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The Anti-Apoptotic Factor GADD45B Regulates the Nuclear Receptor CAR-Mediated Transcription

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Running title: GADD45B as a CAR co-activator

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Abbreviations: CAR, constitutive active/androstane receptor; PB, phenobarbital; TCPOBOP, 1,4 bis[2-(3,5-dichloropyridyloxy)]benzene; MKK7, mitogen-activated protein kinase kinase 7; GADD45B, growth arrest and DNA-damage-inducible 45 beta; JNK, c-Jun N-terminal kinase.

Abstract

The nuclear receptor CAR up-regulated expression of the apoptotic GADD45B gene in HepG2 cells. Over expression of GADD45B augmented CAR-mediated induction of the human *CYP2B* gene by the CAR activator TCPOBOP and co-activated CAR-dependent transcription of the NR1-Luc reporter gene. siRNA knock down of GADD45B resulted in repression of both the induction and the co-activation. Induction of the mouse *Cyp2b10* gene by TCPOBOP was profoundly attenuated in the primary hepatocytes prepared from GADD45B-KO mice compared with those from wild type mice. Since CAR is a key transcription factor that activates the genes that encode for xenobiotic metabolizing enzymes and transporters, GADD45B, acting as a CAR co-activator and co-regulating CAR-target genes, may be involved in hepatic drug metabolism and excretion of xenobiotics.

Introduction

The nuclear receptor CAR is a member of the nuclear steroid/thyroid hormone receptor superfamily (Timsit and Negishi, 2007). CAR is characterized as a xenobiotic-sensing nuclear receptor that can be activated by a large number of xenobiotics including therapeutic drugs such as phenobarbital, phenytoin and various statins (Honkakoski et al., 1998; Jackson et al., 2004; Kobayashi et al., 2005). The activated CAR translocates from the cytoplasm to the nucleus of hepatocytes (Kawamoto et al., 1999). Once in the nucleus the CAR forms a heterodimer with RXR and binds to the promoters of its target genes and activates their transcription: these genes include the large set of hepatic genes that encode enzymes and transporters that are involved in xenobiotic metabolism and excretion (Sueyoshi et al., 1999; Sueyoshi et al., 2001). Human cytochrome P4502B6 (CYP2B6) is the classic CAR-regulated enzyme that metabolizes an ever increasing number of therapeutic drugs (*e.g.* bupropion, efavirenz, ifosfamide, methadone and meperidine) and activates the anti-cancer pro-drug cyclophosphamide (Zanger et al., 2007; Rodriguez-Antona and Ingelman-Sundberg, 2006; Schwartz et al., 2003). Through these processes, CAR constitutes a key part of the cellular defense mechanism against xenobitoic toxicity and carcinogenicity by increasing hepatic capability to detoxify and excrete xenobiotitics. Similar to the other nuclear receptors, the CAR-RXR heterodimer requires co-regulators in this activation (Muangmoonchai et al., 2001; Ueda et al., 2005; Makinen et al., 2003). A number of co-regulators have been demonstrated to be CAR co-activators or co-repressors: for example, GRIP1, SRC1, PGC-1α, PBP, FoxO1, SMRT and N-CoR (Jia et al., 2005; Ueda et al., 2005; Kodama et al., 2003; Shiraki et al.,

2003;Min et al., 2002; Ueda et al., 2002). We have now characterized GADD45B as being a novel CAR co-activator

