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Induction of human UDP-glucuronosyltransferase 1A1 by cortisol-GR

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Abstract

During the course of the study of UGT1A1 induction by bilirubin, we could not detect the induction of the reporter gene (−3174/+14) of human UGT1A1 in HepG2 by bilirubin (Mol. Biol. Rep. 31: 151–158 (2004)). In this report, we show the finding of the induction of the reporter gene of UGT1A1 by cortisol at 1 μM, a major natural cortico-steroid, with human glucocorticoid receptor (GR). RU486 of a typical GR antagonist at 10 μM inhibited the induction by cortisol from 5.9- to 1.8-fold. This result indicates that the induction by cortisol-GR is dependence on ligand-binding. This induction is caused by the UGT reporter gene itself, from the results of noinduction with control vector pGL2 (equal to pGV-C) in the presence of cortisol-GR. We confirmed that the induction of the reporter gene by cortisol is dependent on the position of proximal element (−97/−53) of UGT1A1. From this result, we concluded that the increase of cortico-steroid in neonates must induce the elevation of UGT1A1 after birth and prevent jaundice. With the study of induction by corisol, we studied the influence of co-expression of PXR (pregnenolone xenobiotic receptor) with the UGT1A1 reporter gene and we could not find the induction of UGT1A1 expression in the presence of dexamethasone, rifampicin, or pregnenolone 16α-carbonitrile of the PXR ligands. These results suggest that the induction of UGT1A1 expression by GR is not mediated by PXR, unlike the induction of CYP3A4 through PXR.

Abbreviations: CAR – constitutive androstane receptor; EMSA – electrophretic mobility shift assay; GR – human glucocorticoid receptor; HNF – hepatocyte nuclear factor; PE – proximal element; PXR – human pregnenolone xenobiotic receptor; RIF – rifampicin; UGT – UDP-glucuronosyltransferase

Introduction

UDP-glucuronosyltransferase (UGT) transfers the glucuronic acid in UDPGA to ligands, such as steroid hormones, catecholamines, and bilirubin, to make water-soluble glucuronides [1, 2]. UGT1A1 is an essential enzyme, because only UGT1A1 among several UGTs conjugates bilirubin with glucuronic acid to decrease the bilirubin level in plasma and to prevent jaundice, and the conjugate was excreted in bile by MRP2 (multi-drug resistance-associated protein 2). UGT1A1 is a main enzyme in the conjugation of bilirubin and a defect in UGT1A1 causes hyperbilirubinemia and jaundice, such as Crigler-Najjar syndrome type II and Gilbert's syndrome. We previously analyzed the promoter region (−3174/+14) of the human UGT1A1 gene in relevance to Gilbert's syndrome [3–6]. Gilbert's syndrome of hyperbilirubinemia without liver injury is found in approximately 2–6% of the total population. There are many UGT species and they are classified into the UGT1 and UGT2 families, with a molecular mass of 50–57 kDa [7]. The human UGT1A subfamily gene is found on chromosome 2, and 9 kinds of UGT1 can be made by alternative

splicing between several of exon 1 and a constant of exon 2–5 [4]. Also, the UGT2 family gene is found on chromosome 4 [8]. From a genetic map of the UGT1A subfamily, 4 pseudo genes of exon 1 have been declared [9, 10].

Ueyama et al. [3] reported two transcriptionally regulatory elements in the promoter region (up to −3174) by transient transfection assay; one was the distal element (DE, −1346/−1204) and the other was the proximal element (PE, −97/−54). PE consisted of two regions, E-box (−88/−79) and an HNF-1 site (−75/−63). Bosma et al. and Monaghan et al. [11, 12] reported that one-third of the patients of Gilbert's syndrome had a TATA box mutation, i.e, $(TA)_7TAA$ instead of $(TA)_6TAA$. Sato et al. compared the promoter activity of the two TATA boxes, but did not find a substantial difference [5]. These results suggest that the TATA box mutation itself is not the major cause of the syndrome. Some patients with Gilbert's syndrome had simultaneous mutations in the TATA box and in the coding region (G71R) [5]. Koiwai et al. [6] considered that the higher frequency of the syndrome, compared to the frequency of the gene, might depend on the dominant negative phenomenon.

