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Xenobiotic- and Vitamin D-responsive Induction of the Steroid/bile acid-sulfotransferase Sult2A1 in Young and Old Mice: The role of a Gene Enhancer in the Liver Chromatin

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Abstract

The xenobiotic-activated nuclear receptors PXR (pregnane X receptor) and CAR (constitutive androstane receptor) and the vitamin D₃-activated nuclear receptor VDR regulate steroid and xenobiotic metabolism by inducing the phase I cytochrome P450 monooxygenases, phase II conjugating transferases, and the phase III transporters, which mediate the efflux of water-soluble lipid metabolites from cells. Metabolic stress due to the deviant expression of steroid- and xenobiotic-metabolizing enzymes is known to have severe health consequences including accelerated aging, and increased expression of these enzymes is associated with extended longevity (Gachon et al, 2006; McElwee et al, 2004). Information on the similarities and dissimilarities in drug metabolism between the young and old, as may be uncovered by studying aging regulation of the genes relevant to steroid and xenobiotic metabolism, is likely to have clinical significance. In this report, we examined the VDR- and PXR-mediated gene induction of the phase II sulfotransferase Sult2A1 in the livers of 4-month and 20-month old mice. Sult2A1 converts bile acids, steroids and a number of drugs to the corresponding sulfated metabolites, which are readily eliminated from the body due to increased water solubility. In RT-PCR assay, aging did not change the induction of Sult2A1 mRNAs by the hormonally active vitamin D₃ and the catatoxic synthetic steroid PCN (pregnenolone-16 α -carbonitrile). Chromatin immunoprecipitation (ChIP) from liver nuclei showed that aging had no effect on the activity of an IR0 enhancer in the *Sult2A1* chromatin to recruit VDR, RXR- α (retinoid X receptor) and PXR in mice injected with D₃ or PCN. Thus, mice in late life are as competent as those in early life in responding to the hormonal and xenobiotic signaling for *Sult2A1* induction. This is the first report describing the role of aging in the functional response of an enhancer in the liver chromatin to the nuclear receptor-dependent signaling.

Keywords

Aging; Sulfotransferase; Gene Enhancer; Nuclear Receptor; Tissue ChIP

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1. Introduction

The xenobiotic-regulated nuclear receptors PXR (pregnane X receptor) and CAR (constitutive androstane receptor) play central roles in the induced expression of the enzymes and transporters that mediate the metabolism and clearance of endogenous sterols and steroids and diverse xenobiotics including therapy related drugs (Handschin and Meyer, 2003; Xu et al, 2005). The xenobiotic stress induced by an aberrant metabolome is associated with severe health adversity (Gachon et al, 2006; Green and Takahashi, 2006). Thus, a thorough understanding of the regulatory underpinnings for these enzymes and proteins should be insightful in identifying potential targets amenable to therapeutic modulations. Furthermore, differential phase I/II/III activity profiles between the young and old, including the profiles for drug metabolism and disposition, could arise from the altered expression of steroid- and drug-metabolizing enzymes/transporters during aging. An assessment in this regard is necessary to develop remedial strategies in the elderly. In the present study young and old mice were examined for the induced expression of the phase II sulfotransferase SULT2A1 in the liver upon administration of the synthetic catatoxic steroid PCN (pregnenolone-16 α -carbonitrile), which is an agonist ligand for PXR, or vitamin D₃, which binds and activates the vitamin D receptor (VDR). Vitamin D₃ has a known role in xenobiotic metabolism in addition to its classic function in bone physiology (Makishima et al. 2002; Reschly and Krasowski, 2006; Song et al, 2006). Additionally, we probed the mouse liver chromatin to investigate the association of nuclear receptors and coregulators with a vitamin D- and xenobiotic-responsive enhancer in the *Sult2A1* promoter.

