Activation of cAMP-Dependent Signaling Pathway Induces Mouse Organic Anion Transporting Polypeptide 2 Expression

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ABSTRACT

Rodent Oatp2 is a hepatic uptake transporter for such compounds as cardiac glycosides. In the present study, we found that fasting resulted in a 2-fold induction of Oatp2 expression in liver of mice. Because the cAMP-protein kinase A (PKA) signaling pathway is activated during fasting, the role of this pathway in Oatp2 induction during fasting was examined. In Hepa1c1c7 cells, adenylyl cyclase activator forskolin as well as two cellular membrane-permeable cAMP analogs, dibutyryl cAMP and 8-bromo-cAMP, induced Oatp2 mRNA expression in a time- and dose-dependent manner. These three chemicals induced reporter gene activity in cells transfected with a luciferase reporter gene construct containing a 7.6-kilobase (kb) S′-flanking region of mouse Oatp2. Transient transfection of cells with S′-deletion constructs derived from the 7.6-kb Oatp2 promoter reporter gene construct, as well as 7.6-kb constructs in which a consensus cAMP response element (CRE) half-site CGTCA (−1808/−1804 bp) was mutated or deleted, confirms that this CRE site was required for the induction of luciferase activity by forskolin. Luciferase activity driven by the Oatp2 promoter containing this CRE site was induced in cells cotransfected with a plasmid encoding the protein kinase A catalytic subunit. Cotransfection of cells with a plasmid encoding the dominant-negative CRE binding protein (CREB) completely abolished the inducibility of the reporter gene activity by forskolin. In conclusion, induction of Oatp2 expression in liver of fasted mice may be caused by activation of the cAMP-dependent signaling pathway, with the CRE site (−1808/−1804) and CREB being the cis- and trans-acting factors mediating the induction, respectively.

Organic anion transporting polypeptides (Oatps) belong to a family of uptake transporters for structurally diverse organic solutes, including organic anions, cations, and neutral compounds. In rodent species, Oatp2 (also known as Oatp1a4, Slc21a5) is highly expressed in liver, where it is localized to the basolateral membrane domain of hepatocytes (Hagenbuch and Meier, 2003). Oatp2 transports the common OATPs/Oatps substrates, such as estrone-3-sulfate, ouabain, and dehydroepiandrosterone sulfate (Hagenbuch et al., 2000; Hagenbuch and Meier, 2003). Oatp2 also transports thyroid hormones and the cardiac glycoside digoxin. The latter is not a substrate for other OATPs/Oatps except OATP1B3. Hepatic Oatp2 expression can be induced by xenobiotics via the activation of pregne X receptor and constitutive androstane receptor (CAR) signaling pathways (Hagenbuch et al., 2001; Guo et al., 2002; Wagner et al., 2005), whereas other liver-predominant OATPs/Oatps are not readily inducible. cAMP functions as a second messenger in cells in response to such extracellular stimuli as hormones. Binding of cAMP to the regulatory subunit of protein kinase A (PKA) results in the release of the catalytic subunit of PKA. PKA phosphorylates serine 133 of the transcription factor CAMP response element binding protein (CREB). Phosphorylated CREBs form a homodimer that binds to a cAMP response element (CRE) within the promoter region of target genes, controlling gene expression at the transcription level (Parker et al., 1996; Mayr and Montminy, 2001).

In liver, the maintenance of energy homeostasis during starvation and stress is controlled in part by a cAMP-PKA signaling pathway. In addition, several pathways for the metabolism and disposition of endobiotics and xenobiotics are regulated via cAMP-PKA pathways. For example, hepatic expression of heme oxygenase-1 and 5-aminolevulinate synthase, both of which are involved in heme metabolism, is induced via cAMP-PKA signaling pathways (Immenschuh et al., 2007). This work was funded by grant ES09649 from the National Institutes of Health.

