K phosphorylation by ~2-fold and that LY294002 and wortmannin (Fig. 4A) as well as rapamycin (Fig. 4B) completely prevented the activation of p70S6K in response to AA. To determine the time-dependent effects of AA on p70S6K activation, cells were treated with AA for various time intervals (3–24 h) (Fig. 4C). Although AA was without effect on the phosphorylation of p70S6K at 3 h, the phosphorylation of p70S6K increased by ~2-fold at 6-h treatment (Fig. 4C). The activation of p70S6K in response to AA was maintained up to 9 h and declined to ~1.5-fold at 24 h (Fig. 4C). Taken together, these data demonstrate for the first time that AA activates p70S6K, which is a downstream target of PI 3-K and mTOR signaling and that activation of this signaling pathway by AA regulates CYP2E1 mRNA expression.

The activation of PI 3-K may also result in the activation of PKC (Campbell et al., 2004), although PKC activation may also occur independent of this pathway. We have reported that PKC plays a role in the AA-mediated activation of ERK1/2 and p38 MAPK in primary cultured rat hepatocytes (Abdelmegeed et al., 2004), suggesting a role for PKC activation in response to AA. In this study, we wanted to confirm that AA activates PKC in primary cultured rat hepatocytes by directly examining its effect on PKC (Ser662) phosphorylation and by examining the effect of both the broad spectrum PKC inhibitor bisindolylmaleimide (10 μM) and the PI 3-K inhibitors LY294002 (20 μM) and wortmannin (500 nM) on AA-mediated activation of PKC following 6-h treatment (Fig. 5, A and B). As illustrated in Fig. 5, A and B, AA increased PKC (Ser662) phosphorylation by ~2.5-fold. In addition, our

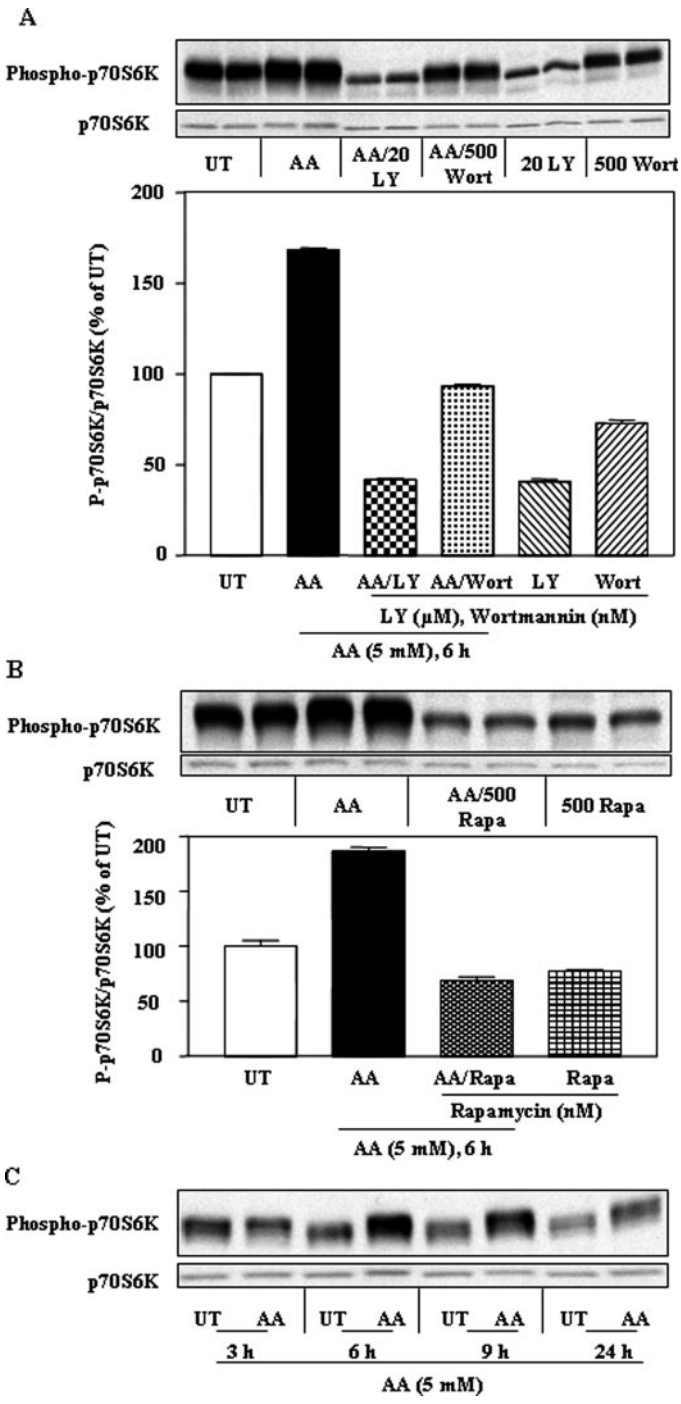

Fig. 4. The role of PI 3-K and mTOR in the activation of p70S6K by AA. Hepatocytes were treated with the PI 3-K inhibitors LY294002 (LY) (20 μM) or wortmannin (Wort) (500 nM) (A) or with the mTOR inhibitor rapamycin (Rapa) (500 nM) (B) for 1.5 h before treatment with 5 mM AA for 6 h, or with AA for various time intervals (3–24 h) (C). Untreated hepatocytes (UT) were cultured in the absence of AA, PI 3-K inhibitors, and mTOR inhibitor. Phosphorylation of p70S6K was monitored by immunoblot analysis, and band density was normalized to total p70S6K. Data are means ± range of phospho-p70S6K band densities from two preparations of cell lysate.