SULT2A1 is a sulfo-conjugating enzyme with substrate predilection for hydroxysteroids, bile acids and certain medicinal compounds. The enzyme is expressed at high abundance in the first-pass tissues (liver and intestine) and in the steroidogenic tissue of the adrenal gland. Multiple nuclear receptors including PXR, CAR and VDR can induce SULT2A1 in cultured cells and *in vivo* in the mouse liver (Song et al, 2001; Sonoda et al, 2002; Echchgadda et al, 2004a; Assem et al, 2004; Maglich et al, 2004; Fang et al, 2005; Seeley et al, 2005; Chatterjee et al, 2005; Song et al, 2006). We had shown earlier that the vitamin D₃- and xenobiotic-mediated induction of the mouse *Sult2A1* promoter in transfected liver and intestinal cells is dependent upon binding of the corresponding receptors to a 21-base pair inverted repeat (IR0) enhancer (Echchgadda et al, 2004b). Chromatin immunoprecipitation (ChIP) assay showed that the IR0 in the *Sult2A1* chromatin was enriched for VDR in the livers of vitamin D₃-injected mice, thus validating the enhancer's role in a tissue context (Song et al, 2006). In the present report we show that aging has no significant influence on the qualitative and quantitative profiles of the Sult2A1 mRNA induction by vitamin D₃ or PCN. Also, young and old mice responded similarly to the vitamin D₃-/PCN-induced nuclear receptor recruitment at the IR0. This is the first report in which the liver chromatin from young and old mice was analyzed for the functional response to the nuclear receptor signaling.

2. Materials and Methods

2.1 Hormone and drug injections, semi-quantitative RT-PCR, PCR primers

The hormonally active vitamin D (1 α ,25-dihydroxy vitamin D₃, hereon D₃) at 50 μ g hormone/kg body weight/day or pregnenolone-16 α -carbonitrile (PCN) at 50 mg/kg body weight/day was injected intraperitoneally into male mice (C57Bl/6J) for four days. As vehicle, control mice received 0.05M propylene glycol in PBS (for vitamin D₃) or DMSO (for PCN). Mice were purchased from the National Institute on Aging (Bethesda); they were handled as per the approved institutional animal care protocol. Total liver RNAs, isolated by Trizol (Invitrogen), were analyzed semi-quantitatively for Sult2A1 mRNAs using reverse transcription-polymerase chain reaction (RT-PCR). Reverse transcription of RNAs and PCR amplification

of the reverse transcribed cDNAs were as described (Echchgadda et al, 2004b). PCR primers were selected from the N-terminal end of the mouse *Sult2A1* cDNA in order to avoid cross reactivity with any other SULT family members. The following primer sets were used: *Sult2A1*, 5'-GAAGGCATACCTTTT-CCTGCCAT (at +51, forward); 5'-GTAACCA-GACACAAGAATATCTCT (at +419, reverse). *Gapdh*, 5'-GTATTGGGCGCCTGGTCACCAG (at + 121, forward); 5'-CCTTCTCCATGG-TGGTGAAGAC (at + 410, reverse).

2.2 Chromatin immunoprecipitation (ChIP) with liver samples

i) Preparation of solubilized liver chromatin fragments—ChIP was performed on the mouse liver nuclear lysate using the procedure of Sandoval et al, 2004, with minor modifications. Fresh liver (~2 gm) was placed on ice-cold phosphate buffered saline, pH 7.5 (PBS) in a Petri dish, chopped in small pieces (~0.2 gm each), rinsed with fresh PBS and then shaken gently in 1% formaldehyde (in PBS) using an orbital shaker (10 min, room temp.). Cross linking was stopped by adding 125mM glycine, pH 3.0 (10 min, room temperature). The fixed tissue, rinsed once (ice-cold PBS), was incubated in 2 volumes of ice-cold lysis buffer (5mM HEPES pH8.0, KCl 85mM, NP40 0.5% and a protease inhibitor cocktail from Sigma at 1:1000 dilution) for 30 min at 0°C. The lysed tissue was homogenized for 30 seconds on ice (Polytron™ homogenizer, 10 mm diameter), incubated further on ice (15 min) and the homogenate was centrifuged (3500× g, 5 min) to pellet nuclei. The nuclei were lysed with 10 mM EDTA, 1% SDS, 50mM TrisHCl, pH 8.1 at 1:1 (initial tissue weight/volume). The lysate was incubated on ice (10 min), spun quickly (4°C) and the supernatant was sonicated using 3 separate sonication bursts at 30% amplitude (Vibra-Cell VCX-500 sonicator from Sonics and Materials). Each burst lasted 25 sec (at a pulse-grade 7) with a 2-minute resting interval in-between. The sonicated and centrifuged samples (4°C, 10 min, 13,000 rpm) were analyzed on a 1% agarose gel to ensure a 300–800 base pair size range of the fragmented DNAs. The supernatant was diluted 10-fold in a buffer containing the protease inhibitor cocktail and the DNA concentration was measured at OD₂₆₀.