ABBREVIATIONS: OATP/Oatp, human/nonhuman organic anion transporting polypeptide; CAR, constitutive androstane receptor; PKA, protein kinase A; CREB, CAMP response element binding protein; CRE, CAMP response element; dbcAMP, di-butyryl cAMP; 8-Br-cAMP, 8-bromo cAMP; PPAR, peroxisome proliferator-activated receptor; PCR, polymerase chain reaction; bp, base pair(s); Gapdh, glyceraldehyde-3-phosphate dehydrogenase; kb, kilobase(s); ACREB, dominant-negative CREB; DPM 904, 4-(3-pentylamino)-2,7-dimethyl-8-(2-methyl-4-methoxyphenyl)-pyrazolo[1,5-a]pyrimidine.
Results

Oatp2 mRNA in Liver of Fasted Mice. As shown in Fig. 1A, hepatic expression of Oatp2 was increased approximately 2-fold in mice that were fasted for 24 h compared with fed mice. The magnitude of Oatp2 induction in mice that were fasted for 24 h only. Oatp2 induction was attenuated in mice that were re-fed for 48 h after a 24-h fasting. A 2-fold induction of hepatic Oatp2 expression was also observed in PPARα-null mice that were fasted for 24 h only. Oatp2 induction was attenuated in mice that were re-fed for 48 h after a 24-h fasting.

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The presence and orientation of the cloned promoter fragment were verified by sequencing into the insert from both the 5' and 3' end of the pGL3 multiple cloning site. Synthesis of PCR primers and DNA sequencing were carried out by the Biotech Support Facility, University of Kansas Medical Center (Kansas City, KS).

5'-Deleted Mouse Oatp2 Reporter Gene Constructs. Four 5'-deleted Oatp2 reporter gene constructs (designated as 1.2, 2.6, 3.8, and 5.3 kb) were produced by PCR, using the original 7.6-kb mouse Oatp2 promoter reporter gene construct as a template. The forward primer was 5'-CTT CTC GAG GTG AGA AGT CCA CAC ATG AAG GAG-3'. The reverse primers were 5'-ACG CGT AAT ATC TCA GCT TTT CTT CTT CCT GA-3' (1.2 kb), 5'-ACG CGT TGT GTC TTT GGA CTT GTG TGC G-3 (2.6 kb), 5'-ACG CGT CAG CAA CAG CCT TGT GTA GCC GCT C-3 (3.8 kb), and 5'-ACG CGT CTC TGG CTA GGA CTT CAA CCA TTA-3 (5.3 kb). The PCR products were gel-purified and ligated to pGL3-basic luciferase reporter gene vector. The presence and orientation of the cloned promoter fragment were verified by sequencing into the insert from both the 5' and 3' end of the pGL3 multiple cloning site.

Site-Directed Mutagenesis. Site-directed mutagenesis was performed using QuickChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). The consensus CRE half-site (~1805/~1804 bp, 5'-CGTCA-3') in the 7.6-kb Oatp2 promoter identified by in silico analysis was deleted (designated as 17.6 kb) or mutated (designated as M7.6 kb). The complementary primers (-5'-GAACCTCGGCTGT-GTCTTTTACATGGTAAC-3’ and -5’-GTACTCAACGGAACGAGGGGGAGG-3’) were used to delete the CRE site. The underlines represent the positions of the deleted CRE site in the primers. The complementary primers (-5’-GAACCTCGGCTGT-GTCTTTTACATGGTAAC-3’ and -5’-GTACTCAACGGAACGAGGGGGAGG-3’) were used to produce AC to GT transition in the CRE site. The mutations and orientation of the cloned promoter fragment were verified by sequencing.

Transient Transfection and Luciferase Assay. Cells were seeded onto 24-well plates at a density of 5 x 10^4 cells/well and transfected using Lipofectamine 2000 reagent (Invitrogen) when reaching approximately 50 to 80% confluence. Where indicated, cells were transfected with mouse Oatp2 reporter gene constructs (500 ng) and pRL-TK vector encoding Renilla reniformis luciferase (50 ng), or reporter gene constructs along with expression plasmid encoding wild-type or mutant PKA catalytic subunit (50 ng) (Orellana and McKnight, 1992), as well as dominant-negative CREB (ACREB) (Ahn et al., 1998) or its empty vector CMV200 (2 and 10 ng). After transient transfection for 18 h, cells were treated with forskolin (5 µM) or cAMP analogs (0.5 mM) for 24 h. Cells were lysed and dual luciferase assays were performed according to the manufacturer’s instructions (Promega).

Materials and Methods

Materials. Forskolin was obtained from Calbiochem (La Jolla, CA). Di-butylryl cAMP (dbcAMP), 8-bromo cAMP (8-Br-cAMP), and dimethyl sulfoxide were purchased from Sigma (St. Louis, MO).