GADD45B, an anti-apoptotic factor, is known to directly bind to MKK7 and inhibit MKK7-dependent phosphorylation of JNK, thereby repressing JNK-mediated apoptosis (Papa et al., 2004). Also the *Gadd45b* gene is one of the CAR-regulated genes that are induced by drugs such as PB and TCPOBOP (Columbano et al., 2005; Yamamoto et al., 2008). In addition, we have shown that CAR directly binds to the GADD45B protein, increasing the ability of GADD45B to inhibit MKK7 activity (Yamamoto et al., 2008). Consistent with the CAR-dependent inhibition of MKK7 activity, treatment with TCPOBOP attenuates JNK-mediated apoptosis in wild type mouse primary hepatocytes but not in GADD45B-KO primary hepatocytes. Thus, protein-protein interactions of CAR with GADD45B confer upon CAR the ability to determine GADD45B activity. Given their interactions, here we now examine whether or not GADD45B can regulate the trans-activation activity of CAR. To this end, we performed Real Time PCR and cell-based transient transfection assays to examine co-activation activity of GADD45B, employed siRNA to knock down GADD45B and used primary hepatocytes of GADD45B-KO mice to determine the role of GADD45B in CAR-mediated gene induction. The experimental observations obtained are consistent with the conclusion that GADD45B co-activates CAR-mediated transcription and is critically involved in drug induction of both human and mouse *CYP2B* genes.

Materials and methods

Materials

TCPOBOP was purchased from Sigma-Aldrich, Androstenol from Steraloids, HepatoZYME-SFM and Superscript First-Strand Synthesis System from Invitrogen, fetal bovine serumfrom Atlanta biological, and Complete mini protease inhibitor cocktail tablet from Roche. All other reagents were purchased from Sigma, unless indicated otherwise.

Plasmids

Using proper sets of primers, the full length of mouse GADD45B was amplified from mouse liver cDNA library using LA taq DNA polymerase (Takara, Japan) and was cloned into pcDNA3.1-TOPO plasmid, designated pcDNA3.1-GADD45B. The sequences of insert were verified by DNA sequencing. pGL3-tk-$(NR1)_{x5}$-Luc was previously constructed (Kawamoto et al., 2009) and pcDNA3.1 and PhRL-TK were purchased from Invirogen and Promega, respectively.

Animals

GADD45B-KO and the corresponding C57BL/6 mice, provided by Dr. Binfeng Lu at University of Pittsburgh, were housed in a temperature-controlled environment with 12 hr light/dark cycles with access to standard chow and water *ad libitum* under the protocols and procedures approved by the National Institutes of Health Animal Care and Use Committee in accordance with National Institutes of Health guidelines.

Cell culture

Ym17 cells, a stable cell line that expresses mCAR tagged with V5-His, were established from HepG2 cells (Swales et al., 2005). HepG2 and Ym17 cells were cultured in Minimal Essential Medium (MEM) supplemented with 10 % fetal bovine serum and antibiotics (100 units/ml of penicillin and 100 µg/ml of streptomycin). Cells in a 24-well plate were transfected with a pGL3-TK-NR1-luciferase reporter plasmid (0.2 µg/well) and phRL-TK plasmid (0.05 µg/well) by LipofectAMINETM 2000 (Invitrogen) according to the manufacture's instruction, with in additional co-transfection of 0.2 µg/well of pcDNA3.1-GADD45B or pcDNA3.1 empty plasmids. After 16 hours, the cells were subjected to treatment with chemicals; DMSO, 250 nM TCPOBOP, or 10 µM Androstenol for additional 24 hours. Luciferase activity was measured using the Dual-Luciferase reporter assay system.

siRNA transfection

GADD45B SMARTpool siRNAs were obtained from Dharmacon Research, Inc (Lafayette). Liposome solutions of one hundred pmol of GADD45B siRNA or control siRNA (siCONTROL Non-Targeting siRNA#1) were mixed with a separately prepared liposome solution with the pGL3-TK-NR1-luciferase reporter and phRL-TK plasmids. Subsequently, these liposome mixtures were transfected into Ym17 cells for LUC reporter assays. After 24 hours, the cells were treated with chemicals. Reduction of GADD45B expression was confirmed by Real Time PCR.

Mouse primary hepatocytes

Mouse primary hepatocytes were prepared from male mice by a two-step collagenase perfusion method and cultured as previously described (Honkakoski et al., 1997). Hepatocytes were suspended in Williams medium E supplemented with 7% fetal bovine serum, 1 X Liquid Media Supplement (5 μg/ml insulin, 5 μg/ml transferrin, 5 μg/ml selenite; Sigma-Aldrich), 2 mM L-glutamine and 30 mM pyruvate and were allowed to adhere to 24-well plates (1 X 10^5 cells) for 1 h in a CO2 incubator at 37°C. These hepatocytes were treated with DMSO or 250 nM TCPOBOP for 16 h in Williams medium E supplemented with 5 nM Dexamethasone and 1 X Liquid Media Supplement.

