Acetoacetate Induces Cytochrome P450 (CYP) 2E1 Protein and Suppresses CYP2E1 mRNA in Primary Cultured Rat Hepatocytes

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Running Title: CYP2E1 Regulation by Ketone Bodies

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List of abbreviations: AA, lithium acetoacetate; 3HB, DL-β-hydroxybutyrate; PI 3-K, phosphatidylinositol 3-kinase; p70S6K, p70 ribosomal S6 kinase; mTOR, mammalian target of rapamycin; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase kinase; LY294002, 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; bisindolylmaleimide, 2-[1-(3-Dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide; PD98059, 2′-Amino-3′-methoxyflavone; SB203580, 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole; DMSO, dimethyl sulfoxide.

Cellular and Molecular

Abstract

The ketone body acetoacetate (AA) in the absence of insulin or in the presence of diabetic insulin levels decreases cytochrome P450 (CYP) 2E1 mRNA expression in a concentration- and time-dependent manner in primary cultured rat hepatocytes. AA activates p70S6K and PKC by ~ 2 - 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 or wortmannin, in agreement with p70S6K being downstream of PI 3-K. Inhibition of PI 3-K, or mTOR with rapamycin, or PKC with bisindolylmaleimide, ameliorated the AA-mediated downregulation of CYP2E1 mRNA expression. Neither the MEK inhibitor PD98059, nor the p38 MAPK inhibitor SB203580 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 h 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-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.

Introduction

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) 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.

AA and 3HB effects on cytochrome P450 (CYP) 2E1 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 as compared to 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 time by insulin, and all of which have been reported to alter CYP2E1 expression (reviewed in 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,

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translational and post-translational events (reviewed in 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, nitrosamines, as well as therapeutic agents and endogenous fatty acids and ketones (Reviewed in 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 which 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 appears 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, 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), p70 S6K 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 microsomes isolation, with 3 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 post-plating 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%-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 (10µg/ml) and the protein synthesis inhibitor

cycloheximide (10µg/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 non-toxic 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 chemiluminescene 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 (hnRNA)

Analysis. 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 Perkin Elmer Cetus DNA Thermal Cycler using CYP2E1-specific forward and reverse primers (5'-CGGGATCCAGACACGGTCTATCTAAAGCA-3' and 5'-CGGGATCCAGAGTGGGAAAATTAGCCCAC-3', respectively) containing *Bam*HI 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, *Bam*HI digested, ligated into *Bam*HI digested pBluescript KS+T7/A90 and transformed into JM109 cells. The resulting plasmid was digested with *Eco*RI and *Nco*I, which created a 75 bp internal deletion within intron 2. This construct was linearized with *Hind*III 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% non-denaturing polyacrylamide gel with Tris-base/Boric acid/EDTA (TBE) 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 as 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

is also helpful in comparison between different samples and different experiments.

Immuno-blot 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 DTT, and 0.1% bromophenol

blue). Cell lysates (10-15 µl) were separated by SDS-PAGE on 10% gels and electrophoretically

transferred to nitrocellulose membrane (Bio-Rad Inc., Hercules, CA). 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 1:20000 in 5% milk powder in TBS-T). Proteins were detected by enhanced

chemiluminescence on Kodak X-OMAT film (Sigma Chemical Co.) and quantified by

densitometry with a Molecular Dynamics scanning laser densitometer and ImageQuant analysis

program (Amersham Biosciences, Piscataway, NJ). Following probing with phospho-specific

antibodies, blots were stripped and reprobed for total levels of the respective kinases or GAPDH,

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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 immuno-blot 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 PBS). For immnunodetection, 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 4 dishes per treatment. To harvest, cells were placed on ice, washed twice with cold PBS 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

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min to remove nuclei and debris. One ml of the supernatant was then placed onto 11 ml of a 10-60% 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 at 4°C. Gradients were collected as 0.8 ml fractions, using an ISCO fraction collection system, into disposable 15 ml conical tubes at 0.375ml/min flow rate and collection at ~144 sec intervals. Fractions were adjusted to 2 ml with DEPC-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 mls 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 (TE) pH 7.5. A volume of 156 µl of 3M 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 TE 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 ANOVA 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 to 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 (5mM) 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 to 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 to 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 2-3 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 h, 12 h, 24 h, 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. 3A). The inhibition of the AA-mediated decrease in CYP2E1

expression in cells pre-treated 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). Pre-treatment 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 pre-treated with rapamycin was dose-dependent, although the maximal effect on CYP2E1 mRNA occurred with 500 nM rapamycin, with CYP2E1 mRNA achieving levels comparable to 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. 4A and B). As shown in Fig. 4A and B, AA

increased p70S6K phosphorylation by an ~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 an ~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. 5A and B). As illustrated in Fig. 5 A and B, AA

increased PKC (Ser662) phosphorylation by an ~2.5-fold. In addition, our data shows that while

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bisindolylmaleimide (10 µM), at a concentration sufficient to inhibit PKCα, βI, δ, ε, 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 an ~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. I). Rather, the inhibitors PD98059 and SB203580 appeared 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 ~20-25%, 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.