Regarding the enhancer region of UGT1A1, which is activated by phenobarbital (PB), a drug for the treatment of jaundice, PB response enhancer element (PBREM) was found at position −3483/−3194 as a functional site in upstream region of UGT1A1 [13]. Meanwhile, we found the induction of the reporter gene (−3174/+14) of UGT1A1 in HepG2 by dexamethasone [14]. However, we could not find stimulation by β-estradiol, phenobarbital and rifampicin [14]. We investigated the position playing a role in the induction by dexamethasone in the promoter region of UGT1A1 using deletion mutants. The region −97/−53 is essential for induction by dexamethasone. This region contains HNF1 element, therefore, we suggest that dexamethasone directly and/or indirectly stimulates UGT1A1 expression through this HNF1 region in the promoter region of UGT1A1. Thus, we clarified that UGT1A1 was induced by dexamethasone and the key position was the region (−97/−53) in UGT1A1 promoter [3, 14].

UGT is not expressed in embryos and neonates but is found after birth (neonatal jaundice disappears after expression of UGT1A1). It has been suggested that the expression of UGT1A1 after birth depend on the increase of some hormones, such as corticosteroids and thyroxine [15–17]. In the course of clarifying the promoter region of human UGT1A1, we studied the stimulation of UGT1A1 by certain drugs, and found the stimulation by dexamethasone of a typical drug corticosteroid [14]. In this report, we show the induction of UGT1A1 expression by cortisol-GR. Cortisol is a major natural glucocorticoid.

Materials and methods

Materials

The drugs used were dexamethasone (DEX) from Wako Chemicals, rifampisin (RIF), cortisol from Sigma Chemicals, pregnenolone 16α-carbonitrile (PCN) and RU486 [18] (mifeprostone) from CaHK, Japan. These were dissolved in DMSO and added to culture medium at appropriate times for transfection assays. Fetal bovine serum (FBS)(Trace) treated with charcoal was used for cell culture in the stimulation with drugs. This treatment was carried out to remove endogenous stimulaters, such as steroids, as follows [3, 14]. The charcoal solution, made of 0.25% Norit A SX-II (Wako Chemicals) and 0.025% Dextran T70 (Amersham) in 0.25 M sucrose, 1.5 mM $MgCl_2$, and 10 mM Hepes (pH 7.4), was mixed overnight at 4 °C. The charcoal was collected by centrifugation at $500 \times g$ for 10 min and was mixed with FBS. The mixture was centrifuged at $500 \times g$ for 10 min, and then the supernatant containing FBS was treated once more with charcoal and centrifuged. The supernatant after centrifugation was treated at 56°C for 45 min and sterilized by filtration through a 0.45-μM membrane (Becton–Dickinson). FBS not treated with charcoal was used in the general cell culture medium.

Plasmid

The plasmids used were pSV-β-galactosidase vector (Promega), pGV-B (Wako), pGV-C (Wako), pSG5 basic vector (Stratagene), pSG5-hGR vector (a gift from Dr. Pierre Chambon of IGBMC, France) [19], and pSG5-hPXR vector (a gift from Dr. Steven A Kliewer of Dallas Southwestern Medical Center, USA) [20]. Deletion mutants

containing the 5′-upstream promoter region of human UGT1A1 in the luciferase reporter plasmid pGV-B (Wako) were prepared as described in a previous report [3, 14]. DNA from these constructions for transfection was prepared on a large scale by ultra-centrifugation in CsCl-ethidium bromide and the DNA concentration was adjusted to 1 μg/μl.

Culture of HepG2

The human cell line used in this study was the hepatoma-derived HepG2 [14]. HepG2 cells were maintained at 37 °C in 95% air and 5% CO_2 in sterilized Dulbecco's modified Eagle's medium (DMEM, phenol red-free) supplemented with 10% FBS. Transplantation of cells was carried out at 70–80% confluence. The dish was washed with PBS (-, Mg-free) and then the cells were collected by treatment with PBS (-) containing 0.02% EDTA and 0.05% trypsin. The solution of cells was diluted to one-third and transplanted to dishes.