Real time PCR

Total RNA was isolated from HepG2, Ym17 and mouse primary hepatocytes using the TRIzol (Invitrogen). cDNAs were synthesized using the Superscript First-Strand Synthesis System and random hexamer primers. Real Time PCR measurement of individual cDNAs was performed with the ABI prism 7700 sequence detection system. For the probes used, assay ID number of pre-designed TaqMan Gene expression Assay (gene, assay ID number, PE Applied Biosystems) are: human GADD45B, Hs00169587_m1; human GADD45A, Hs00169255_m1; human CYP2B6, Hs00167937_g1; mouse CYP2B10, Mm00456591_m1; mouse CAR, Mm00437986_m1; mouse GADD45B, Mm00435123_m1. The TaqMan rodent GAPDH and human β-actin controls (PE Applied Biosystems) were used as internal control.

Results

CAR regulates the GADD45B gene: We previously characterized the *GADD45B* gene as being CAR-regulated in liver and primary hepatocytes using *Car*$^{-/-}$ mice (Yamamoto et al., 2008). In order for us to use HepG2 cells as an experimental system, we first tested whether CAR can regulate the *GADD45B* gene in HepG2 cells. Ym17 cells are derived from HepG2 cells and stably express mouse CAR tagged with V5-His and the mCAR-V5, spontaneously present in the nucleus, can be activated by the CAR ligand 1,4 bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) (Swales et al., 2005). Treatment with TCOPBOP increased the levels of GADD45B mRNA in a time-dependent manner, approximately 2.5-fold at 72 hr in Ym17 cells (Fig. 1A). On the other hand, treatment with the CAR antagonist androstenol slightly decreased the mRNA levels (Fig. 5B). These increases and decreases of GADD45B mRNA were not observed in HepG2 cells, indicating the direct involvement of CAR in the activation of the *GADD45B* gene. The *CYP2B6* gene was induced by TCPOBOP in the Ym17 but not in HepG2 cells (Fig. 5C and fig. 5D).

GADD45B co-activates CAR-mediated transcription: Given our previous observation that GADD45B and CAR interact (Yamamoto et al., 2008), we examined whether or not this affects CAR and how CAR activates enhancers. CAR activated a Luc reporter gene bearing the CAR binding site NR1 in Ym17 cells following treatment with TCPOBOP, whereas androstenol treatment repressed transcription (Fig. 2A). Subsequently, co-expression of GADD45B was found to up-regulate TCPOBOP-activated reporter activity 3- to 4-fold. To provide further evidence supporting this co-activation by GADD45B, we employed siRNA to knock down endogenous GADD45B in Ym17 cells. In cells

transfected with siRNA GADD45B, the CAR-mediated activation of NR1-Luc reporter gene as well as the expression of the endogenous *CYP2B6* gene were attenuated approximately 50% compared with those in cells treated with control siRNA (Fig. 2B and Fig. 2C). Under the conditions used, siRNA GADD45B down-regulated GADD45B mRNA approximately 60% and appeared to be specific to GADD45B since no decrease of the GADD45A mRNA occurred (Fig. 2D). These results are consistent with the conclusion that GADD45B is the co-activator of CAR.

