AMP-activated Protein Kinase Mediates Phenobarbital Induction of CYP2B Gene Expression in Hepatocytes and a Newly Derived Human Hepatoma Cell Line*

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Phenobarbital (PB) administration is known to trigger pleiotropic responses, including liver hypertrophy, tumor promotion, and induction of genes encoding drug-metabolizing enzymes. The induction of human CYP2B6 and the rat (CYP2B1) and mouse (Cyp2b10) homologues by PB is mediated by the nuclear receptor constitutive androstane receptor (CAR). The study of CYP2B gene regulation and CAR activity by PB has been difficult due to the lack of a cellular model. In this study, we describe a novel differentiated human hepatoma cell line (WGA), derived from HepG2, which expresses CYP2B6 and CAR. WGA cells represent a powerful system to study the regulation of CYP2B6 gene expression by PB. There is evidence that CAR activity is regulated by phosphorylation and that regulation of some CYP genes depends on the nutritional status of cells. The AMP-activated protein kinase (AMPK) functions as an energy sensor and is activated when cells experience energy-depleting stresses. In this report, we show that addition of 5-amino-imidazole carboxamide riboside, an AMPK activator, to WGA and human hepatocytes induces CYP2B6 gene expression. Expression of a constitutively active form of AMPK mimics the PB induction of CYP2B6 and CYP2B1 gene expression. Conversely, the expression of a dominant negative form of AMPK inhibits the induction of these genes by PB. Finally, we demonstrate, for the first time, that AMPK activity increases in cells cultured with PB. Our data strongly support a role for AMPK in the PB induction of CYP2B gene expression and provide new insights into the regulation of gene expression by barbiturate drugs.

The cytochrome P450 (CYP) gene family plays a crucial role in the biotransformation of structurally diverse classes of xenobiotics including drugs and endogenous compounds such as steroid hormones, vitamins, and fatty acids (1). Preferentially expressed in the liver, members of the CYP1, CYP2, and CYP3 families exhibit a broad substrate specificity and metabolize the majority of administered drugs. Recent studies demonstrated that the CYP2B6 isoform comprises 2–10% of total human liver P450 content. In addition, CYP2B6 has been reported to be involved in the metabolism of almost 25% of current pharmaceuticals (2), and expression of CYP2B6 is strongly induced by structurally diverse compounds, such as phenobarbital (PB), rifampicin, and clotrimazole (3, 4). Phenobarbital has been used in the treatment of epilepsy and causes pleiotropic responses in the liver including hypertrophy, tumor promotion, and the induction of genes encoding drug-metabolizing enzymes (5, 6). The hepatic expression of many cytochrome P450 genes, including members of the CYP1A, CYP2A, CYP2B, CYP2C, and CYP3A subfamilies, is induced by phenobarbital. The barbiturate induction of human CYP2B6 and murine Cyp2b10 and rat CYP2B1 has been shown to be mediated by constitutive androstane receptor (CAR) (7). CAR is a 49-kDa member of the nuclear receptor family NR1I3 and is expressed preferentially in human liver. It was first shown to activate a DR5-type of retinoid acid response element (βRARE) in a ligand-independent manner (8, 9) and is now considered a constitutively active receptor. CAR binds to and activates the PB response element found in promoters of PB-inducible genes (7). The induction of CYP2B gene expression by PB is controlled by changes in CAR intracellular localization. Upon PB treatment, CAR translocates from the cytoplasm into the nucleus, where it binds and transactivates target genes (10). Translocation is inhibited by addition of okadaic acid, suggesting that phosphorylation controls this process (11, 12).

In addition to P450 regulation by exogenous chemicals, there is evidence that nutritional status can be a significant factor in determining cytochrome P450 levels. For example, an enhancement of CYP2B1 gene expression is observed in the liver of streptozotocin-induced diabetic rats, and this diabetic state enhances the PB-induced CYP2B1 gene expression (13). However, PB does not induce CYP2B1 gene expression in obese diabetic Zucker rats (14). Interestingly, the low level of activated protein kinase; PB, phenobarbital; CAR, constitutive androstane receptor; AICAR, 5-amino-imidazole carboxamide riboside; MOI, multiplicity of infection; PEPCK, phosphoenolpyruvate carboxykinase; tk, thymidine kinase; DMEM, Dulbecco’s modified Eagle’s medium; RT-PCR, reverse transcription-PCR; PBREM, phenobarbital-responsive enhancer module.

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CYP2B1 gene expression observed in these animals was associated with the absence of CAR (15). In addition, CYP2B and CYP3A gene expression is induced by ketone bodies, such as 3-hydroxybutyrate, in primary rat hepatocytes (16). These studies suggest that CYP2B genes can also be regulated by hormones and nutrients and that the energy status of the hepatocyte may be important.