Transfection assay

Transfection of the deletion mutants in the promoter region of UGT1A1 in luciferase reporter plasmid was done by the standard calcium phosphate method [3]. HepG2 cells were plated in a 12-well culture plate at 30–40% confluence and transfected after 18 h. In general, the transfection mixture contained 2 μg of hUGT1A1 deletion mutants, 400 ng of β-galactosidase expression vector (pSV-β-galactosidase), and either 400 ng of pSG5, pSG5-hGR or pSG5-hPXR in 0.2 ml of Hepes-buffered saline and 0.125 M $CaCl_2$ per well. After 6 h, the medium was replaced with DMEM supplemented with 10% FBS, treated with charcoal, and the cell were treated with various chemicals for 48 h. Chemicals were dissolved in DMSO and diluted in DMEM-FBS medium. The HepG2 cells in the dish were washed with PBS. The cells were lysed, and analyzed for luciferase and β-galactosidase activities [3, 14]. Luciferase activity was measured with a luminometer (ARVO 1420) as follows. HepG2 cells were harvested in 100 μl of lysis buffer (25 mM Tris–phosphate (pH 7.8), 2 mM DTT, 2 mM *trans*-1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid, 10% glycerol, 1% TritonX-100). Cell lysates (25 μl) were mixed with 50 μl of luciferin reaction mixture (20 mM Tricine, 1.07 mM $(MgCO_3)_4$ $Mg(OH)_2$5-H_2O, 2.67 mM $MgSO_4$, 0.10 mM EDTA, 0.53 mM ATP, 33.3 mM DTT, 0.27 mM coenzyme A, 0.47 mM luciferin). Light output was measured for 5 s, and luciferase activity was expressed as relative light units. β-galactosidase activities were determined using a standard *o*-nitro-phenyl-β-D-galactopyranoside assay.

Results

In the previous report, we showed the induction of the reporter gene of UGT1A1 with dexamethasone of a synthetic glucocorticoid [14]. In this report, we show the induction of the reporter gene (−3174/+14) of UGT1A1 with cortisol of a major natural glucocorticoid. Figure 1 shows that GR itself (DMSO) did not induce, and the induction of UGT1A1 expression was done through the cooperation of cortisol at 1, 10 and 100 μM with GR. The levels at 1 and 10 μM cortisol were significantly increased.

Figure 2 shows the effect by the GR antagonist RU486 on the induction of UGT1A1 expression by cortisol at 10 μM in the presence of GR.

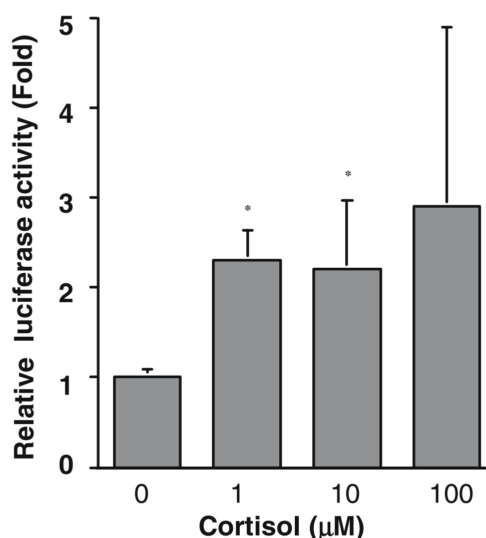

Figure 1. Induction of the reporter gene of UGT1A1 by cortisol in the presence of GR. Dose-dependent stimulation of the reporter gene by cortisol in the presence of GR. The values indicate the means ± SD of three determinants. Statistical significance is; *, $p < 0.05$.

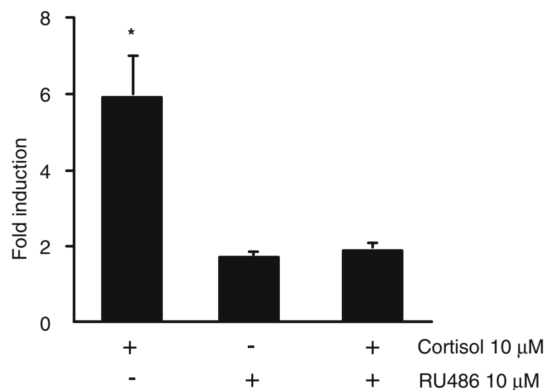

Figure 2. In hibition of cortisol induction of the reporter gene of UGT1A1 by RU486. Influences of RU486 (10 μM) (GR antagonist) on the induction by cortisol at 10 μM. The values indicate the means ± SD of three determinants. Statistical significance is; *, $p < 0.05$.

RU486 inhibited the induction by cortisol by from 5.9- to 1.8-fold. These results indicate that the induction by GR depends on the ligand binding manner of GR. We ascertain that this induction is caused by the UGT reporter gene itself, from the results of noinduction with control vector pGL2 in the presence of corisol-GR.

Figure 3a shows the position responsible for the induction of the reporter gene by cortisol in the presence or absence of GR. We found clear induction with plasmids #2 (−2598/+14), #5 (−1351/+14) and #15 (−97/+14), but not with plasmid #16 (−53/+14). Plasmid #16 did not contain the position (−97/−53) responsible for the induction by cortisol-GR. Ueyama et al. have previously shown two functional positions, DE (−1344/−1204) and PE (−97/−54), on the 5′-upstream promoter region of UGT1A1[3]. This result shows that the PE region is essential for induction by cortisol. PE region is composed of PE1 site (−88/−79) and PE2 (−75/−63) as shown in Figure 3b. PE2 is the DNA element for biding of HNF1 and HNF1 site might be contained in the site that interacts with cortisol-GR.