GADD45B-KO attenuates CYP2B expression: If, in fact, GADD45B regulates CAR-mediated trans-activation in hepatocytes, the expression of CAR target gene by CAR activators such as TCPOBOP should be attenuated in hepatocytes that lack GADD45B. To this end, primary hepatocytes were prepared from wild type and GADD45B-KO mice and were treated with TCPOBOP. The *Cyp2b10* gene is the CAR-regulated CYP2B in mouse liver. TCPOBOP treatment increased the levels of CYP2B10 mRNA in wild type primary hepatocytes approximately 7-fold. In GADD45B-KO primary hepatocytes, both basal and induced levels of CYP2B10 mRNA were sharply repressed to approximately 20% of those as observed in the wild type primary hepatocytes (Fig. 3A). Despite the repression in GADD45B-KO primary hepatocytes, the fold induction of CYP2B10 mRNA by TCPOBOP remained approximately 9-fold, which is similar to that observed in wild type primary hepatocytes. These results suggest that GADD45B regulates, possibly acting as a co-activator, the maximal induction of the *Cyp2b10* gene. GADD45B did not regulate the expression of the CAR gene in the primary hepatocytes and, as expected (Fig. 3B). GADD45B mRNA was increased by TCPOBOP in wild type primary

hepatocytes and was not detected in the GADD45B-KO primary hepatocytes (Fig. 3C). Thus, the effective repression of the CYP2B10 mRNA in the GADD45B-KO primary hepatocytes confirmed that GADD45B performs the critical role in the CAR-mediated induction by TCPOBOP of the *Cyp2b10* gene.

Discussion

The anti-apoptotic factor GADD45B has now been shown to regulate CAR-mediated transcription of the *CYP2B6* gene in HepG2 cells as well as the *Cyp2b10* in mouse primary hepatocytes. Together with our previous finding of direct CAR interaction with GADD45B (Yamamoto et al., 2008), present cell-based transfection assays have demonstrated that GADD45B is a new transcriptional co-activator of CAR. Since GADD45B is a factor that may transfer signals into various cellular pathways, an indirect effect of GADD45B to co-activate CAR can not totally be eliminated as the possible regulatory mechanism at this present time. Nevertheless, the fact that expression levels of CYP2B10 mRNA by TCPOBOP in GADD45B-KO primary hepatocytes was reduced to 20% to the corresponding levels observed in wild type primary hepatocytes indicates that co-activation by GADD45B appears to be critical for maximal CAR activation of transcription of the *Cyp2b10* gene.

Responding to various endogenous stimuli, the *Gadd45b* gene is activated and GADD45B protein is involved in transducing various cell signals (Abdooahi et al., 1991). It is now found that CAR enables the *Gadd45b* gene to respond to xenobiotics. The biological consequences of xenobiotic activation of the *Gadd45b* gene remain an attractive subject for future research. CAR activators PB and TCPOBOP are non-genotoxic carcinogens and chronic CAR activation by these activators promotes development of hepatocellular carcinoma (HCC) in rodents (Yamamoto et al., 2004; Huang et al., 2005). We have demonstrated that treatment with TCPOBOP attenuates

TNFα-induced cell death of mouse primary hepatocytes only in the presence of both CAR and GADD45B, suggesting that CAR may promote HCC by down-regulating apoptotic JNK pathway (Yamamoto et al., 2008). PB induction of xenobiotic metabolizing enzymes such as CYP2B has also been suggested to be a factor responsible for PB promotion of HCC (Diwan et al., 2001), although this suggestion has not yet been experimentally demonstrated. Our present finding that GADD45B is a CAR co-activator raises an intriguing question as to whether the role of CYP enzymes in HCC development should be revisited. When rodents are treated with CAR activators, liver GADD45B increases and perhaps put the animals into a vicious cycle of continuous induction of the CYP2B enzyme (Fig. 4). Thus, in response to CAR activation, GADD45B co-ordinates attenuation of apoptosis and induction of the CYP2B enzyme and/or other CYP enzyme that may synthesize chemicals assisting HCC development.

The liver is endowed with the metabolic capability to detoxify xenobiotics including therapeutic drugs in order to counter toxicity and carcinogenicity caused by xenobiotics. Microsomal CYP enzymes provide this metabolic capability with the flexibility and adaptability for the liver to deal with an unlimited number of xenobiotics and their structurally diversity. This flexibility and adaptability comes from various unique characteristics of the CYP enzymes and *CYP* genes, one of which is the capability of *CYP* genes to exhibit altered transcriptional activity in response to xenobiotic exposure, thereby regulating the levels of CYP enzymes. Human CYP2B6 is one such CYP enzyme. CYP2B6 exhibits extreme individual variations in its basal as well as induced levels in human livers, which may affect how patients respond to drug therapy and