A key sensor of cellular energy change is the AMP-activated protein kinase (AMPK). This kinase is activated when cells experience energy-depleting stresses (17). Recently, AMPK has been implicated in the regulation of glucose uptake in skeletal muscle during physical exercise and also in regulation of hepatic glucose output through the inhibition of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase gene expression (18). Thus, AMPK has been proposed as a target for the development of anti-diabetic drugs (19) and has been shown to be activated by the existing anti-diabetic drugs metformin and rosiglitazone (23, 50). In the present study, we identify AMPK as a novel PB-regulated signaling molecule and identify AMPK as a novel PB-regulated signaling molecule that plays an important role in the regulation of CYP2B gene expression by PB. In addition, we describe a newly derived human hepatoma cell line that will be beneficial in the study of both CAR function and CYP2B gene regulation.

EXPERIMENTAL PROCEDURES

Animals—Animal studies were conducted according to United Kingdom guidelines for care and use of experimental animals. Male Sprague-Dawley rats (200–300 g) used for isolation of hepatocytes were purchased from Charles River Ltd. (Margate, UK).

Plasmids/Viruses—The human phenobarbital-responsive elements linked to luciferase (PBREM-tk-luciferase) and (NR1)5-tk-luciferase reporter gene plasmids, respectively. The pcDNA3 vector was a kind gift from Dr. M. Negishi (Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, NC). The complementary oligonucleotides of human PBREM and the quintuplet human NR1 (5'-gatc-ACTGACTTTCCTCAGCTCTGatc-3') sequences were synthesized and cloned in front of the tk promoter (BglII site), resulting in PBREM-tk- and (NR1)5-tk-luciferase reporter gene plasmids, respectively. The pcdNA3-o1DN vector was generously provided by Prof. J. Ha (Department of Molecular Biology, Kyoung Hee University, Seoul, Korea). The adenosine vectors ad-o1 (22) (ad-1-activating) and ad-DN-o1-AMPK were a kind gift from Dr. D. Carling (Cellular Stress Group, University of Sheffield, UK) (20). PGL3-basic-luciferase was purchased from Promega (Madison, WI).

Antibodies—Polyclonal antibodies raised against the rat P450s CYP2B1/2 were as described previously (21). Antibodies against rat AMPK-α1 and -α2 (22) phosphorylated Ser-79 of acetyl-CoA carboxylase (aACC) and phosphorylated Thr-172 of AMPK were described as prepared previously (23, 24). The polyclonal antibody against rat AMPK was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), whereas a monoclonal antibody to e-MyC was purchased from Chemicon International Ltd. (Harrow, UK).

Cell Culture—Culture media and antibiotics were obtained from Invitrogen, unless noted otherwise. The human hepatoma cell line HepG2 was obtained from the European Collection of Cell Culture (ECACC 85011430). WGA cells were derived from HepG2 as described under "Results." HepG2 and WGA cells were cultured in a mixture of DMEM/DMEM:F12/L15 (50%/25%/25%, respectively) supplemented with 7% NuSerum IV (Becton Dickinson Europe, Le Pont-De-Clair, France), sodium butyrate (400 μM), fructose (4 mM), and a mixture of antibiotics and antimycotics (1% of a 100× stock) in a humidified atmosphere at 37 °C in the presence of 5% CO2.

Rat hepatocytes were prepared by the two-step collagenase perfusion as described previously (25). Cell viability was estimated by trypan blue exclusion, and it was always >90%. Hepatocytes were cultured in DMEM supplemented with 5% fetal bovine serum (Invitrogen) and 1× ITS (insulin transferrin selenium; Becton Dickinson Europe) for 4 h to allow cell attachment. After the attachment period, cells were cultured in DMEM with 5% fetal bovine serum or as detailed under "Results."

Isolation of WGA Hepatoma Cells (a Differentiated Variant of HepG2)—The WGA cell population was isolated from HepG2 after metabolic selection as described by Armbuster et al. (26) with some modification. HepG2 cells were first adapted to a new growth medium consisting of Leibovitz's L15 medium (Invitrogen) supplemented with 2% Ultrorser G (Bioespera S.A., Cergy-Saint-Christophe, France). Cells were shifted to a L15 glucose- and galactose-free medium (Specialty Media, Phillipsburg, NJ) supplemented with 10 mM fructose and 2% Ultrorser G (Bioespera S.A.). This culture medium drives the selection of cells expressing a differentiated hepatocyte phenotype, i.e., the metabolism of fructose. In hepatocytes, fructose is transported across the cell membrane by the facilitative transmembrane transporter, Glut2 (27), and is preferentially metabolized by the aldolase pathway (28). After 3 weeks of culture under these conditions, cells were then placed in an L15 galactose-free and arginine-free medium (Specialty Media) supplemented with glucose (20 mM), glutamine (2 mM), dexamethasone (1 μM), and Ulitros G (2%) (Bioespera S.A.). The culture of hepatoma cells in arginine-free medium has also been successfully used to isolate the primary human liver tumor cell line (BC2) that expresses drug-metabolizing enzymes (29). The remaining cell population (WGA) was then expanded in growth medium as detailed under "Cell Culture."