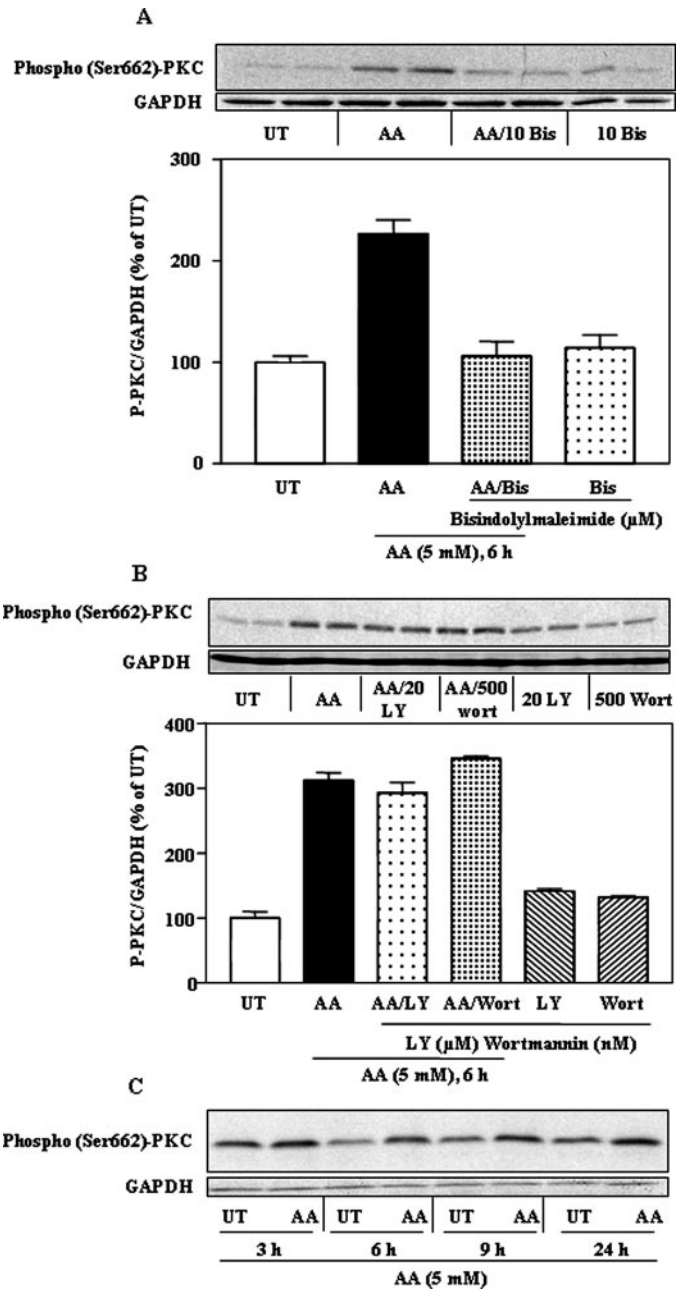

Fig. 5. The role of PI 3-K in the activation of PKC by AA. Hepatocytes were treated with the PKC inhibitor bisindolylmaleimide (Bis) (10 μM) (A) or with the PI 3-K inhibitors LY294002 (LY) (20 μM) or wortmannin (Wort) (500 nM) (B) for 1.5 h before treatment with 5 mM AA for 6 h, or with AA for various time intervals (3–24 h) (C). Untreated hepatocytes (UT) were cultured in the absence of AA, PKC inhibitor, and PI 3-K inhibitors. Phosphorylation of PKC was monitored by immunoblot analysis, and band density was normalized to GAPDH. Data are means ± range of phospho-PKC band densities from two preparations of cell lysate.

data shows that whereas 10 μM bisindolylmaleimide, at a concentration sufficient to inhibit PKCα, βI, δ, ϵ, and ζ, completely prevented the activation of PKC by AA (Fig. 5A), neither LY294002 nor wortmannin inhibited the AA-mediated activation of PKC (Fig. 5B), suggesting that PKC activation is not occurring through the PI 3-K signaling cascade. To determine the time-dependent effects of AA on PKC activation, cells were treated with AA for various time intervals (3–24 h) (Fig. 5C). AA was without effect on the phosphorylation of PKC at 3 h while increasing PKC phosphorylation by ~2.5-fold at 6 h (Fig. 5C). The activation of PKC in response to AA was maintained up to 9 h and declined to ~2-fold at 24 h (Fig. 5C). Taken together, these data show

that AA activates PKC and that this activation occurs independently of the activation of the PI 3-K signaling pathway.