human susceptibility to xenobiotic exposure (Zanger et al., 2007). We previously found that insulin represses the mouse *Cyp2b* gene by down-regulating CAR-mediated transcription indirectly through the forkhead transcription factor FoxO1, but not by directly regulating CAR expression (Kodama et al., 2004). Here we have now identified GADD45B as being an example of indirect up-regulation of CAR-mediated transcription of the *CYP2B* genes. Thus, factors such as GADD45B and FoxO1 provide CAR with a molecular mechanism by which endogenous hormones and stimuli become the critical determinants of hepatic metabolic capability of xenobiotics.

Inflammatory stimulation by lipopolysaccharide (LPS) treatment increases GADD45B through a NF-kB pathway in mouse liver (Zhang et al., 2005). LPS treatment is known to strongly repress expression of the *Cyp2b* gene and its induction by PB in mouse liver and primary hepatocytes (Morgan, 1997; Li-Masters and Morgan, 2001). These responses to LPS treatment appear to contradict our conclusion that GADD45B co-activates CAR-mediated transcription. However, a more recent report demonstrated that LPS treatment greatly reduces the levels of CAR protein in mouse liver, resulting in the liver's inability to activate the *Cyp2b10* gene (Beigneus et al., 2002). Although its molecular mechanism is not known, this LPS-dependent reduction seems to be a nonspecific event since LPS reduces not only the levels of CAR but also those of PXR and RXRs as well. Once the non-specific reduction of CAR is eliminated, increases of GADD45B can be expected to be involved in co-regulation of CAR-mediated induction of the CYP2B enzyme. The extent to which the inflammation signal via GADD45B modulates the hepatic levels of

CYP2B genes and also the other CAR-target genes remains an intriguing question at the present time.

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Footnote

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Figure legends

Figure 1. CAR regulates expression of the *Gadd45b* gene. Ym17 and HepG2 cells were treated with DMSO, TCPOBOP and androstenol. At various time points after treatments, total RNAs were prepared from these cells and were subjected to Real Time PCR to measure the levels of GADD45B and CYP2B6 mRNAs. The levels of mRNA were normalized to β-actin mRNA levels and were shown in relative to mRNA levels in cells treated with DMSO set at one. Error bars show SD values from at least 3 experiments.

Figure 2. GADD45B co-activates CAR-mediated transcription. (A) Ym17 cells were co-transfected with pcDNA3.1-GADD45B and NR1-lucifearse reporter plasmid and were subsequently treated with DMSO, TCPOBOP and androstenol. These cells were homogenized to prepare cell extracts for measuring luciferase activity. The levels of luciferase (Luc) activity were normalized by with renilla luciferase activities. Fold activation was calculated relative to those in cells transfected with pcDNA3.1 and treated DMSO as one. (B) Prior to co-transfection with pcDNA3.1-GADD45B and NR1-lucifearse reporter plasmid, Ym17 cells were treated with GADD45B siRNA. Luciferase activities were measured and calculated by taking those in control siRNA- and DMSO-treated cells as one. (C) Total RNAs were prepared from the siRNA-treated cells and subjected to Real Time PCR to measure CYP2B6 mRNA. The levels of mRNA were normalized to β-actin mRNA levels and were shown in relative to mRNA levels in cells transfected with control siRNA and treated with DMSO as one. (D) Using total RNAs prepared from Ym17 cells treated with control and GADD45B siRNAs, the levels of

GADD45A and GADD45B mRNAs were measured by Real Time PCR and were shown relative to mRNA levels in cells transfected with control siRNA and treated with DMSO set at one. Error bars show SD values from at least 3 experiments

Figure 3. GADD45B-KO attenuates expression of the *Cyp2b10* gene. Primary hepatocytes were prepared from wild type and GADD45B-KO mice and were treated with DMSO (open bars) and TCPOBOP (closed bars). Total RNAs were prepared from these hepatocytes and were subjected to Real Time PCR. The levels of CYP2B10, CAR and GADD45B mRNAs are shown relative to those of corresponding mRNAs in DMSO-treated wild type primary hepatocytes set at one. Error bars show SE values from at least 3 experiments.