Animals. Male 6-week-old C57BL6 mice were purchased from Charles River Laboratories (Wilmington, MA) and maintained on a 12-h light/dark cycle. After acclimation for 10 days, mice were fasted for 24 h starting at 10:00AM with free access to water, or fasted for 24 h and re-fed for the following 24 h or 48 h. Fed mice were used as control subjects.

Breeding pairs of homozygous peroxisome proliferator-activated receptor (PPAR) α-null mice on an SV129 background were obtained from Dr. Frank Gonzalez (NCI, Bethesda, MD). Male PPARα-null mice (3–4 months old) were divided into two groups (n = 5 for each group): a 24-h fasting group and a control feeding group. At the end of experiments, liver was excised and rapidly frozen in liquid nitrogen. Samples were stored at −80°C until use.

Total RNA Isolation. Total RNA was isolated from frozen tissue samples using RNA-Bee reagent (Tel-Test, Friendswood, TX) according to the manufacturer’s instructions. After spectrophotometric quantification of RNA concentrations, samples were diluted with diethyl pyrocarbonate-treated water to a final concentration of 1 µg/µl. The integrity of the diluted RNA samples was determined by visual examination of the 18 and 28 S rRNAs separated on 1.2% denaturing agarose gel.

Cell Culture and Treatment. Hepa-1c1c7 cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F12 medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (Hyclone, Logan, UT), 100 µg/ml penicillin, and 10 µg/ml streptomycin (Invitrogen) at 37°C in a humidified incubator maintained at 5% CO2. For treatment, cells were seeded onto 24-well plates at a density of 5 x 10^4 cells/well and allowed to grow to 90 to 95% confluence. Cells were treated with various concentrations of forskolin, dbcAMP, 8-Br-cAMP, or vehicles (dimethyl sulfoxide for forskolin, PBS for dibutyryl cAMP and 8-bromo cAMP) for 4, 8, 18, or 24 h. At the end of treatment, cells were lysed with lysis mixture provided in the branched DNA assay kit (Genospectra, Fremont, CA). Cellular lysate was stored at −80°C until use.

Quantification of Mouse Oatp2 mRNA. Mouse Oatp2 mRNA was quantified using Quantigene branched DNA signal amplification assay (Genospectra). Branched DNA probe set for mouse Oatp2 was reported previously (Cheng et al., 2005). Branched DNA assay was performed per manufacturer’s instructions, using cellular lysate directly or total RNA isolated from liver tissue samples. Oatp2 mRNA expression was normalized to Gapdh mRNA expression. Results were presented as arbitrary units.

Mouse Oatp2 Promoter Reporter Gene Construct. A BAC clone (RP24-309B5) containing mouse Oatp2 gene was used as template to produce a 7.6-kb fragment containing the 5'-flanking region of mouse Oatp2 (from −7561 bp to +39 bp), using PCR (forward primer, 5'-CTT CTC GAG GTG AGA AGT CCA CAC ATG AAG GAG-3' ; reverse primer, 5'-CTT ACG CGT CAC ATG TTG CCA CTT ATA GGG TTG-3'). Underlined letters indicate XhoI and MluI restriction sites introduced into the primers. The 7.6-kb PCR product was gel-purified and ligated to luciferase reporter gene vector, pGL3-basic (Promega, Madison, WI). The presence and orientation of the cloned promoter fragment were verified by sequencing into the insert from both the 5’ and 3’ end of the pGL3 multiple cloning site. Synthesis of PCR primers and DNA sequencing were carried out by the Biotech Support Facility, University of Kansas Medical Center (Kansas City, KS).
Oatp2 Induction in Hepa1c1c7 Cells. Hepa-1c1c7 cells were treated with various concentrations of the adenylyl cyclase activator, forskolin (0.5, 1, 2, and 10 μM), or two membrane-permeable analogs of cAMP, dbcAMP and 8-Br-cAMP (0.01, 0.1, 0.5, and 1 mM) for 18 h. forskolin, dbcAMP, and 8-Br-cAMP dose dependently induced Oapt2 expression in Hepa-1c1c7 cells (Fig. 2A). The maximal induction was approximately 4-fold.

Hepa-1c1c7 cells were also treated with forskolin (5 μM), dbcAMP or 8-Br-cAMP (0.5 mM) for 4, 8, 18, or 24 h. As shown in Fig. 2B, Oatp2 expression in Hepa-1c1c7 cells was induced in a time-dependent manner. Maximal induction was observed after 18 h of treatment.