We next investigated whether PKC plays a role in the AA-mediated decrease in CYP2E1 levels (Fig. 6). Bisindolylmaleimide (5 μM) inhibited the AA-mediated suppression of CYP2E1 mRNA levels by ~18%, which was not significant (Fig. 6). Bisindolylmaleimide at 10 μM, however, resulted in a significant 61% inhibition of the AA-mediated decrease in CYP2E1 mRNA levels, achieving 89% of the CYP2E1 mRNA levels monitored in untreated hepatocytes (Fig. 6). Bisindolylmaleimide alone was without significant effect on CYP2E1 mRNA levels (not shown). Thus, these results support the conclusion that PKC plays a role in effecting the AA suppression of CYP2E1 mRNA expression.

We next investigated the possible role of Ras/Raf/MEK/ERK1/2 or p38 MAPK signaling pathways in mediating AA effects on CYP2E1 mRNA levels (Table. I). Our data revealed that neither the mitogen-activated kinase kinase (MEK) inhibitor PD98059 at concentrations sufficient to inhibit MEK1/2 (Abdelmegeed et al., 2004), nor SB203580 at concentrations well established to inhibit p38 MAPK activation, ameliorated the AA-mediated suppression of CYP2E1 mRNA levels (Table 1). Rather, the inhibitors PD98059 and SB203580 seemed to promote further the AA-mediated suppression of CYP2E1 mRNA levels (Table 1). Both PD98059 (100 μM) and SB203580 (20 μM) alone, decreased CYP2E1 mRNA levels by ~20 to 25%, respectively (Table 1), which may be due to the release of the opposing effects on the basal activity levels of PI 3-K signaling pathway. These results, however, suggest that neither ERK1/2 nor p38 MAPK signaling pathways are involved in the AA-mediated inhibition of CYP2E1 expression.

TABLE 1

Effect of PD98059 and SB203580 on AA-mediated decrease in CYP2E1 mRNA levels

Forty-eight hours after plating, hepatocytes were treated with PD98059 (50–100 μM) or SB203580 (10–20 μM) for 1.5 h before treatment for 24 h with 5 mM AA. UT hepatocytes were cultured in the absence of AA and PD98059 and SB203580 compounds. CYP2E1 mRNA levels were monitored by Northern blot analysis, and band density was normalized to 7S RNA. CYP2E1 mRNA levels are plotted as a percentage of the CYP2E1 mRNA levels monitored in untreated hepatocytes. Data are means ± S.E.M. of Northern blot band densities of three preparations of total RNA.

	CYP2E1 mRNA Levels
	% of UT
UT	100 ± 4.2
AA	55 ± 2.1*
5 AA/50 PD	54 ± 1.4*
5 AA/100 PD	39 ± 4.1*
100 PD	80 ± 1.5*
5 AA/10 SB	37 ± 2.2*
5 AA/20 SB	34 ± 1.3*
20 SB	73 ± 1.1*

PD, PD98059; SB, SB203580.
* Significantly different from levels monitored in UT ($P < 0.05$).

The decline of CYP2E1 mRNA levels in response to AA could be the result of two molecular mechanisms: 1) inhibition of CYP2E1 gene transcription and/or 2) enhancement of CYP2E1 mRNA degradation. We have reported previously that the decrease in CYP2E1 mRNA in response to insulin was associated with the concerted action of inhibition of CYP2E1 gene transcription and increased CYP2E1 mRNA degradation (Woodcroft et al., 2002). Thus, studies on both mechanisms were pursued.

To address the effect of AA on CYP2E1 gene transcription in primary cultured rat hepatocytes, analysis of CYP2E1 hnRNA (a nascent unspliced RNA), was used (Fig. 7). AA-treated hepatocytes exhibited an ~50% suppression of