The decline of CYP2E1 mRNA levels in response to AA could be the result of two molecular mechanisms: (A) inhibition of CYP2E1 gene transcription, and/or (B) 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 employed (Fig. 7). AA-treated hepatocytes exhibited an ~50% suppression of CYP2E1 gene transcription compared to 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 pre-treated with bisindolylmaleimide (10 μM), there was also a complete abrogation of the AA inhibitory effect on CYP2E1 gene transcription relative to that monitored

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in AA-treated cells (Fig. 7). These results, in combination with the results obtained from Figures 2 and 3, suggest that AA significantly inhibits CYP2E1 gene transcription and that both 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 an ~1.3-, 2-, and 2.5-fold at 12 h, 24 h, and 48 h, respectively, as compared to 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: (A) an increase in the translation of the existing CYP2E1 mRNA, and/or (B) 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-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 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 to untreated cells with the half-life for AA-treated cells ~24 h compared to ~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 appears 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 (Peng and Coon, 1998; 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

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. In order 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 which 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 which 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 and, 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-3ß, and HNF4α on the decreased DNA-binding

activity, as well as the activation of negative regulatory factors such as nuclear factor kappa-B

(NF-KB), 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

appears 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 (AP)-1, NF-KB, nuclear factor of activated T cells

(NFAT) and CCAAT enhancer binding protein (C/EBP) (Abdel-Razzak et al., 2004). PI3-K and

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PKC signaling pathways have previously been implicated in the inhibition of gene transcription, possibly through modulation of transcription factors (Nowak et al., 2005; Cheng et al., 2000). In addition, PI3-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 there could exist a co-ordination 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 multi-step process. For example, it has been reported that the inhibition of CYP2A5 gene transcription resulted in relocalization of a heterogeneous nuclear ribonucleoprotein (hnRNP) 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 hnRNP 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 even though AA decreased CYP2E1 mRNA levels, AA, but not 3HB, induced CYP2E1 protein in a time-dependent manner in primary cultured rat hepatocytes. Studies which utilized 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 PI3-K/Akt/mTOR and PKC signaling pathways might play a role in increased translational efficiency through these RNA complexes (Wu et al., 2005; Angenstein et al., 2002). 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 PI3-K and PKC signaling are involved in AA-mediated CYP2E1 mRNA translation.

Little is known about CYP2E1 protein degradation mechanism(s). The decreased turn-over of CYP2E1 protein seems to occur mainly through the interaction of the substrate with the protein, change its configuration and hence its identification by the proteosome, however, this is not always the case (Reviewed in Novak and Woodcroft, 2000). PI3-K and PKC have been reported to play a role in protein stabilization (Zhou et al., 2004; Yamauchi et al., 2003).

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 (Lieber et al., 2004; Leclercq et al., 2000), 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 to 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 while 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.

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Acknowledgments

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Footnotes

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Legends for Figures

Figure 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 normalized to 7S RNA. CYP2E1 mRNA levels are plotted as a percentage of the CYP2E1 mRNA levels monitored in untreated hepatocytes. Data are means ± SEM of Northern blot band densities of 3 preparations of total RNA. *Significantly different from UT; ** Significantly different from corresponding hepatocytes treated with insulin only (*P<0.05).*

Figure 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 normalized to 7S RNA. CYP2E1 mRNA levels are plotted as a percentage of the CYP2E1 mRNA levels monitored in untreated hepatocytes. Data are means ± SEM of Northern blot band densities of 3 preparations of total RNA. *Significantly different from UT (*P<0.05).*

Figure 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 normalized to 7S RNA. CYP2E1 mRNA

levels are plotted as a percentage of the CYP2E1 mRNA levels monitored in untreated

hepatocytes. Data are means ± SEM of Northern blot band densities of 3 preparations of total

RNA. *Significantly different from UT, ** significantly different from hepatocytes treated with

AA only (*P<0.05)*.

Figure 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 immuno-blot analysis and band density

normalized to total p70S6K. Data are means ± range of phospho-p70S6K band densities from

two preparations of cell lysate.

Figure 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 immuno-blot analysis and band density normalized to GAPDH. Data are

means ± range of phospho-PKC band densities from two preparations of cell lysate.

Figure 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 normalized to 7S RNA. CYP2E1 mRNA levels are plotted as a percentage of the CYP2E1 mRNA levels monitored in untreated hepatocytes. Data are means ± SEM of Northern blot band densities of 3 preparations of total RNA. *Significantly different from UT, ** significantly different from hepatocytes treated with AA only ($P<0.05$).

Figure 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 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 ± SEM of band densities of 2 or 3 preparations of total RNA. *Significantly different from UT, ** significantly different from hepatocytes treated with AA only ($P<0.05$).

 Figure 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 immuno-blot

analysis. CYP2E1 protein levels are plotted as a percentage of the CYP2E1 protein levels

monitored in untreated hepatocytes. Data are means ± SEM of Western blot band densities of 3

preparations of microsomes. *Significantly different from UT (*P<0.05).*

Figure 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-60S untranslated fractions (fractions 3 to 7). The data presented in panels B represent the

mean values monitored in two separate experiments.