RNA Extraction and Reverse Transcription—Cells were washed twice in ice-cold phosphate-buffered saline, and total RNA was extracted using the Qiagen RNaseasy mini kit (Qiagen, Crawley, UK) as described by the manufacturer. Total RNA concentration was estimated by A260, and the purity of RNA was verified by the A260/A280 ratio. RNA integrity was evaluated by denaturing gel electrophoresis in agarose (1%), formaldehyde (0.66 × 105) gels. Bands were visualized using ethidium bromide staining.

Total RNA (1 μg) was reverse-transcribed using murine leukemia virus reverse transcriptase (PerkinElmer Life Sciences) in the presence of random hexamers in a final volume of 20 μl. Reactions were carried out using in a thermal cycler (Peltier PTC225; MJ Research, Inc., Waltham, MA). The cycles were 10 min at room temperature and 15 min at 42 °C, and then the reaction was stopped by incubation at 99 °C for 5 min.

Real-Time RT-PCR (TaqMan®)—Gene-specific primers and probes (Table I) were designed using Primer Express (PerkinElmer Life Sciences) and synthesized by MWG-Biotech (Ebersberg, Germany). All primers and probes were entered into the NCBI BLAST® program to ensure specificity. The amplification of target genes by real-time PCR was performed from 1 μl of cDNA using a Master Mix (PerkinElmer Life Sciences) containing Taq DNA polymerase in a final volume of 20 μl. The reaction was performed as described by the manufacturer (PerkinElmer Life Sciences). Samples were loaded on a 96-well plate (Micro-Amp Optical; PerkinElmer Life Sciences), and fluorescence intensity was monitored employing an ABI PRISM 7700 sequence detector. All reactions were standardized with 18 S rRNA using TaqMan® rRNA control reagents (PerkinElmer Life Sciences).

Adenoviral Transfection—Primary rat hepatocytes were prepared as described above. After attachment (4 h), medium was replaced by DMEM supplemented with 10% fetal bovine serum. Hepatocytes were infected with adenovirus at the multiplicity of infection (MOI) described in the figure legends for 24 h and then incubated with or without 2.5 mM PXB for an additional 24 h. WGA cells were infected at 60–70% confluence.

Dual Reporter System Assay—WGA cells were seeded at 5 × 105 cells/well into black-walled and clear-bottomed 96-well plates (Corning Inc., Corning, NY). Cells were incubated for 24 h at 37 ºC before transfection. Transfection was performed overnight in growth medium with FuGENE 6 (Roche Applied Science), according to the manufacturer's instructions. Reporter plasmids PBREM/pGL3-tk (700 ng/well) and (NR1)5/pGL3-tk (600 ng/well) were co-transfected with pcDNA3-o1DN expression vector (60–70 ng/well). All plasmids were co-transfected

| Gene         | Sequence                                      |
|--------------|-----------------------------------------------|
| CYP2B1       | 5'-CACCTCAGGCGTACAGAGATC-3'                  |
|              | 5'-CCCAGGCGTACAGAGATC-3'                     |
|              | 6FAM-5'-ACCAGGACCTCCCTCCCCCTCCC-3'TMRA      |
| CYP2B6       | 5'-CACCTCAGGCGTACAGAGATC-3'                  |
|              | 5'-CCCAGGCGTACAGAGATC-3'                     |
|              | 6FAM-5'-ACCTTTGCAAGGAAACCGCCTGGAAG-3'TMRA    |
| CAR          | 5'-CACAGGCGCCATCTTTTGA-3'                    |
|              | 5'-AGAGGGCTGTTATGGAAG-3'                     |
|              | 6FAM-5'-TTGTTGCAATGTTAGGCTCCGACTCTACT-3'-TMRA|

*6FAM, 6-carboxyfluorescein; TMRA, 6-carboxytetramethylrhodamine.
with pRL-CMV (Renilla luciferase; 1 ng/well). After transfection, fresh growth medium was added with or without P450 inducers for 36 h as stated in the figure legends. Cells were rinsed twice in ice-cold phosphate-buffered saline and lysed in 20 μl of passive lysis buffer (Promega) for 15 min at room temperature. Firefly and Renilla luciferase activity was assayed in an Orion microplate luminometer (Berthold Technologies, Bad Wildbad, Germany). Data were analyzed with Simplicity 2.1 software (Berthold Detection System), and results were expressed as the ratio of firefly/Renilla activities. Each determination was carried out in triplicate.