Figure 4 shows the influence of co-expression of PXR. These results indicated that PXR did not induce the luciferase reporter gene of UGT1A1 in the presence of dexamethasone. PXR-RIF and PXR-PCN also did not induce the reporter gene, although RIF and PCN are good ligands for PXR. These results clarified that the luciferase reporter gene binding UGT1A1 promoter gene (−3174/+14) did not contain the elements for the binding of ligands for PXR. Meanwhile, it has been reported that the element for PXR is present at the position −3430/−3285 in PBREM [21, 22].

Thus, we confirmed the induction of the reporter gene of UGT1A1 by cortisol. This result suggests the role of endogenous gluco-corticoid

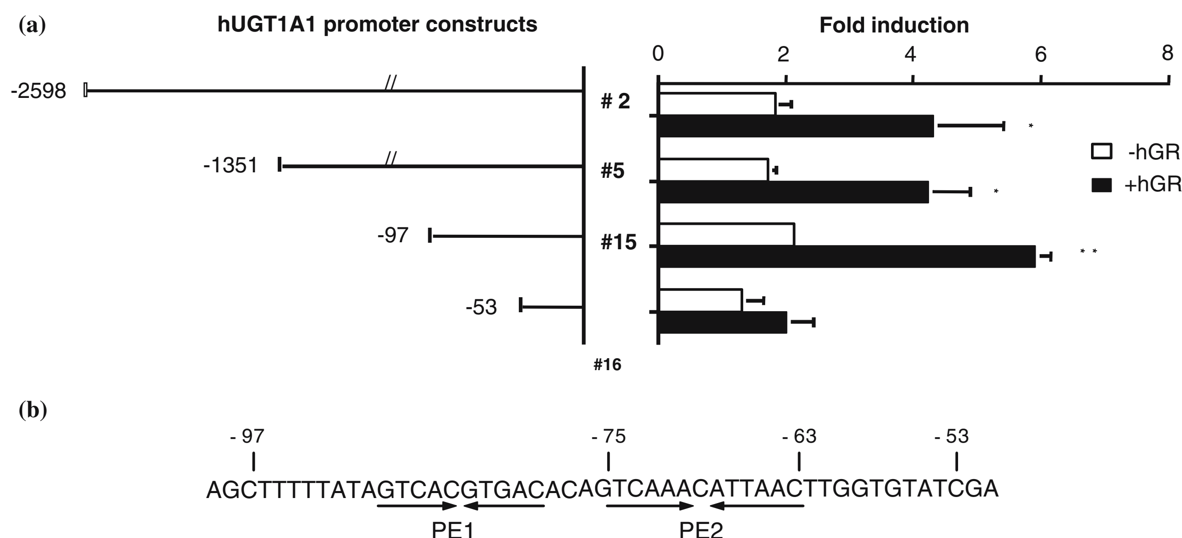

Figure 3. (a) The assignment of the functional site for the stimulation by cortisol-GR. The left side shows the length of the promoter region upstream UGT1A1. The right side shows the induction level by cortisol in the presence (black) or absence (white) of GR. Statistical significance is; *, $p < 0.05$: **, $p < 0.01$. (b) The sequence of the functional site (−97/−53) for the stimulation by corisol-GR.

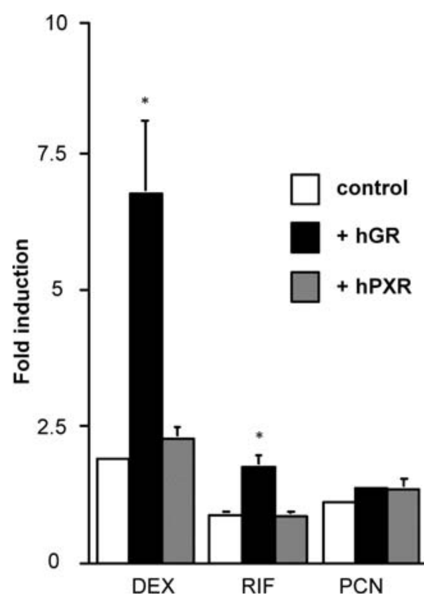

Figure 4. Influences of certain drugs on the induction of UGT1A1 in the absence (white) or presence of GR (black), or the presence of PXR (shade). The concentrations of DEX, RIF, and PCN were 100, 10, and 10 μM, respectively. The vector used for transfection was #12 (−542/ + 14). The values indicate the means ± SD of three determinants. Statistical significance between control and + receptor is; *, $p < 0.05$.

hormones in the regulation of the expression of UGT1A1 and endogenous corticoid hormones might play a role of induction of UGT1A1 expression by steroid shower after birth and prevention of neonatal jaundice [15–17].