ii) Specific immunoprecipitation of the chromatin DNA and PCR amplification—Immunoprecipitation of the solubilized chromatin, recovery of the cross-linked protein-DNA complex, reversal of the cross link, and recovery of the immunoprecipitated DNA were carried out as described (Song et al, 2006). All antibodies were from the Santa Cruz Biotechnology (Santa Cruz, CA). Briefly, the immunoprecipitated cross-linked complexes were incubated with protein A-agarose (Upstate Biotechnology, Lake Placid, NY), washed sequentially with low salt buffer, high salt buffer and LiCl buffer and eluted from the protein A-agarose bead. After reversing the cross-linking, DNA was isolated from proteinase K-digested samples and PCR was performed to amplify the mouse *Sult2A1* promoter region that spans the IR0 enhancer. The denatured template (100 ng) was amplified for 33 cycles (95°C, 30 sec; 55°C, 30 sec; 72°C, 40 sec) in the presence of: 5'-GCATTTCTATGTCCTATTAC (forward primer at -231) and 5'-GATATGATATGGCAGG-AAAAGGT (reverse primer at + 81).

3. Results

3.1. Induction of *Sult2A1* mRNAs and the IR0 enhancer in the mouse liver chromatin

Sult2A1 mRNAs were induced in the mouse liver upon the administration of 1,25 (OH)₂D₃ (D₃) and concomitantly, the IR0 enhancer in the *Sult2A1* chromatin was enriched for VDR, RXR-α (the retinoid X receptor), RNA polymerase II and the p160 coactivator SRC-1, as shown in Figure 1. *Sult2A1* mRNAs were induced ~3-fold in RT-PCR analysis when young-adult male mice (4-month-old) were injected with D₃ (Fig. 1A). *Gapdh* mRNA expression was the normalization control. To determine fold induction, the amplified DNA bands of the RT-PCR

assay corresponding to the control and experimental mice were quantified using a Gel documentation system (Gel Doc™ EQ, Bio-Rad, Hercules, CA). A standard curve was generated from a plot of the PCR band density (Y axis) against the volume of the reverse transcribed reaction mixture used in the PCR amplification (X-axis). The X axis parameter is proportional to the level of *Sult2A1* mRNAs in the reaction mixture. The relative levels of *Sult2A1* mRNAs in the hormone-injected and control mice were determined from the linear range of the standard curve and fold induction was calculated. The RT-PCR data is in line with our earlier Northern blot results, which showed 3.8 to 6-fold induction of *Sult2A1* mRNAs in D_3 -injected mice (Chatterjee et al, 2005; Song et al, 2006). Animal-to animal variation can explain the 3- to 6-fold induction range.

A direct role of VDR in *Sult2A1* induction was demonstrated by immunoprecipitation (IP) of solubilized chromatin fragments from the livers of mice with or without the hormone treatment (Fig. 1B). Hormone injection caused significant enrichment of the immunoprecipitable VDR at the IR0-spanning *Sult2A1* chromatin. The retinoid X receptor (RXR- α) was also enriched at the IR0 in a D_3 -dependent manner. RXR- α is a heterodimer partner of VDR and thus binds to VDR target genes at the vitamin D response element. Specificity of the receptor association with the IR0 is evidenced by the lack of a PCR signal in the lanes corresponding to chromatin IP with the Cox-2 antibody.

The D_3 -responsive transcriptional induction paralleled recruitment of RNA polymerase II to the target IR0 site (Fig. 1B). Furthermore, among the p160 coactivators only SRC-1 showed enrichment at the IR0 in response to D_3 . SRC-2 and SRC-3 did not show hormone-dependent enrichment at IR0. The weak signals observed in the absence of the hormone injection are likely due to *Sult2A1* expression promoted by the normally circulating D_3 . The enrichment of SRC-1 at the IR0 appears to be specifically responsive to the acute increase in the D_3 level. However, our earlier ChIP data on the human *SULT2A1* chromatin showed that upon D_3 treatment of HepG2 human hepatoma cells, both SRC-1 and SRC-2 were inducibly associated with the vitamin D response element that we had identified in the human *SULT2A1* gene. On the other hand, SRC-3 was not detected at this site either basally, or in D_3 -treated cells (Song et al, 2006).