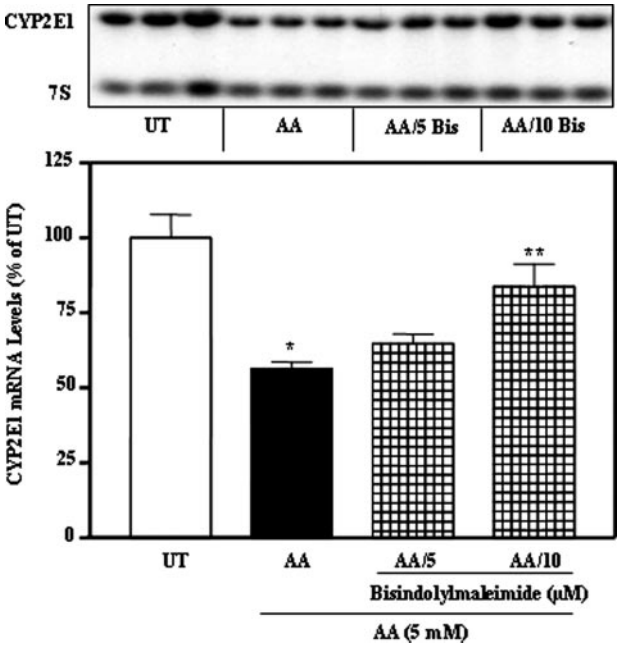

Fig. 6. The effect of the PKC inhibitor bisindolylmaleimide (Bis) on the AA-mediated decrease in CYP2E1 mRNA levels. Hepatocytes were treated with the PKC inhibitor bisindolylmaleimide (5–10 μM) for 1.5 h before treatment with 5 mM AA for 24 h. Untreated hepatocytes (UT) were cultured in the absence of AA and PKC inhibitor. CYP2E1 mRNA levels were monitored by Northern blot analysis, and band density was normalized to 7S RNA. CYP2E1 mRNA levels are plotted as a percentage of the CYP2E1 mRNA levels monitored in untreated hepatocytes. Data are means ± S.E.M. of Northern blot band densities of three preparations of total RNA. *, significantly different from UT; **, significantly different from hepatocytes treated with AA only ($P < 0.05$).

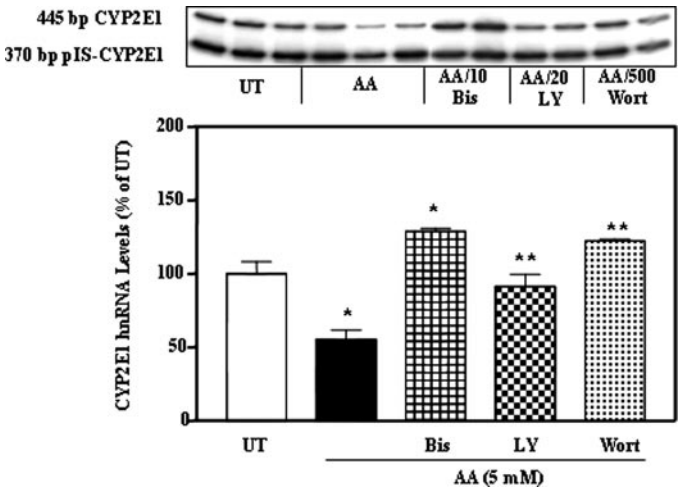

Fig. 7. The effect of AA on CYP2E1 gene transcription. Hepatocytes were treated with PI 3-K inhibitors, LY294002 (LY) (10–20 μM) or Wortmannin (Wort) (100–500 nM), or the PKC inhibitor bisindolylmaleimide (Bis) (10 μM), for 1.5 h before treatment for 24 h with 5 mM AA. Untreated hepatocytes (UT) were cultured in the absence of AA, PI-3 kinase inhibitors, and the PKC inhibitor. CYP2E1 gene transcription was monitored by hnRNA analysis, and band density was normalized to the 370-bp CYP2E1 internal standard. CYP2E1 hnRNA levels are plotted as a percentage of the CYP2E1 hnRNA levels monitored in untreated hepatocytes. Data are means ± S.E.M. of band densities of two or three preparations of total RNA. *, significantly different from UT; **, significantly different from hepatocytes treated with AA only ($P < 0.05$).

CYP2E1 gene transcription compared with untreated cells (Fig. 7). We also examined the role of PI 3-K and PKC signaling pathways in mediating the AA inhibitory effect on CYP2E1 gene transcription (Fig. 7). The treatment of cells with PI 3-K inhibitors LY294002 (20 μM) or wortmannin (500 nM) prior to the addition of AA resulted in an ~180% (LY294002) or complete prevention (wortmannin), respectively, of the AA-mediated suppression of CYP2E1 gene transcription (Fig. 7). In addition, when hepatocytes were pretreated with 10 μM bisindolylmaleimide, there was also a complete abrogation of the AA inhibitory effect on CYP2E1 gene transcription relative to that monitored in AA-treated cells (Fig. 7). These results, in combination with the results obtained from Figs. 2 and 3, suggest that AA significantly inhibits CYP2E1 gene transcription and that both the PI 3-K/Akt/mTOR/p70S6K and PKC signaling pathways play a role in mediating this effect.

The effect of AA on CYP2E1 mRNA degradation was examined to explore the role of this process in the AA regulation of CYP2E1 mRNA levels (data not shown). The RNA synthesis inhibitor actinomycin D (10 μg/ml) was added to hepatocytes 12 h following treatment with medium alone (UT) or with AA, and cells were harvested following the addition of actinomycin D at various time intervals (0–10 h). Interestingly, the CYP2E1 mRNA half-life slightly increased from ~24 h in untreated cells to ~32 h in AA-treated cells. Thus, the decrease in CYP2E1 mRNA levels in response to AA is not a result of enhanced CYP2E1 mRNA degradation.

We examined the effects of AA and 3HB on CYP2E1 protein levels. Interestingly, our results show that AA (5 mM) following 24-h treatment increased CYP2E1 protein levels by ~2-fold, whereas 3HB (5 mM) was without effect, relative to untreated cells (not shown). In addition, the time course of AA on the expression of CYP2E1 protein was investigated. As illustrated in Fig. 8, AA was without significant effect on CYP2E1 protein levels up to 6 h, whereas AA induced CYP2E1 protein expression by ~1.3-, 2-, and 2.5-fold at 12, 24, and 48 h, respectively, compared with untreated hepatocytes (Fig. 8). These data show that AA induces CYP2E1 protein levels and that this increase in protein levels parallels the time course for the AA-mediated depletion of CYP2E1 mRNA. Given the AA-mediated decrease in CYP2E1 gene transcription, this suggests that the existing cytoplasmic stores of CYP2E1 mRNA may be used in translation. Thus, the increase on CYP2E1 protein levels in response to AA could be associated with two molecular mechanisms: 1) an increase in the translation of the existing CYP2E1 mRNA and/or 2) a decrease in the CYP2E1 protein degradation.