Discussion

We found the induction of UGT1A1 by cortisol in the presence of GR in this report. The general finding that cortisol induction of UGT1A1expression for bilirubin conjugation plays a major role after birth is significant. The fact that cortisol production and the need for UGT1A1 expression are coordinated at the same time is also important finding. The finding of the promoter region for the cortisol induction just a few bases upstream between −97 and −53 is also important manifest. The result of the inhibition with RU486 is good evidence to confirm the role of the GR receptor for the induction of UGT1A1 expression. In embryos, UGT is absent and UGT1A1 is expressed after birth with an increase in corticosteroid hormones. Our results suggest that the increase of cortisol after birth is a trigger of the induction of UGT1A1, which plays a role in the disappearance of neonatal jaundice. Huang et al. [23] suggested that bilirubin with CAR might relate to neonatal jaundice. Thus, we speculated that cortisol could be another partner for the regulation of the production of UGT1A1 to prevent neonatal jaundice.

We found the high induction of human UGT1A1 expression by cortisol in the presence of GR. The level of constitutive GR in HepG2 is low and the induction by cortisol is low in the absence of GR. Meanwhile, the level of UGT1A1 expression was increased by cortisol in the presence of GR. This induction by cortisol-GR was inhibited by RU486, a GR antagonist. This clarified that the elevation of UGT1A1 is dependent on a cortisol-GR complex. It has been reported that dexamethasone induces PXR and RXR (retinoid xenobiotic receptor) expression in human hepatocytes, which indicates a synergistic increase of CYP3A4 induction by PXR activators [9]. In addition, transcription of the CYP2C9 gene is regulated by recognition of GRE (−1684/−1648) and CAR (constitutive androstane receptor) element (−1856/−1783) by GR and CAR, respectively [18]. The orphan nuclear factor CAR controls UGT1A1 and other factors involved in drug metabolism and bilirubin clearance [24]. In this work, RIF, a PXR activator, did not stimulate the transcription of UGT1A1, therefore, the signal of cortisol might not pass through PXR, but cortisol may be directly bound to GR.

PBREM was found in the CYP2B gene, which is induced by PB. PBREM (−3483/−3194) in human UGT1A1 has been found to be an important module for stimulating the transcription of UGT1A1 [13]. PBREM contains PXR (SXR) for induction by steroids. We could not find induction by β-estradiol, esterone or ethynylestradiol [14], because our vector DNA of UGT1A1 did not contain PBREM [3]. Our promoter region (−3174/ + 14) was well regulated by DEX, from results using certain deletion mutants [3] and those of this study. We confirmed that the region for enhancing the luciferase reporter gene is PE, which does not contain GRE but the element for HNF1 (PE2). We showed the relation of GR with HNF1 element on UGT1A1. GR did not induce the level of HNF1 but increased the binding of HNF1 protein to HNF1 element by the EMSA method and this may not depend on the amount of HNF1 (unpublished).

There have been some reports of the synergistic stimulation of GRE and HNF1 element by phanylalanine hydrolase [25] and IGFBP [26]. The synergistic stimulation of PXR and GRE was also reported for CYP2C9 [13] and CYP3A4 [11]. HNF1 is expressed in the liver and the deletion of HNF1 is lethal. HNF1 is related to the transcription of many liver proteins such as albumin and fibrinogen, whose genes have the element for binding HNF1α and HNF1β. HNF1 also plays a role in expression of UGT1A1 in mice, and the mutation in the HNF1 gene causes hyperbilirubinemia in mice [27, 28]. Ueyama et al. [3] and Bernard et al. [27] have shown that the HNF1 sequence in UGT1A1 is important for the expression of UGT1A1, and in this paper we showed that GR induced UGT1A1 through the HNF1 site on the UGT1A1 promoter region. The induction mechanism at the HNF1 site in the PE region (−97/−54) of the UGT1A1 promoter by DEX remains to be resolved.

Another importance of cortisol induction of UGT1A1 expression is on carcinogen metabolism and conjugation. The fact that the UGT1A1 plays a role to conjugate aromatic amine and PAH may be related to induction of UGT1A1 by cortisol. The risk from exposure to tobacco smoke or overcooked meat carcinogen in breast milk may be impacted by cortisol and its timing of induction. This would be an important link of cortisol and exposure of carcinogen after birth.

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