Figure 4. Cross talk regulation of GADD45B to co-activate CAR. Whether CAR directly activates the *Gadd45b* gene remains a subject for future investigations.

Figure 1

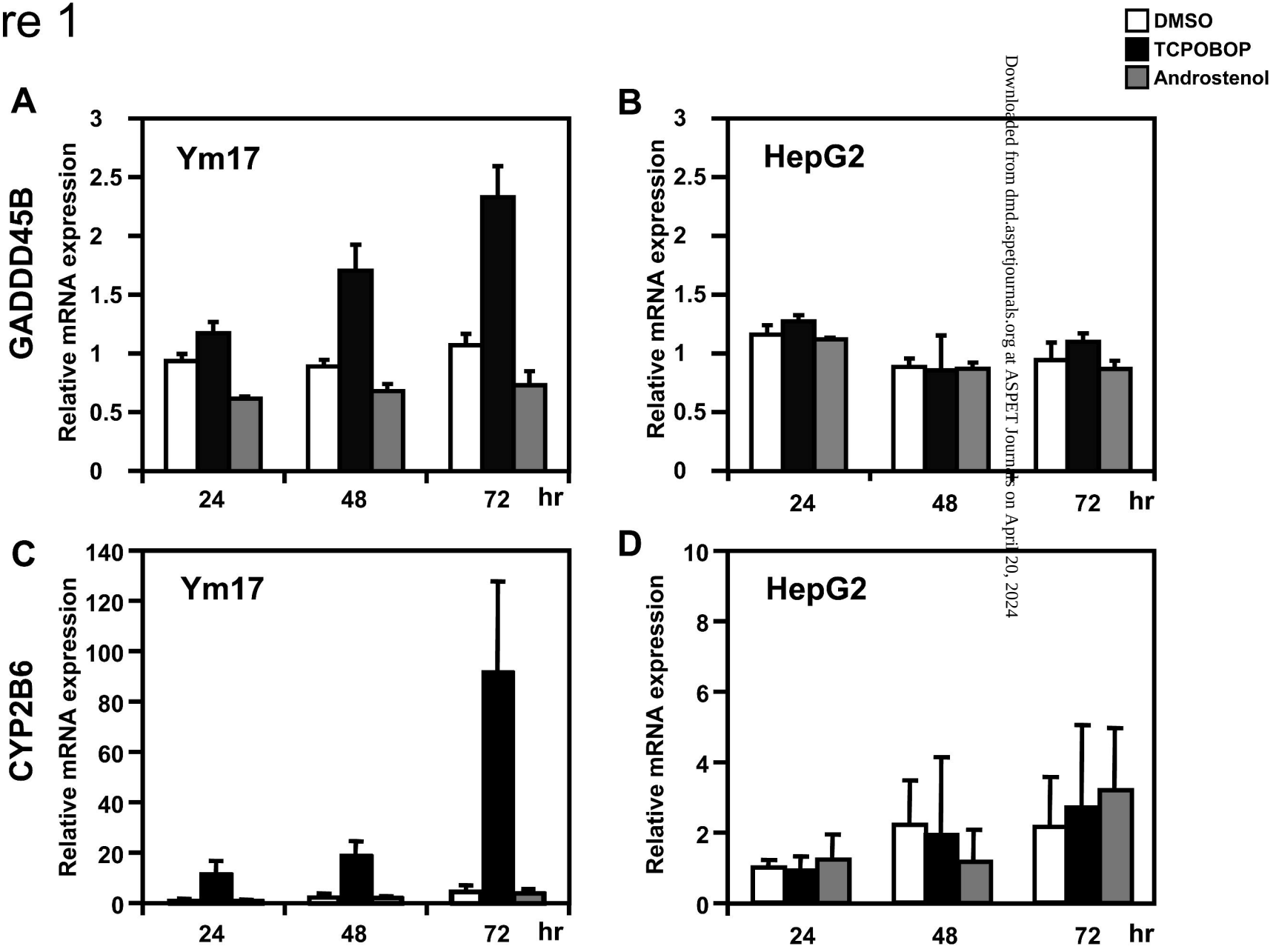