Because the elevation of CYP2E1 protein following AA treatment at 24 h was clearly significant, we used this time period for all subsequent experiments. To address whether AA may actually increase the translation of CYP2E1 mRNA, we examined the effect of AA treatment on the polysomal distribution of CYP2E1 mRNA in primary cultured rat hepatocytes (Fig. 9). AA treatment caused a shift in the polysomal distribution of CYP2E1 mRNA relative to untreated hepatocytes (Fig. 9A). The proportion of RNA associated with the actively translated polysomal fractions (fractions 8 to 14) versus the 40S to 60S untranslated stored fractions (fractions 3 to 7) was increased by ~40% in AA-treated hepatocytes relative to untreated cells (Fig. 9B). These data suggest that

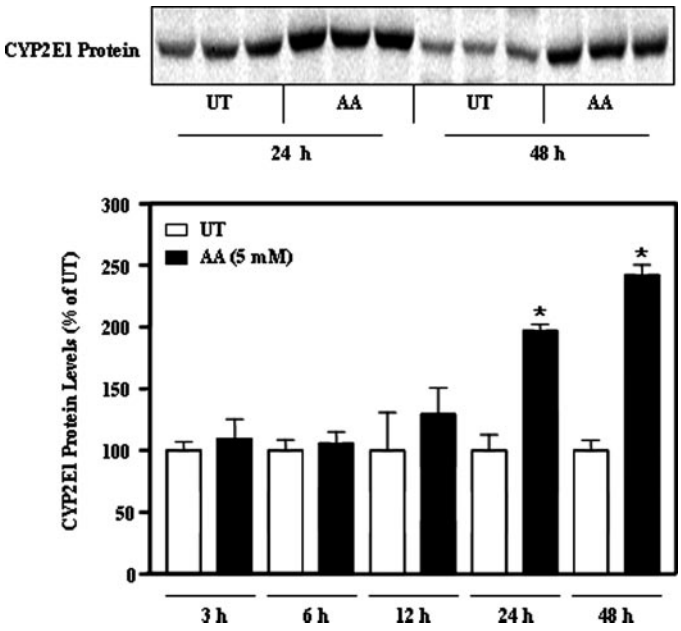

Fig. 8. AA effect on CYP2E1 protein expression. Hepatocytes were treated with AA (5 mM) for various time periods (3–48 h). Untreated hepatocytes (UT) were cultured in the absence of AA. Microsomes were isolated, and CYP2E1 protein levels were monitored by immunoblot analysis. CYP2E1 protein levels are plotted as a percentage of the CYP2E1 protein levels monitored in untreated hepatocytes. Data are means ± S.E.M. of Western blot band densities of three preparations of microsomes. *, significantly different from UT ($P < 0.05$).

the AA-mediated increase in CYP2E1 protein levels is due to, at least in part, an increase in the translation of CYP2E1 mRNA in primary cultured rat hepatocytes.

We investigated the effect of AA on microsomal CYP2E1 protein degradation (Fig. 10). The protein synthesis inhibitor cycloheximide (10 μg/ml) was added to hepatocytes 12 h following treatment with AA or medium alone (UT) (Fig. 10). Cells were then harvested following the cycloheximide addition every 4 h for 12 h, for a total period of 24 h following AA treatment (Fig. 10). AA decreased the rate of microsomal CYP2E1 degradation compared with untreated cells with the half-life for AA-treated cells ~24 h compared with ~8 h monitored in untreated cells (Fig. 10). Thus, the decreased CYP2E1 protein degradation in response to AA may also play a role in the AA-mediated induction of CYP2E1 protein levels. Taken together, the AA-mediated induction of CYP2E1 protein levels seems to be the result of enhanced CYP2E1 translation and decreased CYP2E1 protein degradation.

Discussion

Hyperketonemia plays a role in the induction of CYP2E1 in diabetes (Dong et al., 1988) and in cellular oxidative stress (Jain et al., 2003; Abdelmegeed et al., 2004). Previously, we showed that the addition of ketone bodies (AA or 3HB) to primary cultured rat hepatocytes was without effect on CYP2E1 mRNA or protein levels (Zangar and Novak, 1997). However, the high concentration of insulin (1 μM) used in this study, was found to markedly lower CYP2E1 mRNA and protein levels (Woodcroft and Novak, 1997; Woodcroft et al., 2002). Woodcroft et al. (2002) reported that AA, but not 3HB, significantly decreased CYP2E1 mRNA in the absence of insulin. In this study, we showed that the presence of high