Figure 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 ± SEM of Western blot band densities of 3 preparations.

Figure 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) which activate the intracellular signaling

cascade.

Table 1. Effect of PD98059 (PD) and SB203580 (SB) on AA-mediated decrease in CYP2E1 mRNA levels. Forty-eight hours after plating, hepatocytes were treated with PD (50-100 µM) or SB (10-20 µM) for 1.5 h before treatment for 24 h with 5 mM AA. Untreated hepatocytes (UT) were cultured in the absence of AA and PD and SB compounds. CYP2E1 mRNA levels were monitored by Northern blot analysis and band density normalized to 7S RNA. CYP2E1 mRNA levels are plotted as a percentage of the CYP2E1 mRNA levels monitored in untreated hepatocytes. Data are means ± SEM of Northern blot band densities of 3 preparations of total RNA.

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

* Significantly different than levels monitored in UT, $P < 0.05$.

Figure 1

Figure 2

Figure 3

A

B

Figure 4

Figure 5

A

B

C
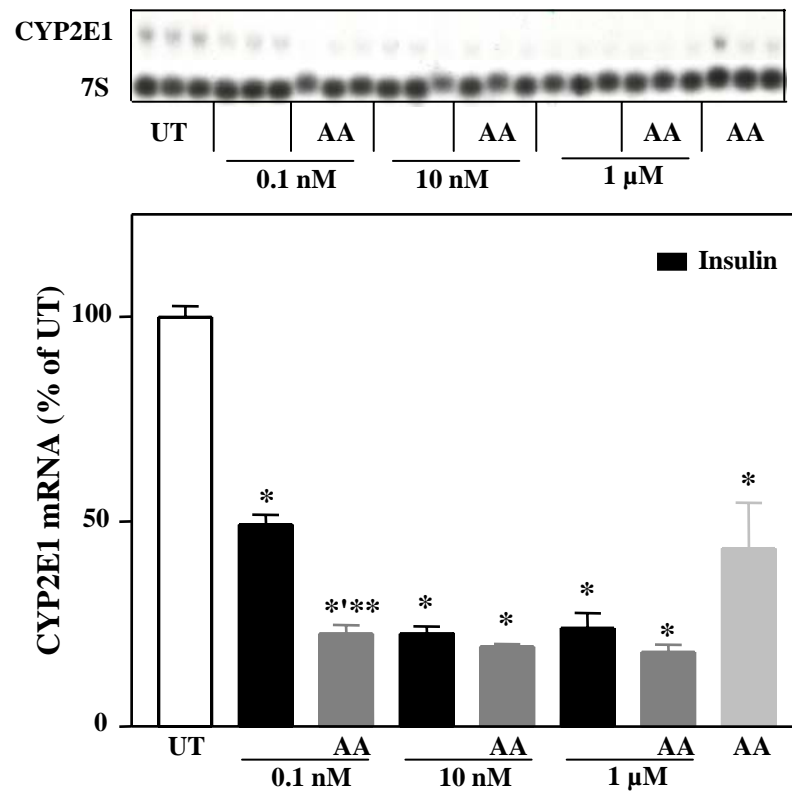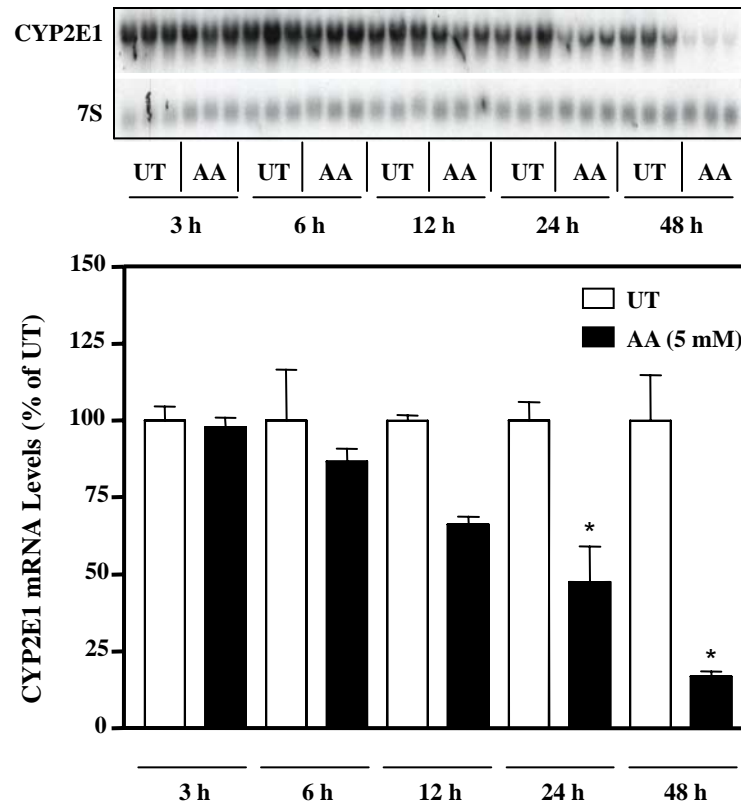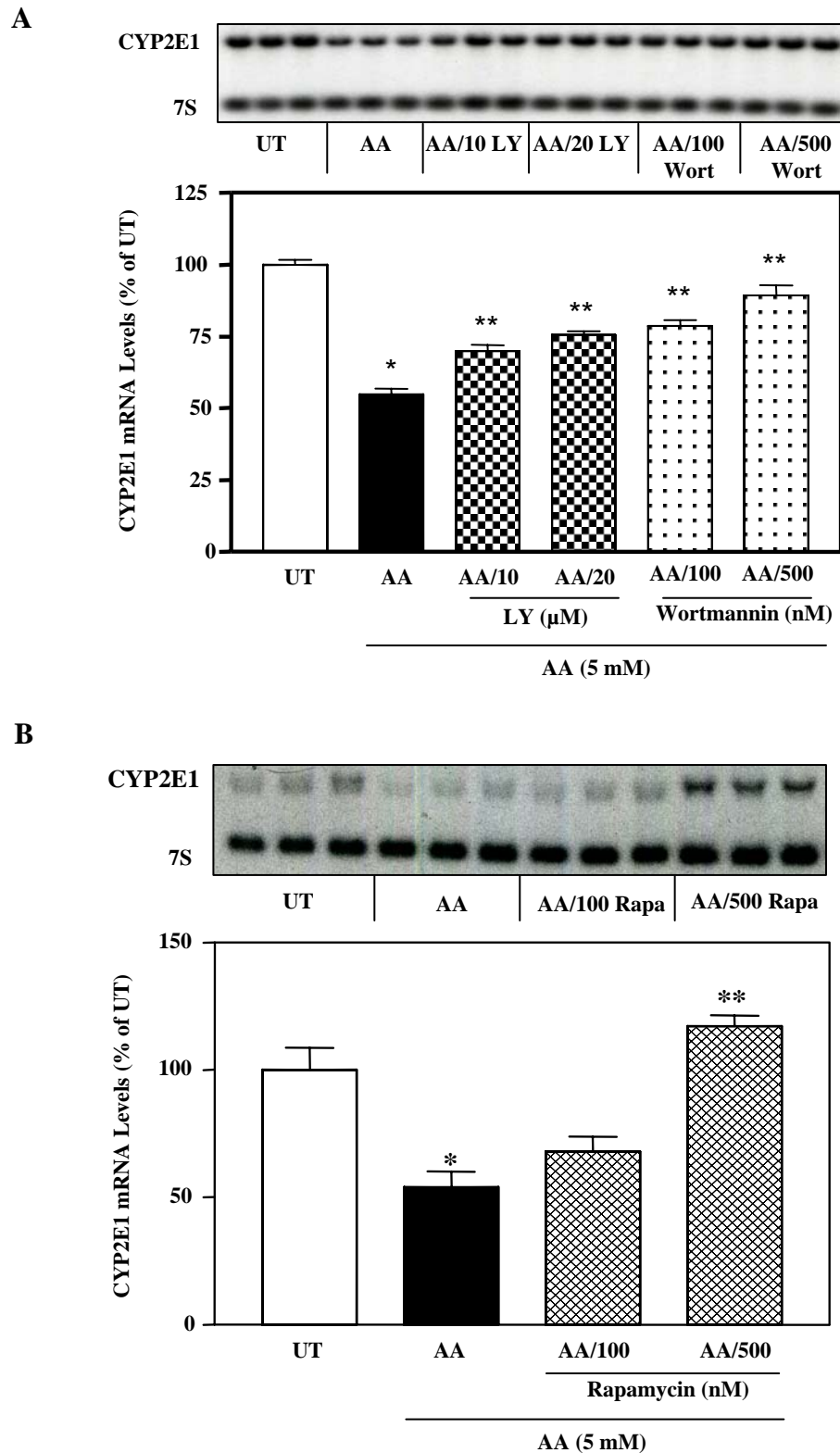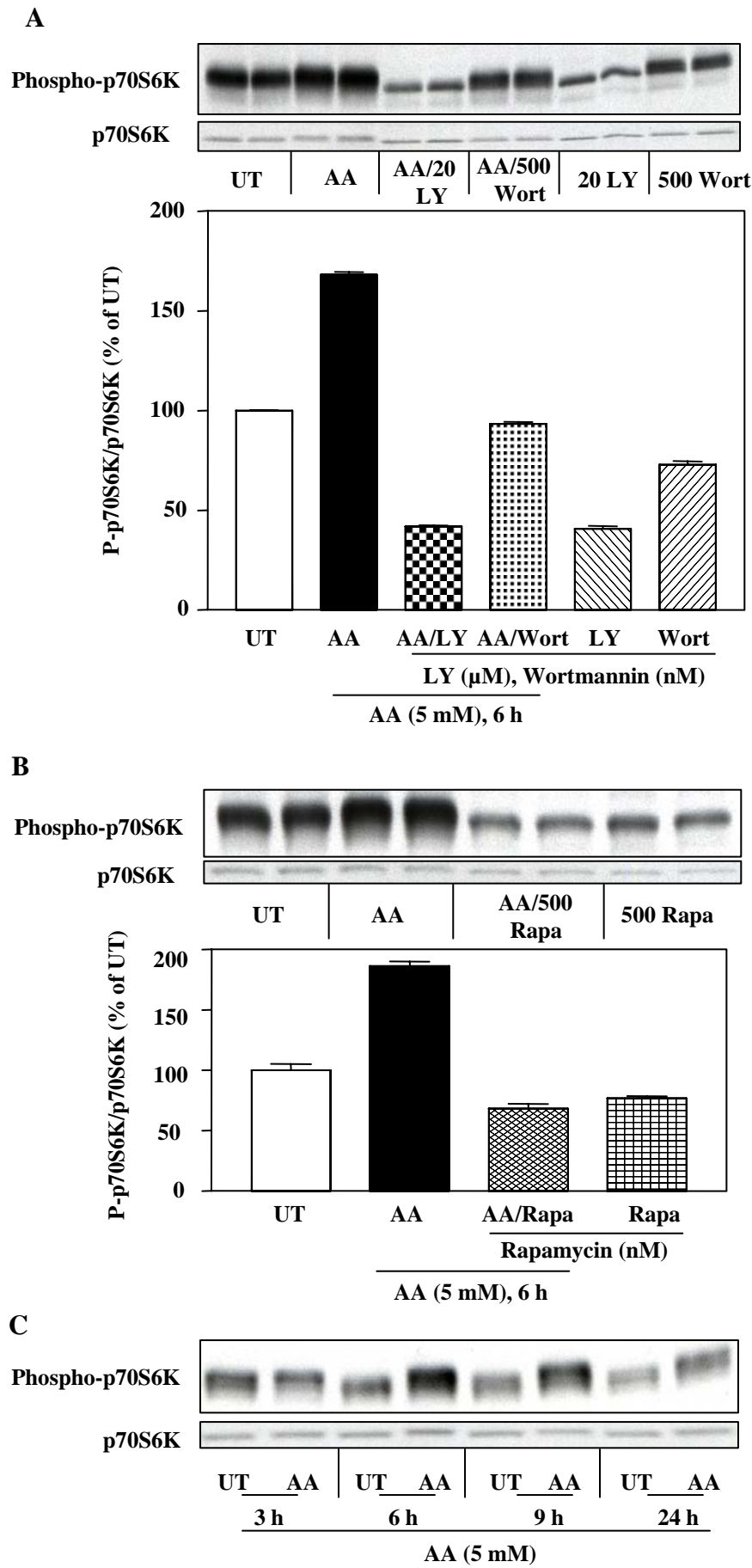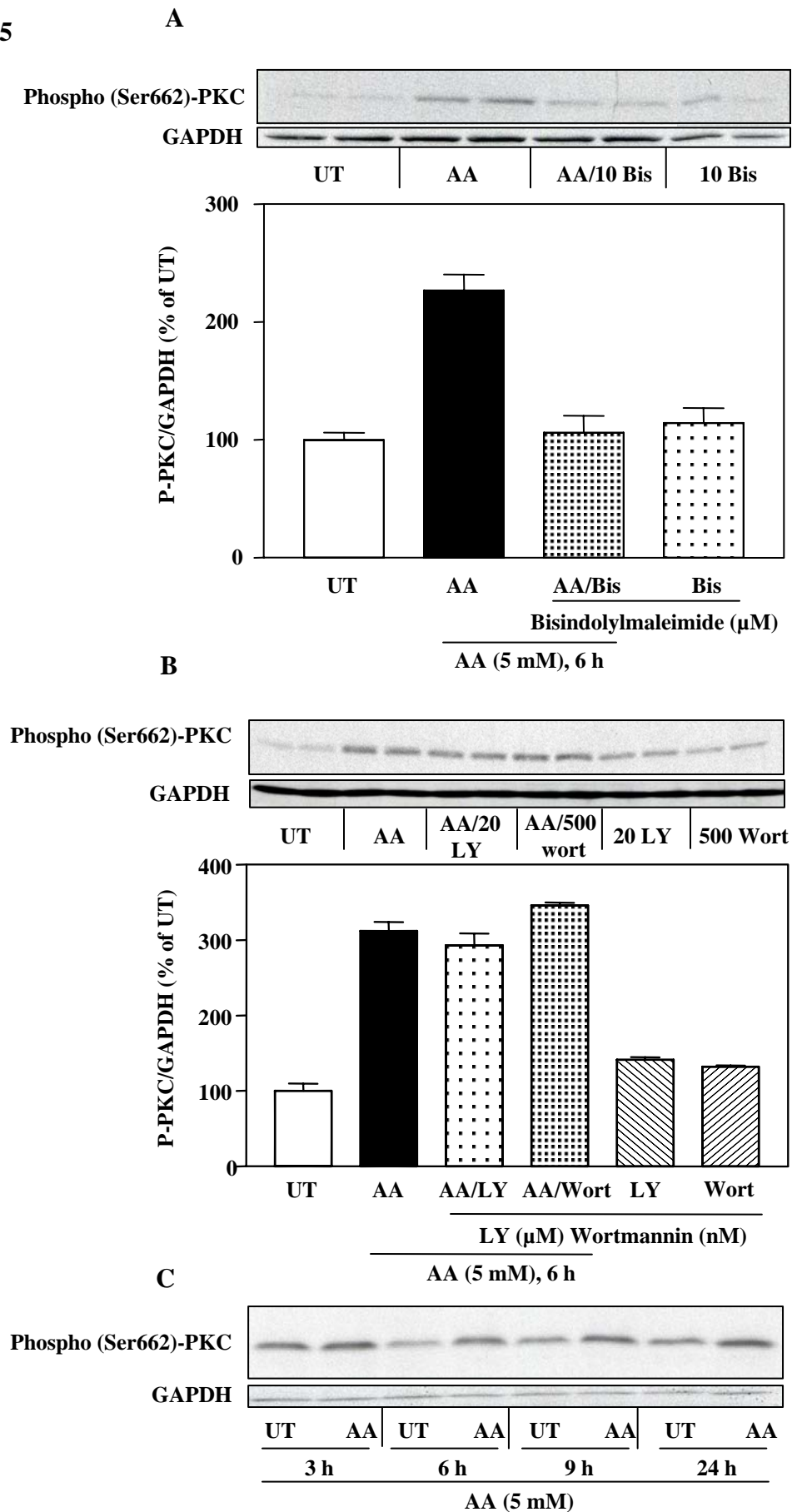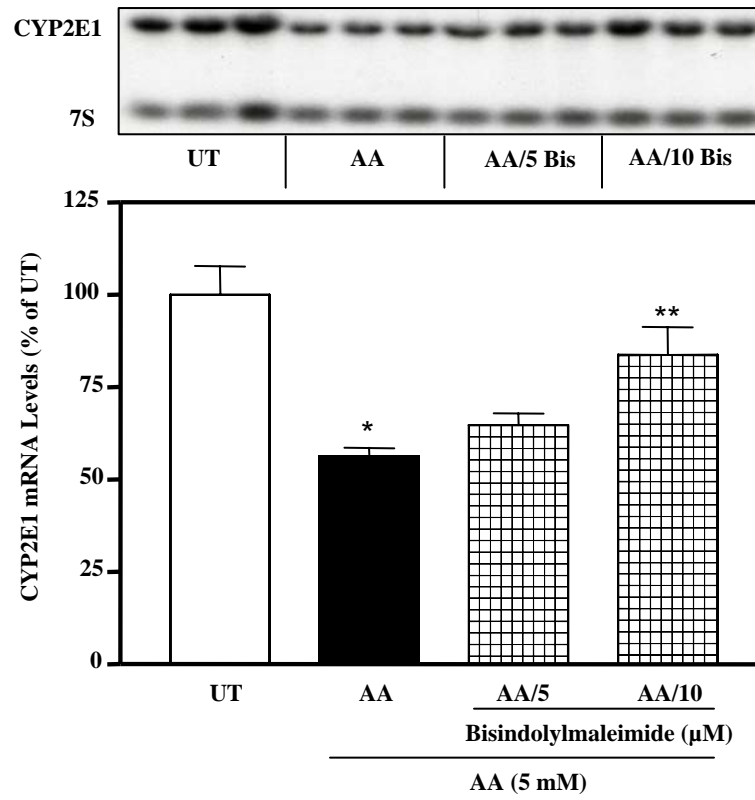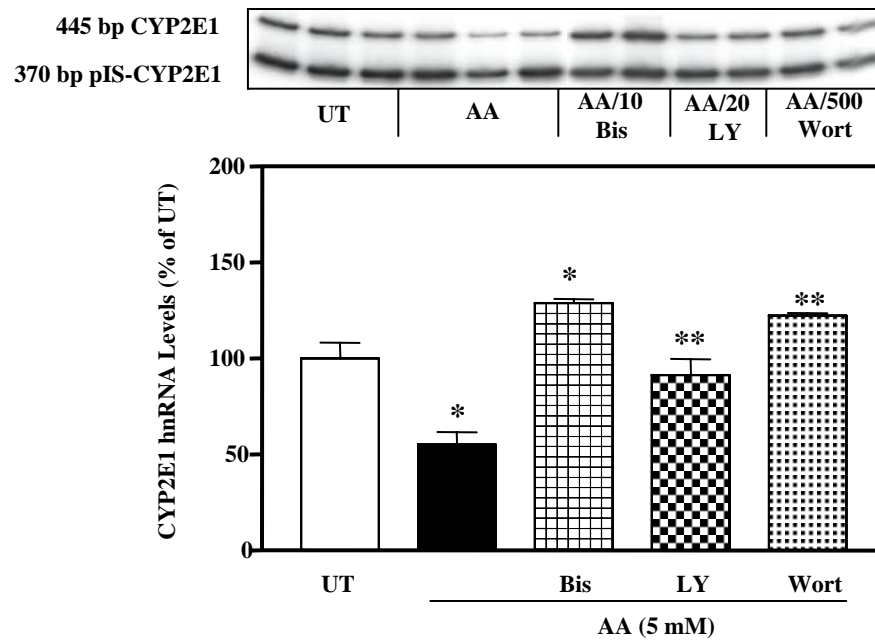
Figure 6