Figure 2

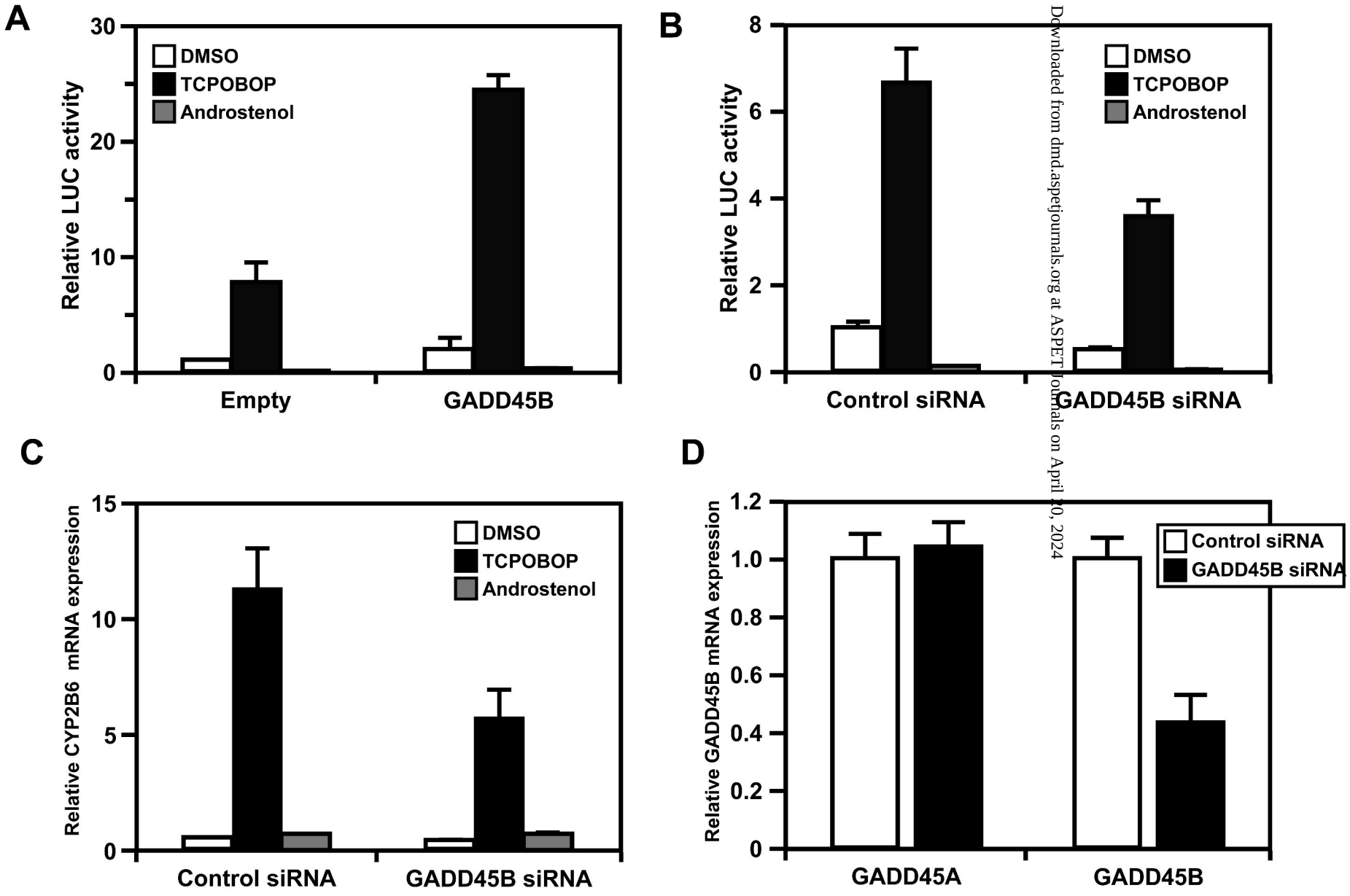

Figure 3

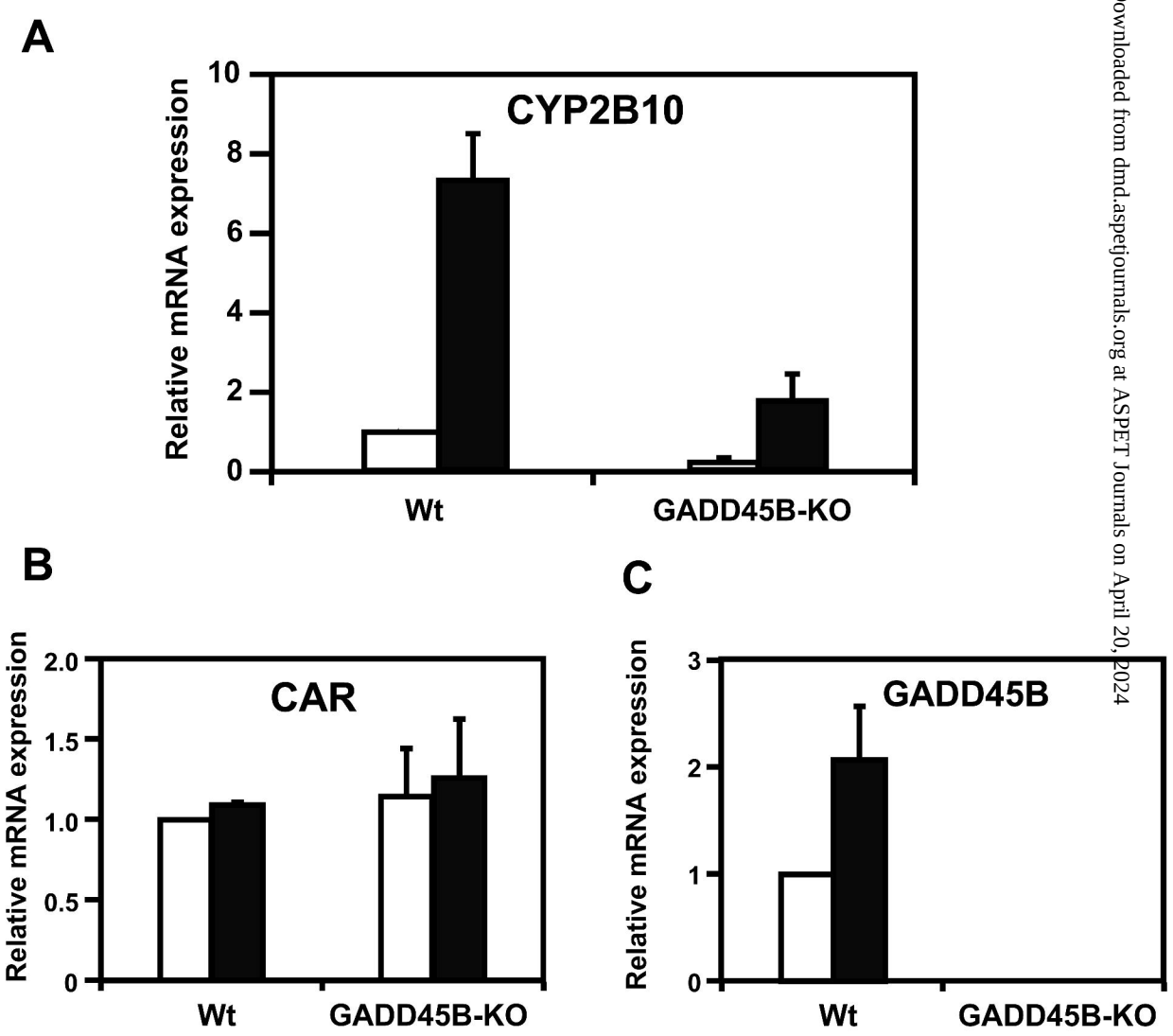

Figure 4