Induction of Luciferase Activity Driven by Oatp2 Promoter. A luciferase reporter gene construct containing a 7.6-kb promoter region of mouse Oatp2 gene (from −7561 bp to −39 bp) was generated as described under Materials and Methods. To determine whether the 7.6-kb Oatp2 promoter region contains the DNA sequences that mediate induction of Oatp2, Hepa-1c1c7 cells were transfected with this Oatp2 reporter gene construct. Then, transfected cells were treated with forskolin (5 μM), dbcAMP (0.5 mM), 8-Br-cAMP (0.5 mM), or vehicle. Luciferase activity driven by the 7.6-kb Oatp2 promoter was induced by 4- to 6-fold after treatment (Fig. 3). These results indicate that the regulatory DNA sequences that mediate Oatp2 induction are located within the 7.6-kb promoter.

Oatp2 Promoter Deletion Analysis. To further determine the location of the DNA sequences that mediate Oatp2 induction, a series of 5′-deletion constructs of the 7.6-kb Oatp2 reporter gene construct were generated as described under Materials and Methods. The size of the deletion constructs ranges from 1.2 to 5.3 kb. Hepa-1c1c7 cells were transfected with the reporter gene constructs of different sizes. Transfected cells were treated with forskolin for 18 h. forskolin did not affect the reporter gene activity driven by the 1.2-kb Oatp2 promoter (Fig. 4A). Luciferase activity driven by the other Oatp2 promoters (2.6, 3.8, 5.3, and 7.6 kb) was induced by forskolin.

Protein kinase A is a key component of the cAMP-dependent pathway. The PKA catalytic subunit phosphorylates the transcription factor CREB in response to elevated intracellular cAMP levels. Therefore, the effects of PKA catalytic subunit on gene expression were determined to further establish the role of cAMP-dependent pathway in the induction...
of Oatp2. Hepa-1c1c7 cells were cotransfected with expression plasmid encoding PKA catalytic subunit and the Oatp2 promoter reporter gene constructs. As shown in Fig. 4B, cotransfection of wild-type PKA expression plasmid did not induce luciferase activity driven by the 1.2-kb promoter. However, cotransfection of wild-type PKA expression plasmid caused a 3-fold induction of luciferase activity driven by 2.6-, 3.8-, 5.3- and 7.6-kb Oatp2 promoter, respectively. The results from this experiment support our hypothesis that a cAMP-dependent pathway plays a role in Oatp2 induction.

**Reporter Assay Using Oatp2 Reporter Gene Constructs in Which a Putative CRE Is Deleted or Mutated.** In silico analysis of the Oatp2 promoter identified a consensus CRE half-site, CGTCA, located at approximately 1.8 kb upstream from the transcription starting site. This element is not present in the 1.2-kb promoter construct but is present in the other larger constructs. Based on the results of the promoter deletion study (Fig. 4), it is possible that this consensus CRE half-site may be involved in Oatp2 induction.

To further determine whether this CRE half-site is needed for the induction of gene expression, transient reporter gene assays were performed using Oatp2 promoter reporter gene constructs in which this putative CRE was deleted or mutated. As shown in Fig. 5A, deletion or mutation almost completely abolished the inducibility of luciferase activity after forskolin treatment. Deletion or mutation of the same element also abolished the inducibility of luciferase activity in cells that were cotransfected with expression plasmid encoding wild-type PKA catalytic subunit (Fig. 5B).

**Effects of ACREB on Inducibility of Luciferase Activity Driven by Oatp2 Promoter.** To establish CREB as the trans-acting factor that mediates Oapt2 induction, transient reporter gene assays were performed using the dominant-negative CREB described previously. This dominant-negative CREB can dimerize with the wild-type CREB and prevents the wild-type CREB from binding to DNA. It is clear that cotransfection of the dominant-negative CREB (2 and 10 ng) abolished the inducibility of luciferase activity after forskolin treatment (Fig. 6). The results strongly indicate that CREB is the trans-acting factor mediating Oatp2 induction.