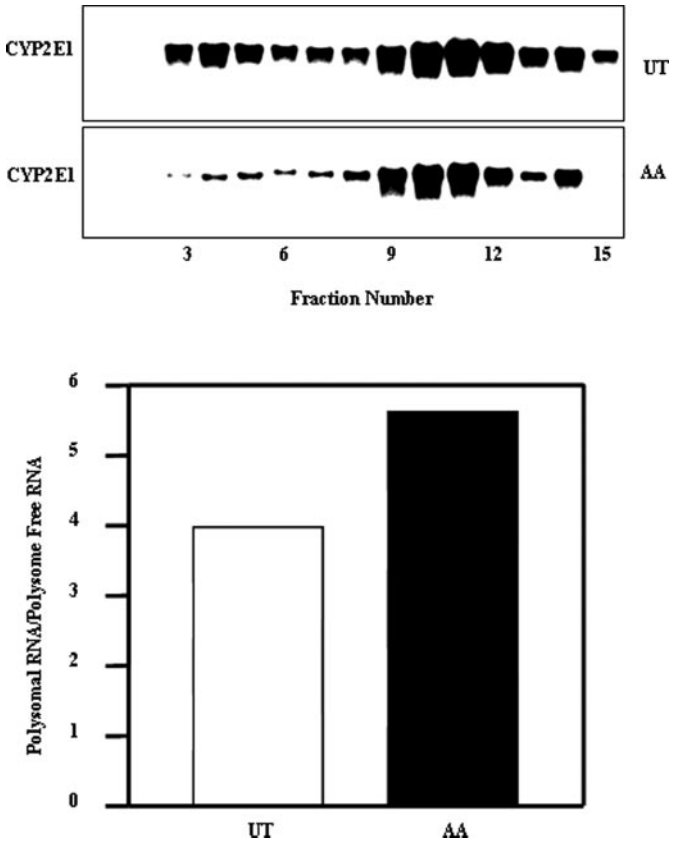

Fig. 9. The effect of AA on polyribosomal distribution of CYP2E1 mRNA. Hepatocytes were either untreated (UT) or treated with AA (5 mM) for 24 h. A, representative autoradiograph showing polyribosomal distributions of CYP2E1 mRNA following fractionation on sucrose density gradients, as monitored by Northern blot analysis. B, ratio of CYP2E1 mRNA associated with polysomal fractions (fractions 8 to 14) relative to that associated with 40S to 60S untranslated fractions (fractions 3 to 7). The data presented in B represent the mean values monitored in two separate experiments.

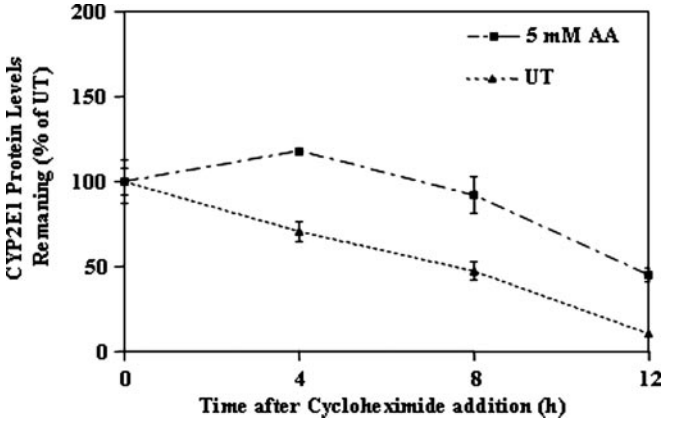

Fig. 10. The effect of AA on CYP2E1 protein degradation. Hepatocytes were either treated with medium alone (UT) (▲) or AA (5 mM) (■). Cycloheximide (10 μg/ml) was added to the two groups of hepatocytes (UT and AA) 12 h following the treatment of cells without change of medium. Microsomes were isolated at various time intervals (0–12 h) following cycloheximide addition. CYP2E1 protein levels were monitored by Western blot analysis. CYP2E1 protein levels are plotted as a fraction of beginning (t = 0) CYP2E1 protein levels for each treatment. Data are means ± S.E.M. of Western blot band densities of three preparations.

concentrations of insulin completely abolish the AA-mediated inhibition of CYP2E1 mRNA levels in primary cultured rat hepatocytes. However, cells treated with a diabetic concentration of insulin (0.1 nM) still exhibited a significant inhibition of CYP2E1 mRNA expression in response to AA. Thus, the difference in the results between the two studies is associated with differences in the insulin concentrations in the media. To examine the singular effects of ketone bodies on CYP2E1 mRNA and protein expression, and because the inhibition of CYP2E1 mRNA by AA in the absence of insulin and in the presence of diabetic concentrations of insulin was comparable, we examined the effects of AA on CYP2E1 mRNA and protein in the absence of insulin.