**Western Blot Analysis**—Cells were washed in ice-cold phosphate-buffered saline and harvested in 300 μl 25-cm² flask of extraction buffer (100 mM KCl, 25 mM Heps, 7.5 mM MgCl₂, and 20% glycerol, pH 7.4) supplemented with protease inhibitors (4 mM dithiothreitol, 2 mg/ml aprotonin, and 1 mM β-mercaptoethanol). The cell suspension was sonicated, cellular debris was removed by centrifugation (1000 × g, 10 min, 4 °C), and protein concentration in the supernatant was estimated by the method of Bradford (30).

Thirty micrograms of total cellular protein were separated by SDS-PAGE and blotted onto nitrocellulose membrane. The following primary antibodies were employed: anti-P450 CYP2B1, anti-CAR, monoclonal anti-Myc, antiphospho-T172/AMPK, or antiphospho-Ser-79 acetyl-CoA carboxylase. Horseradish peroxidase-conjugated secondary antibodies were as follows: anti-goat IgG (Sigma; 1:5000), anti-rabbit IgG (DAKO; 1:1000), and anti-mouse IgM (Sigma; 1:2000). Blots were developed using ECL reagent (Amersham Biosciences) and exposure to x-ray films (Kodak X-OMAT).

**AMPK-activated Kinase Activity**—Immunoprecipitation kinase assays of AMPK were performed using anti-α1 or anti-α2 AMPK antibodies as described previously (31). Results were expressed as a percentage of control for two independent experiments, with each determination carried out in duplicate.

**RESULTS**

**Induction of CYP2B6 Expression in the Human Hepatoma Cell Line WGA**—WGA was derived in the mechanism of CYP2B gene expression have been hampered by the lack of a cell system that exhibits phenobarbital-induced changes in gene expression. In order to address the need for such a system, we derived WGA cells from HepG2 cells by metabolic selection using the ability to grow on fructose as the selection system. The morphology of WGA cells was similar to HepG2, with a dense granulated cytoplasm, clear nucleus, and dense nucleoli. Interestingly, the CYP2B6 mRNA concentration in the WGA cells was higher than that detected in HepG2 cells (Fig. 1A).

**Fig. 1. Regulation of CYP2B6 and CAR mRNA levels in HepG2 and WGA hepatoma cells.** Cells were cultured for 24 h with or without 2.5 mM PB. Total RNA was extracted, and CYP2B6 mRNA (A) and CAR mRNA (B) were quantified by real-time RT-PCR as described under “Experimental Procedures.” Values are expressed as ratio of the copy number of each mRNA to that of 18 S rRNA, as described under “Experimental Procedures.” Results are the mean ± S.D. of three or four experiments performed in triplicate.

**Fig. 2. Induction of CYP2B6 mRNA expression in WGA and HepG2 hepatoma cells by phenobarbital and AICAR.** WGA (A) and HepG2 (B) cells were cultured for 24 h in the presence of either PB (2 mM) or AICAR (200 μM). Real-time RT-PCR was used to quantify CYP2B6 mRNA as described under “Experimental Procedures.” Results are expressed as the ratio of the copy number of CYP2B6 mRNA to that of 18 S rRNA. Results are the mean ± S.D. of four experiments, with each determination performed in triplicate.
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Fig. 3. Expression of α 1-AMPK in WGA cells. WGA cells were infected with ad-active-α1-AMPK (MOI, 0–9). Thirty micrograms of total protein was separated on a 10% polyacrylamide gel, and the expression of Myc-tagged-α1-AMPK (Myc-α1) in infected cells was determined by Western blot using an anti-Myc antibody.

Additional 24 h. In the absence of virus, PB induced CYP2B6 expression 4-fold (Fig. 4A). An MOI of 1.5 of the ad-active-α1-AMPK increased constitutive CYP2B6 expression 5-fold (Fig. 4A). This was not increased further by PB administration (Fig. 4A). In contrast, CYP2B6 expression was unaffected by expression of β-galactosidase (Fig. 4A). However, PB or active AMPK expression did not alter the expression of CAR (Fig. 4B).

AICAR and AMPK Induce CYP2B Gene Expression in Primary Rat Hepatocytes—Rat hepatocytes were challenged with PB and AICAR, and the expression of CYP2B1 was quantified by real-time RT-PCR (TaqMan®) (Fig. 5A). The expression of CYP2B2 is strongly induced (almost 50-fold) after a 24-h culture in 2 mM PB. Importantly, CYP2B2 expression was also induced (25-fold) in the presence of 200 μM AICAR (Fig. 5A).

Infection of primary rat hepatocytes with ad-β-galactosidase (MOI, 6) did not alter the basal or PB-induced level of CYP2B2 gene expression (Fig. 5B). However, expression of a dominant negative form of α1-AMPK (α1-AMPK) almost completely abolished the effect of PB on CYP2B2 gene expression (Fig. 5B).