**Discussion**

In this study, a 2-fold induction of hepatic Oatp2 in fasted mice was observed (Fig. 1). Further experiments using Hepa-1c1c7 cell line provided several lines of evidence indicating that activation of cAMP-PKA pathway contributes to this
induction: 1) treatment of cells with forskolin and two cAMP analogs dose- and time-dependently induced Oatp2 mRNA, 2) forskolin treatment induced luciferase activity driven by a 7.6-kb Oatp2 promoter, 3) the location of the DNA sequence mediating the induction was narrowed down to approximately −1200 to −2600 bp by deletion analysis of a 7.6-kb Oatp2 promoter reporter gene construct, 4) deletion and mutation of the consensus CRE half-site, CGTCA, located at −1804 bp, abolished the inducibility of luciferase activity, and 5) cotransfection of a dominant-negative CREB abolished the inducibility of luciferase activity (Figs. 2–6). CREB regulates gene expression by binding to a CRE that exists either as a palindrome (TGACGTCA) or half-site (CGTCA/TGACG) (Mayr and Montminy, 2001). According to a recent study (Zhang et al., 2005), most conserved CREs found in gene promoter regions are located within 200 bp of the transcription start site. It is noteworthy that in this study, a functional CRE half-site was identified at −1804 bp. Taken together, the consensus CRE half-site at −1808/−1804 bp and CREB seem to be the cis-and trans-acting factors, respectively, mediating the induction of Oatp2.

During fasting, the body relies more on fatty acids and ketones than on glucose for energy production. This adaptive metabolic response is controlled in part by the nuclear receptor PPARα. Kok et al. (2003) reported that PPARα activation leads to induction of multidrug resistance transporter 2 in liver of fasted mice. Moreover, phosphorylation of PPARα by PKA has been shown to increase its activity (Lazennec et al., 2000), suggesting that PPARα pathway, to some extent, functions downstream of the PKA pathway. Therefore, the role of PPARα in Oatp2 induction during fasting was examined using PPARα-null mice. Our results indicated that Oatp2 induction is independent of PPARα, although loss of PPARα seems to lead to elevated basal expression of Oatp2 in liver of PPARα-null mice (Fig. 1B). Maglich et al. (2004) reported that fasting induces the expression of CAR target genes such as Cyp2b10 and Ugt1a1 in a receptor-dependent manner. These authors proposed that CAR could be activated without ligand binding in response to metabolic and nutritional stress. Future study using CAR-null mice will help to elucidate the inter-relationship between CAR and cAMP-PKA.
signal pathway in the regulation of Oatp2 and other genes during fasting.

Caloric restriction has been shown to decrease serum thyroid hormone levels in mice and human subjects (Rosenbaum et al., 2000; Maglich et al., 2004). Maglich et al. (2004) attributed this phenomenon to increased hepatic metabolism of thyroid hormones via glucuronidation and sulfation pathways. Wong et al. (2005) reported that induction of hepatic Oatp2 expression in rats treated with a compound known as DPM 904 is associated with increased hepatobiliary clearance of unconjugated thyroid hormones and decreased serum thyroid hormone concentrations. Therefore, it is possible that increased hepatic expression of Oatp2 may be another factor contributing to the accelerated thyroid hormone elimination during caloric restriction. This speculation is in accordance with our observation that Oatp2 was induced in liver of fasted mice (Fig. 1). Besides Oatp2 and other OATPs/Oatps, other types of transporters, such as monocarboxylate transporter 8 and 10, have been shown to transport thyroid hormones according to results from in vitro transport assays (Friesema et al., 2005). However, a correlation between altered expression of thyroid hormone transporters and changes in serum hormone levels has not been previously reported, making it difficult to ascertain the in vivo importance of these various transporters in the disposition of thyroid hormones. Our current findings, together with those of others, suggest that Oatp2 may play a role in the hepatic elimination of thyroid hormones, and that this process is induced via cAMP-PKA pathway during starvation and may cause increased elimination of thyroid hormones and decreased body energy expenditure.

It is well known that fasting often increases susceptibility to chemical-induced liver injury. In some cases, the increase in susceptibility in fasted animals can be attributed to either induction of P450 isozymes or depletion of hepatic glutathione levels. In light of our findings that hepatic Oatp2 expression was induced in fasted mice, it is possible that hepatic induction of Oatp2 during fasting increases hepatic uptake of drugs and hepatotoxins that are Oatp2 substrates, contributing to the increase in susceptibility to liver toxicity.

In conclusion, we found that fasting resulted in induction of hepatic expression of Oatp2 in mouse. Activation of cAMP-PKA pathway seems to be the underlying mechanism for the induction.

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References


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