Figure 7

Figure 8

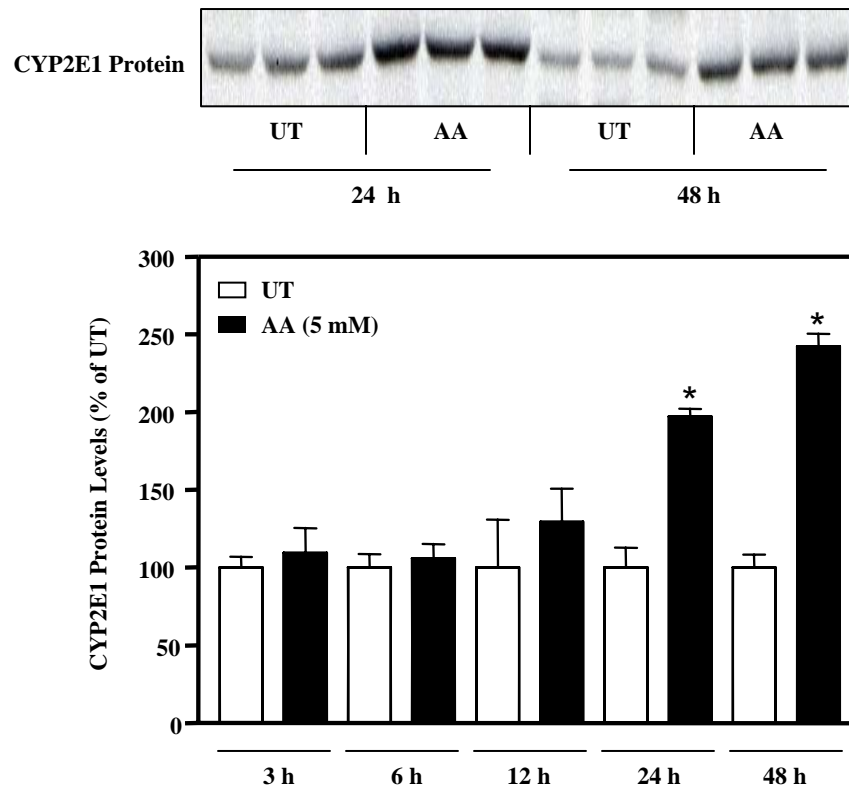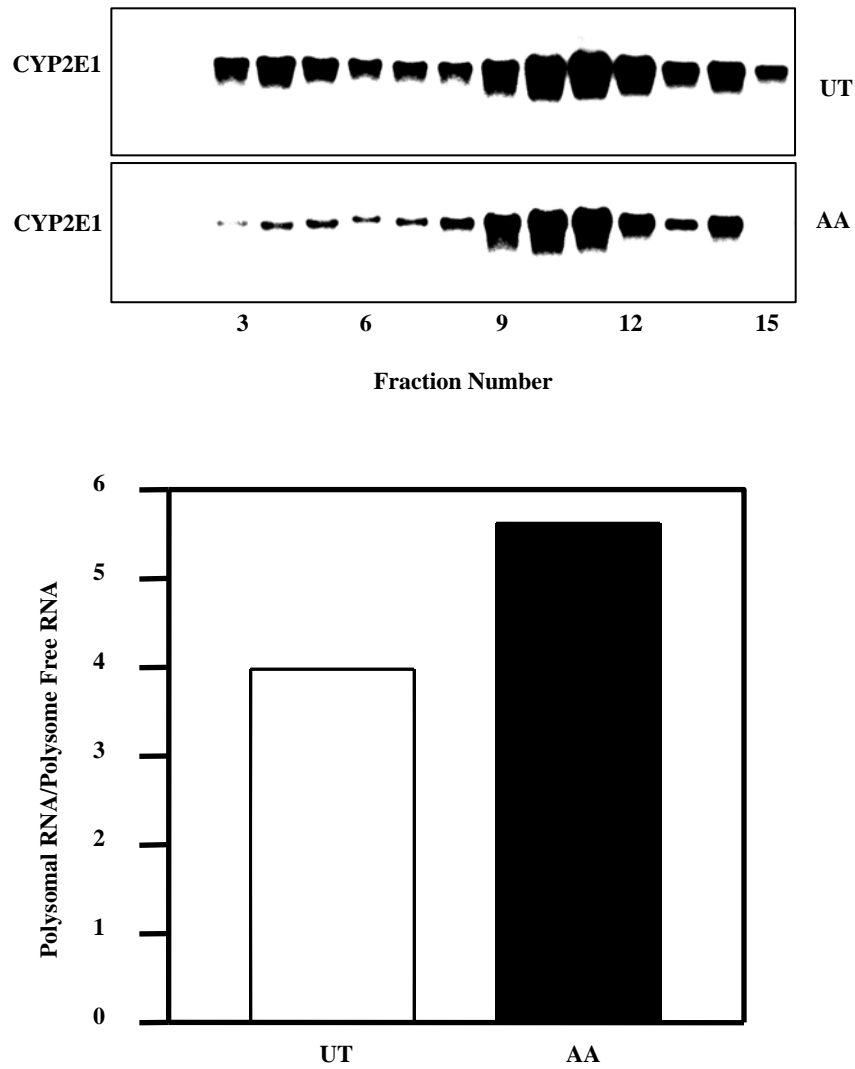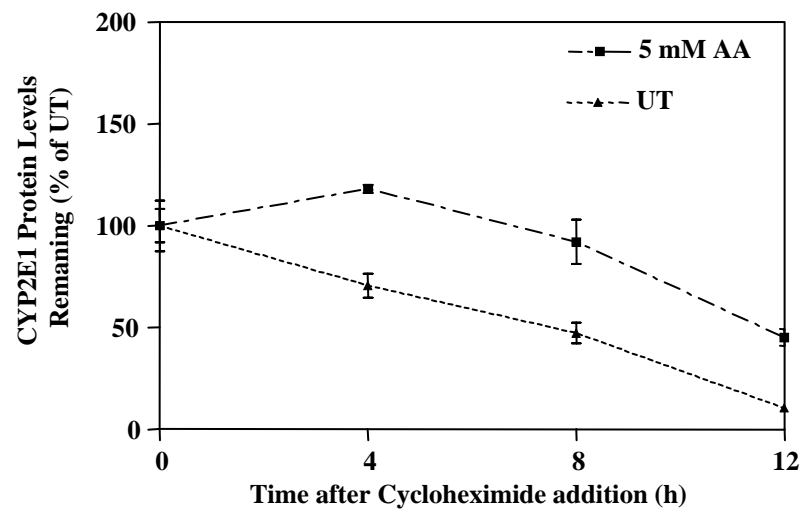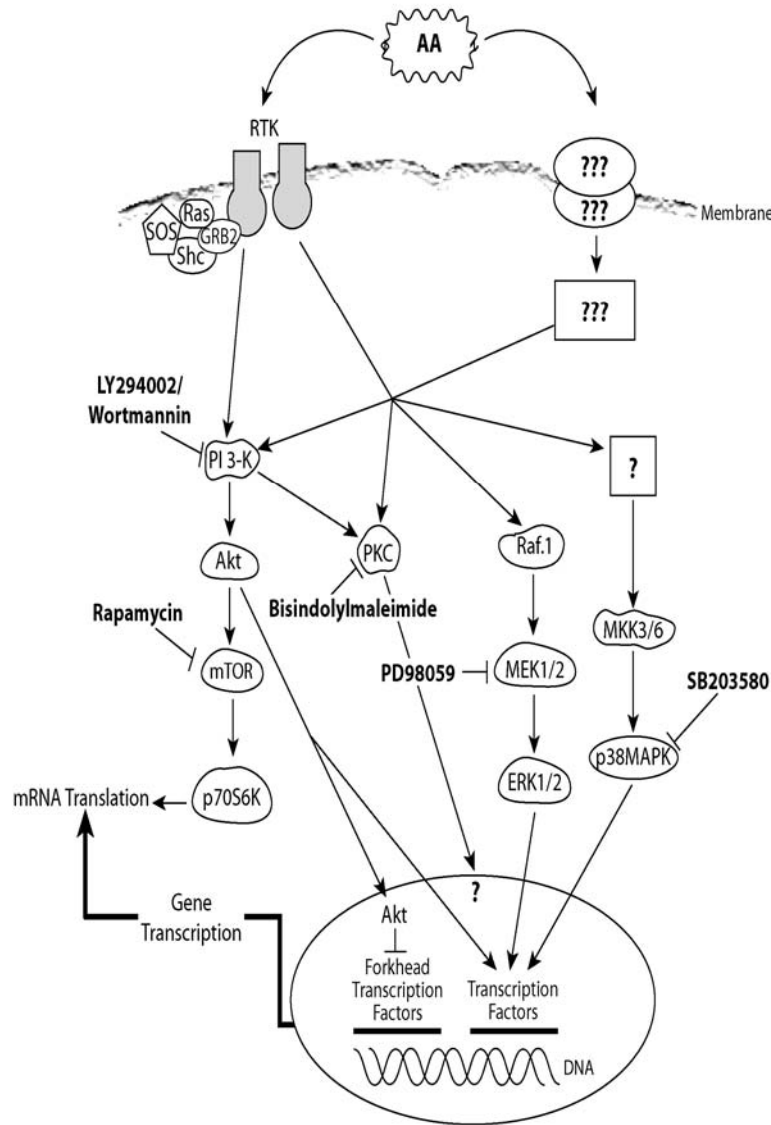**Figure 9**

Figure 10

Figure 11

RTK = Receptor Tyrosine Kinase
Wortmannin/LY294002 = PI 3-K inhibitor
Rapamycin = mTOR inhibitor
mTOR = Mammalian Target of Rapamycin
Bisindolylmaleimide = PKC inhibitor
PD98059 = MEK1/2 (MAP Kinase Kinase) inhibitor
SB203580 = p38 MAPK inhibitor