AICAR Induces P450 2B CYP2B1/2B2 Protein Levels in Primary Rat Hepatocytes—Primary rat hepatocytes were cultured for 48 h in the presence of AICAR (400 μM) (Fig. 6). A strong induction of P450 CYP2B1/2 expression (5) was observed after 48 h, but CAR protein expression was not altered (Fig. 6).

The Induction of PBREM and NR1 Elements from the CYP2B6 Promoter by PB Requires AMPK—A 51-bp enhancer designated PBREM, present in mouse, rat, and human CYP2B promoters, is essential for the response to PB (7). In WGA cells, phenobarbital (2 mM) induced PBREM activity >4-fold compared with control values (Fig. 7A). Moreover, addition of AICAR (200 μM) induced PBREM activity by 2.6-fold (Fig. 7A). Interestingly, co-expression of a dominant negative form of α1-AMPK (pcDNA3-α1DN) inhibited the PB induction of PBREM activity by 60% (PB, 4.2-fold; PB + pcDNA3-α1DN, 1.8-fold). The expression of the dominant negative form of α1-AMPK completely abolished the induction of PBREM activity by AICAR (200 μM) (AICAR, 2.6-fold; AICAR + pcDNA3-α1DN, no effect) (Fig. 7A). The basal activity of PBREM was not altered by transfection of the dominant negative form of α1-AMPK.

The NR1 site of the PBREM is the minimal region required for PB induction of the CYP2B6 promoter, and this is the sequence that binds CAR (9). The NR1-luciferase reporter construct was co-transfected with pcDNA3-α1DN in WGA cells (Fig. 7B). Cells were then cultured in the absence or presence of PB (2 μM) or AICAR (200 μM). Addition of PB induced NR1 activity by 4.8-fold compared with control cells. Meanwhile, AICAR induced NR1 activity by >3-fold (Fig. 7B). The expression of a dominant negative α1-AMPK subunit inhibited the PB induction of (NR1)2-Luc activity by 70% (Fig. 7B). Moreover, expression of the dominant negative α1-AMPK also reduced AICAR induction of (NR1)2-Luc activity by 55% (Fig. 7B).

The inhibitory effects of the dominant negative form of α1-AMPK on the PB response of the PBREM and NR1 promoters is similar. This suggests that the NR1 site within the PBREM is regulated by AMPK activity.

Phenobarbital Activates AMPK in WGA and H4IIE Hepatoma Cells—WGA cells were cultured for 1 and 6 h in the...
presence of AICAR (500 μM) or PB (2.5 mM). As expected, AICAR activates AMPK activity within 1 h with a 45% increase (control, 100 ± 5%; AICAR, 145 ± 13%). More interestingly, the AMPK activity increased by 70% in cells cultured with PB for 1 h (PB, 177 ± 40%). In addition, the AMPK activity in cells incubated with PB increased even more after 6 h of culture (PB, 274 ± 52%). Meanwhile, the AMPK activity decreased somewhat after 6 h of culture in the presence of AICAR (AICAR, 112 ± 16%). Analysis of phosphorylation of Thr-172 of AMPK (activating modification) and Ser-79 of acetyl-CoA carboxylase (AMPK substrate) after PB administration confirmed this rather transient regulation of AMPK in WGA cells and rat hepatocytes (Fig. 8). In WGA cells, AICAR activation was maximal at the past dosing, whereas that of phenobarbital occurred at 6 h. In both cases, activation was attenuated at 24 h. In isolated hepatocytes, activation was also observed at 1 and 6 h and again lost at 24 h after treatment. In agreement with these findings, we found that incubation of WGA or rat H4IIE cells with phenobarbital caused a dose-dependent increase in AMPK activity that was accompanied by a decrease in cellular ATP levels (Fig. 9). Therefore, we have observed PB activation of AMPK in three different liver cell systems.

**DISCUSSION**

The study of the regulation of both CYP2B gene expression and CAR activity has previously been difficult due to the lack of suitable cellular models. Most studies to date have been performed in primary hepatocytes maintained in a differentiated state by using complex medium or extracellular matrix (37, 38). The exogenous expression of CAR in HepG2 hepatoma cells leads to a constitutive nuclear localization of the receptor, despite the absence of any inducers (10). Therefore, this model...
does not allow the study of mechanisms involved in the control of CAR translocation.

In the present study, we have developed a HepG2-derived cell line (WGA) that expresses both CYP2B6 and CAR and, importantly, exhibits an induction of CYP2B6 mRNA expression upon PB treatment. To our knowledge, this is the first report of a human hepatoma cell line expressing CYP2B6 and CAR. The metabolic selection used to obtain WGA cells omits an essential nutrient, thus promoting expression of hepatocyte-specific enzymes in order to allow cell growth and division. In our study, HepG2 cells were cultured for 3 weeks in a medium deprived of glucose but supplemented with fructose. The selection on fructose as a carbon source was also successfully used to isolate the well-differentiated murine hepatoma cell line (mHaT3F) that expresses Glut2 (39, 40). In the liver, Glut2 expression is restricted to differentiated hepatocytes, and Glut2 expression decreases during the progression of tumorigenicity (41).