Our results show that the ketone body AA, but not 3HB, decreased CYP2E1 mRNA levels in a dose- and time-dependent manner. Figure 11 presents an abbreviated diagram for AA activation of signaling pathways and components and the inhibitors that were used to assess the role of individual components in the pathway. We have previously reported that AA produced oxidative stress via the Ras-Raf/MEK-ERK pathway and the p38 MAPK signaling pathways (Abdelmegeed et al., 2004). The present study focused on identification of the pathway and components that regulate *CYP2E1* gene expression. Akt regulates gene transcription and, through mTOR, regulates mRNA translation. Both Akt and PKC have been shown to be active in the phosphorylation of transcription factors and to regulate their transcriptional activity. The AA-mediated CYP2E1-mRNA inhibition is primarily due to inhibition of CYP2E1 gene transcription through PI 3-K/Akt/mTOR/p70S6K and PKC, but not ERK1/2 or p38 MAPK signaling pathways. We also provide evidence that AA activation of PKC is independent of the PI 3-K signaling pathway in this process. The transcriptional regulation of CYP2E1 in response to AA has not been established. The hepatocyte-specific transcription factor (HNF)-1α binding element at the 5′-end of *CYP2E1* gene is the only well characterized element in the rat hepatic *CYP2E1* gene (Liu and Gonzalez, 1995). Elements upstream of the HNF-1α binding site have been reported to negatively regulate CYP2E1 gene transcription (Liu and Gonzalez, 1995). Recently, Cheng et al. (2003) have suggested a possible role of the combined effects of HNF-1α, HNF-3β, and HNF-4α on the decreased DNA binding activity, as well as the activation of negative regulatory factors such as nuclear factor κ-B in the rapid suppression of CYP2E1, CYP2C11, and CYP3A2 in the liver of rats treated with endotoxin. In support of the idea that the regulation of CYP2E1 transcription seems to be complex, Abdel-Razzak et al. (2004) have determined an interleukin 4 (IL-4)-responsive region in the CYP2E1 gene promoter in human hepatocytes. The authors showed that the induction of CYP2E1 by IL-4 was not dependent on a single binding site, but rather on a complex region which includes putative binding sites for signal transducer and activator of transcription (STAT6), activator protein 1, NF-κB, nuclear factor of activated T cells and CCAAT-enhancer binding protein (Abdel-Razzak et al., 2004). PI 3-K and PKC signaling pathways have previously been implicated in the inhibition of gene transcription, possibly through modulation of transcription factors (Cheng et al., 2000; Nowak et al., 2005). In addition, PI 3-K and PKC signaling pathways have been reported to regulate CYP2E1 gene transcription in response to insulin or IL-4 in primary cultured rat hepatocytes or HepG2 cells, respectively (Woodcroft et al., 2002; Abdel-Razzak et al., 2004). Abdel-Razzak et al. (2004) suggested that

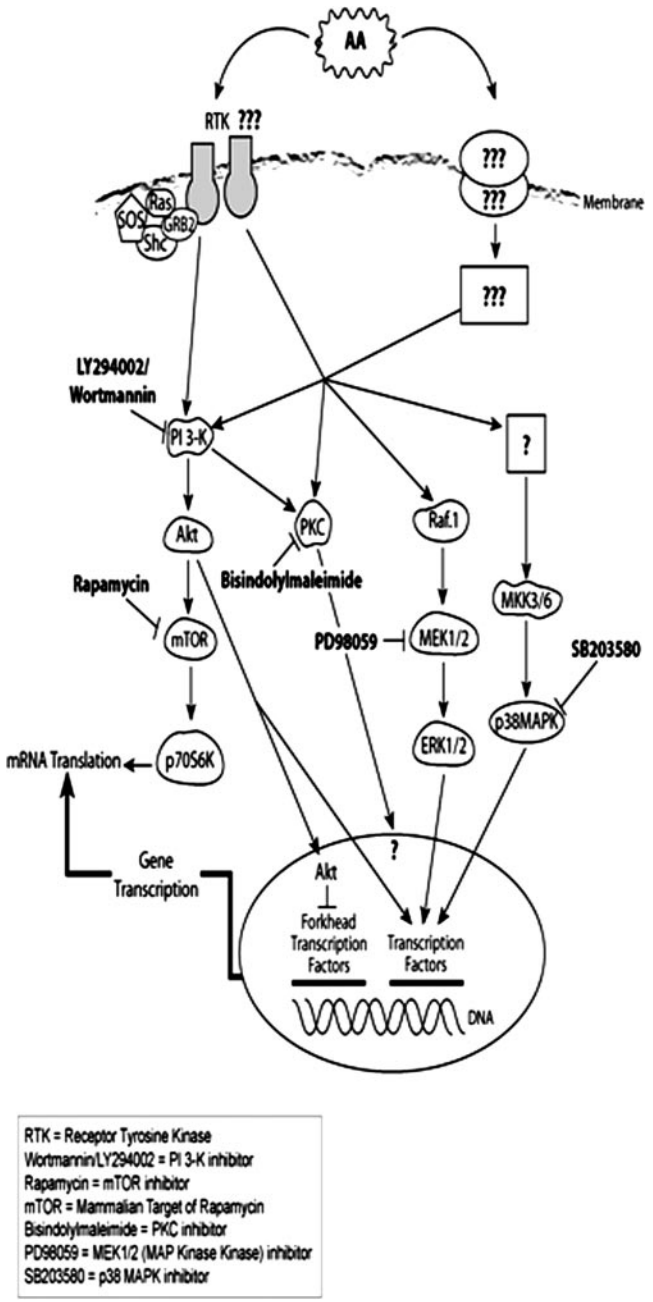

Fig. 11. An abbreviated diagram showing AA activation of intracellular signaling pathways and components in primary cultured rat hepatocytes and effects of inhibitors on the signal components. AA may either bind to or activate a receptor of the large receptor tyrosine kinase family or other membrane protein(s) that activate the intracellular signaling cascade.