The WGA cell line isolated after 3 weeks in fructose also expresses Glut2, and the transcription factor CAR. In addition, a further 4–5-fold increase of CYP2B6 expression was observed after 24 h of culture in PB (Fig. 5), which is similar to the 6–7-fold induction of CYP2B6 gene expression observed in primary human hepatocytes (42). Therefore, WGA cells provide a powerful tool to study regulation of CYP2B6 gene expression as well as CAR translocation and function.

Several studies have demonstrated that intracellular phosphorylation events control the PB induction of CYP2B genes (11, 43–46), although the mechanism involved has not been defined (11, 45, 46). In addition, both energy status and nutritional environment of the cell can influence PB regulation of CYP2B gene expression. Thus, we examined whether PB could regulate a protein kinase considered to play a key role in cellular response to changes in energy status, namely, AMP-activated protein kinase. We observed increased AMPK activity when WGA cells were cultured for 1 and 6 h with PB. This is the first demonstration that PB regulates this enzyme. The degree of AMPK activation observed with PB is similar to that seen when the hepatocytes were incubated with the “classical” pharmacological activator of AMPK, AICAR. We believe that PB induces AMPK activity by increasing AMP levels (Fig. 9). PB could induce ATP depletion by inducing phosphorylation of glucose (47). Alternatively, in a more probable scenario, AMPK is exquisitely sensitive to any inhibition of the respiratory chain, which causes an increase in the cellular AMP/ATP ratio.

AMPK is not the only protein kinase activated by PB. Primary hepatocytes cultured for 12 h with PB exhibit an increase in p42/44 mitogen-activated protein kinase and protein kinase C activity (48). This is related to the tumor-promoting effect of PB observed in liver, and it is unlikely that activation of protein kinase C affects CYP2B gene expression (45). More interestingly, PB transiently increases phosphorylation of a 34-kDa nuclear protein in primary rat hepatocytes, and this correlates with the appearance of CYP2B1 mRNA (42).

In order to assess whether PB regulation of CYP2B gene expression involves AMPK, we performed two experiments. Firstly, we demonstrated that expression of active AMPK or treatment of cells with AICAR was sufficient to induce CYP2B expression (Figs. 2 and 4). Secondly, we blocked AMPK activity by expressing a dominant negative form of AMPK, and we found that PB could no longer induce CYP2B gene expression (Fig. 5). These data strongly argue that PB activates AMPK in order to induce CYP2B expression and, importantly, that activation of AMPK is sufficient to induce this gene. The inducing effect of AMPK on CYP2B gene expression described here was observed in primary rat hepatocytes as well as in the human hepatoma cell line WGA (Figs. 2 and 5). The absence of additive effects on CYP2B6 expression of PB and ad-α1-AMPK suggests that a common mechanism is involved. To our knowledge, this is the first evidence that AMPK induces CYP2B gene expression and indeed that AMPK induces gene expression in hepatocytes, although it has been reported that AMPK activation inhibits expression of hepatic genes coding for enzymes of glucose and lipid metabolism. In primary hepatocytes, AMPK activation inhibits glucose-activated gene expression (fatty acid synthase) (49), liver-type pyruvate kinase, and spot 14 (22). Whereas, in the rat hepatoma cell line H4IIE, PEPCK and glucose-6-phosphatase expression was inhibited by AICAR (18). Interestingly, rosiglitazone induces AMPK activity (50) and CYP gene expression (51) and also represses PEPCK gene expression (52). Thus, at least three agents that activate AMPK (PB, AICAR, and rosiglitazone) also regulate these genes.

The induction of CYP gene expression by PB is mediated by CAR, which binds to the PBREM within these gene promoters (7). The PBREM sequence is strongly conserved between mouse, rat, and human CYP2B genes (9). The 51-bp PBREM is characterized by two nuclear receptor sites (NR1 and NR2) separated by a nuclear factor 1 site (NF1) (7). Mutations in NR1 and NR2 revealed that the NR1 site is predominant over the NR2 site and essential for PBREM activity in response to PB treatment (7). The PBREM activation by PB was blocked in WGA cells transfected with a vector expressing a dominant negative form of α1-AMPK (pcDNA3-α1DN) (Fig. 7). It appears that the NR1 site of the PBREM mediates this effect because the α1-DN completely abolished the PB response of NR1 (Fig. 7). Thus, PB induction of PBREM activity involves the activation of AMPK and the subsequent regulation of the NR1 site. CAR binds to the NR1 site as a heterodimer with the retinoid X receptor αR (7). No changes were observed in CAR expression after AICAR treatment or AMPK overexpression (Figs. 4B and 6). Therefore, AMPK may induce CYP2B promoter activity by promoting CAR nuclear localization or by inducing CAR activity and/or binding to the DNA sequence. We are currently examining whether AMPK activity correlates with changes in CAR localization and whether CAR is directly phosphorylated by AMPK. Recent work has identified CAR as a co-repressor for FOXO1, an insulin-regulated transcription factor, implicating CAR in the regulation of PEPCK transcription (53). Thus, if AMPK does regulate CAR, it is likely to regulate many FOXO-regulated genes and may explain many of the insulin-sensitizing effects of the AMPK activator metformin.

In conclusion, for over a decade, PB was used as a prototypical inducer of CYP2B gene expression. The discovery of the importance of CAR in this process was a major step forward in the understanding of such regulation. We now provide evidence that AMPK links PB to CYP2B expression and suggest that AMPK can regulate CAR activity. The use of gene ablation, as well cDNA microarrays, highlights a large spectrum of genes regulated by CAR (53) including PEPCK and carnitine-palmitoyl transferase 1 (CPT1), genes encoding for key enzymes of glucose and lipid metabolism (53). It will be interesting to determine how many of these CAR-regulated genes are also regulated by AMPK.

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REFERENCES
1. Porter, T. D., and Coon, M. J. (1991) J. Biol. Chem. 266, 13469–13472
2. Xie, W., and Evans, R. M. (2001) J. Biol. Chem. 276, 37729–37742
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3. Goodwin, B., Moore, L. B., Stoltz, C. M., Mckeel, D. D., and Kliwer, S. A. (2001) Mol. Pharmacol. 60, 427–431
4. Pascussi, J. M., Gerbal-Chaloin, S., Fabre, J. M., Maurel, P., and Vilarem, M. J. (2000) Mol. Pharmacol. 58, 1441–1450
5. Conney, A. H. (2003) Annu. Rev. Pharmacol. Toxicol. 43, 1–30
6. Frucht, P. W., Zanger, U. M., and Meyer, U. A. (1997) Mol. Pharmacol. 51, 363–369
7. Waxman, D. J., and Azaroff, L. (1992) Biochem. J. 281, 577–592
8. Honkakoski, P., Zelko, I., Sueyoshi, T., and Negishi, M. (1999) Mol. Cell. Biol. 18, 5652–5658
9. Baes, M., Gulick, T., Choi, H., Martinoli, M. G., Simha, D., and Moore, D. (1994) Mol. Cell. Biol. 14, 1544–1552
10. Sueyoshi, T., Kawamoto, T., Zelko, I., Honkakoski, P., and Negishi, M. (1999) J. Biol. Chem. 274, 6043–6046
11. Kawamoto, T., Sueyoshi, T., Zelko, I., Moore, R., Washburn, K., and Negishi, M. (1999) Mol. Cell. Biol. 19, 6318–6322
12. Honkakoski, P., and Negishi, M. (1998) Biochem. J. 330, 889–895
13. Yoshida, Y., Kimura, N., Oda, H., and Kakinuma, A. (1996) Biochem. Biophys. Res. Commun. 229, 182–188
14. Blouin, R. A., Bandyopadhyay, A. M., Chaudhary, I., Robertson, L. W., Gemzik, B., and Parkinson, A. (1993) Arch. Biochem. Biophys. 303, 313–320
15. Xiong, H., Yoshini, K., Brouwer, K. L., and Carling, D. (2002) Drug Metab. Dispos. 30, 918–923
16. Zangar, R. C., and Novak, R. F. (1997) Arch. Biochem. Biophys. 337, 217–224
17. Winder, W. W., and Hardie, D. G. (1999) Am. J. Physiol. 277, E1–E10
18. Lochhead, P., Salt, I., Walker, K., Hardie, D., and Sutherland, C. (2000) Diabetes 49, 890–893
19. Ferre, F., Azrou-Marniche, D., and Foufelle, F. (2003) Biochem. Soc. Trans. 31, 220–223
20. Woods, A., Azrou-Marniche, D., Foretz, M., Stein, S. C., Lemarchand, P., Ferre, F., Foufelle, F., and Carling, D. (2000) Mol. Cell. Biol. 20, 6701–6711
21. Forrester, L. M., Henderson, C. J., Glancey, M. J., Back, D. J., Park, B. K., Ball, S. F., Kitteringham, N. R., McLaren, A. W., Miles, J. S., Skett, P., and Wolf, C. R. (1992) Biochem. J. 281, 359–368
22. Woods, A., Salt, I., Scott, J., Hardie, D. G., and Carling, D. (1996) FEBS Lett. 397, 347–351
23. Hawley, S., Gaddala, A., Olsen, G., and Hardie, D. (2002) Diabetes 51, 2420–2425
24. Sugden, C., Crawford, R. M., Halford, N. G., and Hardie, D. G. (1999) Plant J. 19, 433–445