there could exist a coordination between the transcription factors and targets of the PKC signaling pathway to regulate CYP2E1 gene transcription in HepG2 cells. Although HepG2 responded to IL-4 (Abdel-Razzak et al., 2004), HepG2 cells are not the best model to examine CYP2E1 gene transcription because they are tumor cells and also lack basal or inducible CYP2E1 activity. Thus, one possible mechanism is that these signaling kinases regulate the transcription factor and/or cofactors involved in cytochrome P450 gene transcription by phosphorylation. Indeed, Guo et al. (2003) and Carriere et al. (2001) have reported that phosphorylation state plays a significant role in the transcriptional activity of HNF1α and HNF4α.

AA-treated cells exhibited a marked inhibition of transcription, with a slightly increased CYP2E1 mRNA half-life. Evidence exists that the transcriptional and post-transcriptional events are linked through multifunctional proteins to ensure the efficient control of this multistep process. For example, it has been reported that the inhibition of CYP2A5 gene transcription resulted in relocalization of a heterogeneous nuclear ribonucleoprotein A1, a multifunctional protein involved in various stages of mRNA metabolism, from the nucleus to the cytoplasm, binding to the CYP2A5 mRNA 3′UTR, and stabilizing the transcript, most likely by controlling the length of its poly(A) tail (Glisovic et al., 2003). Although we did not find potential heterogeneous nuclear ribonucleoprotein A1 binding sites in the proximal CYP2E1 promoter, we cannot exclude the presence of yet unidentified multifunctional protein(s), which can serve the same function.

The induction of CYP2E1 protein is not always accompanied by a corresponding increase in CYP2E1 mRNA levels. Increased CYP2E1 protein levels in response to various nitrogen- and sulfur-containing heterocycles was accompanied by a decrease in poly(A) mRNA (Kim and Novak, 1993). One possible explanation for the discrepancy between CYP2E1 mRNA and protein levels is that CYP2E1 is regulated at the post-transcriptional level. Indeed, our results suggest that although AA decreased CYP2E1 mRNA levels, AA, but not 3HB, induced CYP2E1 protein in a time-dependent manner in primary cultured rat hepatocytes. Studies that used a series of 5′- and 3′-untranslated region constructs suggested a role for translational control in the regulation of CYP2E1 expression (Kocarek et al., 2000). Cytosolic RNAs are retained in protein complexes, and some of them have been implicated in translational regulation (Wu and Bag, 1998). It has been reported that PI 3-K/Akt/mTOR and PKC signaling pathways might play a role in increased translational efficiency through these RNA complexes (Angenstein et al., 2002; Wu et al., 2005). Clearly, Akt plays a major role in the regulation of translation through eukaryotic initiation factor 4E (eIF4E)/ binding protein (4EBP1) (Shen et al., 2005). Thus, it is plausible that PI 3-K and PKC signaling are involved in AA-mediated CYP2E1 mRNA translation.

Little is known about CYP2E1 protein degradation mechanism(s). The decreased turnover of CYP2E1 protein seems to occur mainly through the interaction of the substrate with the protein, changing its configuration and, hence, its identification by the proteosome; however, this is not always the case (reviewed in Novak and Woodcroft, 2000). PI 3-K and PKC have been reported to play a role in protein stabilization (Yamauchi et al., 2003; Zhou et al., 2004). Whether these signaling pathway(s) play a role in stabilizing CYP2E1 protein directly or through interaction with a third party protein(s) remains to be established. Because CYP2E1 metabolism can result in formation of reactive metabolites, and CYP2E1 has been implicated in the generation of tissue-damaging hydroxyl radicals in diabetes and liver disease (Leclercq et al., 2000; Lieber et al., 2004), the AA-mediated increase in CYP2E1 protein levels in conjunction with the AA-induced oxidative stress (Abdelmegeed et al., 2004) represents a substantial risk factor for hepatic damage.

The effects of the two major ketone bodies AA and 3HB on

CYP2E1 mRNA and protein levels are distinct. It has been suggested that the distinctive actions of AA compared with 3HB may be related to the presence of the 2-keto group in AA (Jain and McVie, 1999), although no clear understanding of the differences has been established.

In summary, the present study shows that the AA-mediated decrease in CYP2E1 mRNA levels is due to the inhibition of CYP2E1 gene transcription in primary cultured rat hepatocytes through PI 3-K/Akt/mTOR/p70S6K and PKC signaling events, whereas the AA-mediated increase in CYP2E1 protein levels is due to a concerted action of increased CYP2E1 translation and decreased protein degradation. These studies illustrate the complex mechanisms that regulate CYP2E1 gene expression in response to ketone bodies.

Acknowledgments

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