25. Berry, M. N., and Friend, D. S. (1969) J. Cell. Biol. 43, 606–620
26. Armbruster, L., Cavard, C., Briand, P., and Bertolotti, R. (1992) Differentiation 50, 25–31
27. Thorens, B., Sarkar, H. K., Khasab, H. R., and Lodish, H. F. (1988) Cell 55, 281–290
28. Maenpas, P., Raivio, K., and Kekomaki, M. (1968) Science 161, 1253–1254
29. Gomez de Azua, M., Donato, T., Jover, B., Rodriguez, C., Ponzoda, X., Glaise, D., Castell, J., and Guguen-Guillouzo, C. (2001) Eur. J. Biochem. 268, 1448–1459
30. Bradford, M. (1976) Anal. Biochem. 72, 248–254
31. Hardie, D., Salt, I., and Davies, S. (2000) Methods Mol. Biol. 99, 63–75
32. Sahina, R., Patterson, D., and Holmes, E. W. (1985) J. Biol. Chem. 260, 6107–6114
33. Corten, J., Gillespie, J., Hawley, S., and Hardie, D. G. (1995) Eur. J. Biochem. 229, 558–565
34. Sullivan, J., Carey, F., Carling, D., and Beri, R. (1994) Biochem. Biophys. Res. Commun. 200, 1551–1556
35. Vincent, M. F., Marangos, P. J., Gruber, H. E., and Van den Berghe, G. (1991) Diabetes 10, 1259–1266
36. Longnus, S. L., Wambolt, R. B., Parson, H. L., Brownsey, R. W., and Allard, M. F. (2003) Am. J. Physiol. 284, R936–R944
37. LeCluyse, E. L. (2001) Eur. J. Pharmacol. Sci. 13, 343–368
38. Guillouzo, A., Morel, F., Langouet, S., Maheo, K., and Rissel, M. (1997) J. Hepatol. 26, 73–80
39. Antone, B., Levrat, F., Vallet, V., Berbar, T., Cartier, N., and Khan, A. (1992) Exp. Cell Res. 200, 175–185
40. Renouvel, F., Waeber, G., Antone, B., Rocchiccioli, F., Maudlar, P., Girard, J., and Leturque, A. (1996) Biochem. J. 314, 903–909
41. Grobholz, R., Hacker, H., Thorens, B., and Bannasch, P. (1993) Cancer Res. 53, 4204–4211
42. Wang, H., Faucette, S., Sueyoshi, T., Moore, R., Ferguson, S., Negishi, M., and LeCluyse, E. (2003) J. Biol. Chem. 278, 14146–14152
43. Baffet, G., and Corcos, L. (1995) Biochem. Biophys. Res. Commun. 216, 947–956
44. Sidhu, J., and Omiecinski, C. (1997) J. Pharmacol. Exp. Ther. 282, 1122–1129
45. Marc, N., Galisteo, M., Lagadic-Gossmann, D., Fautrel, A., Jonnard, F., Guillouzo, A., and Corcos, L. (2000) Eur. J. Biochem. 267, 963–970
46. Galisteo, M., Marc, N., Fautrel, A., Guillouzo, A., Corcos, L., and Lagadic-Gossmann, D. (1999) J. Pharmacol. Exp. Ther. 290, 1270–1277
47. Karvonen, I., Stengard, J. H., Saarni, H. U., Stenback, F., and Sotaniemi, E. A. (1987) Diabetes Res. 195–200
48. Hodges, N., Orton, T., Strain, A., and Chipman, J. (2000) Carcinogenesis 21, 2041–2047
49. Foretz, M., Carling, D., Guichard, C., Ferre, P., and Foufelle, F. (1998) J. Biol. Chem. 273, 14767–14771
50. Fryer, L. G., Parbu-Patel, A., and Carling, D. (2002) J. Biol. Chem. 277, 22926–22932
51. Sahi, J., Black, C. B., Hamilton, G. A., Zheng, X., Jolley, S., Rose, K. A., Gilbert, D., LeCluyse, E. L., and Sinz, M. W. (2003) Drug Metab. Dispos. 31, 439–446
52. Davies, G. F., Khandelwal, R. L., Wu, L., Jouurlink, B. H., and Roessler, W. J. (2001) Biochem. Pharmacol. 62, 1071–1079
53. Kodama, S., Koi, C., Negishi, M., and Yamamoto, Y. (2004) Mol. Cell. Biol. 24, 7931–7940
54. Schrauwen, P., Hardie, D. G., Roorda, B., Clapham, J. C., Ahuin, A., Thomas-Hughes, M., Green, K., Frederik, P. M., and Hesselink, M. K. (2004) Int. J. Obes. Relat. Metab. Disord. 28, 824–828
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