3.2. Aging and vitamin D_3 responsiveness of the mouse liver chromatin

Young and old mice responded similarly for *Sult2A1* mRNA expression and for VDR and RXR- α recruitment at the IR0 upon D_3 injection (Figure 2). Thus, the livers of 4-month and 20-month old mice showed ~ 3-fold and ~ 2-fold induction of *Sult2A1* mRNAs, respectively in RT-PCR assay (Fig. 2A). The PCR signals in 20-month-old mouse livers are more intense than those in 4-month-old animals. This increased signal intensity is in line with a higher expression of *Sult2A1* mRNAs during aging (Echchgadda et al, 2004a).

Chromatin IP showed that young and old mice were almost equally effective in the D_3 -mediated enrichment of VDR at IR0. Likewise, the extent of D_3 -responsive RXR- α occupancy at the IR0 was similar in the young and old mice. The specificity of the liver-directed ChIP assay was evident from the absence of PCR signals in lanes where the Cox-2 antibody was used to immunoprecipitate chromatin fragments. These results suggest that both the pre-transcriptional and transcriptional responses of the *Sult2A1* gene to the VDR-induced signaling remain unaltered during physiological aging.

3.3. Xenobiotic-mediated induction of the *Sult2A1* gene

The effect of aging on responses to the xenobiotic signaling was explored in PCN-injected mice (Figure 3). The catatoxic steroid PCN, which is a derivative of the C21 steroid

pregnenolone, renders protection to animals against drugs and toxins by stimulating xenobiotic metabolism (Selye, 1971). PCN is also a glucocorticoid antagonist. The catatonic activity of PCN is based on its ligand agonist activity toward PXR, thus stimulating the expression of the enzymes and proteins involved in the steroid and xenobiotic metabolism. Therefore, PCN-mediated gene expression can be taken as a measure of drug responsiveness. *Sult2A1* mRNAs were induced by PCN to similar extents, i.e. 3-fold versus 2.5-fold in the livers of 4-month- and 20-month-old mice, respectively (Fig. 3A). The mRNA induction is due to promoter activation since we had shown earlier that in PXR cotransfected HepG2 cells, luciferase expression driven by the mouse *Sult2A1* promoter increased several fold by the activated PXR, and this induction was mediated through the IRO enhancer (Echchgadda et al, 2004b).

The IRO's activity to recruit PXR and its heterodimer partner RXR- α in young and old mice was investigated using chromatin IP (Fig. 3B). Higher amounts of PXR and RXR- α specifically associated with the IRO-spanning *Sult2A1* chromatin in the PCN-injected mouse liver compared to the control liver. Aging did not alter the IRO activity in this regard. Fig. 2 and Fig. 3, taken together, lead us to conclude that the responses of *Sult2A1* to the vitamin D₃ and PCN signaling are refractory to the age-associated changes in the metabolic and endocrine milieu of the liver.

4. Discussion

It is of significant interest to know how aging might modulate xenobiotic metabolism, since extended life span is known to be associated with increased expression of the enzymes and proteins that are involved in the metabolism of steroids, drugs and other xenobiotics (McElwee et al, 2004; Amador-Noguez et al, 2004). Transcription profiling has revealed that as in development, physiological aging is associated with differential expression of a wide variety of genes that have important metabolic relevance (Park & Prola, 2005; Darlington, 2004). For example, after age 40 the frontal cortical tissue in humans shows reduced expression of a set of genes involved in synaptic plasticity, vesicular transport and mitochondrial function, whereas genes with roles in the DNA repair, stress response and anti-oxidative response were up regulated (Lu et al, 2004). Nevertheless, specific chromatin events leading to the age-related changes in the transcriptional activity of specific genes, including those for drug-metabolizing enzymes, are not known. The use of tissue-directed ChIP to probe the transcriptional response of the *Sult2A1* gene in young and old mice is a useful advance in this regard. We show that young and aged tissues responded similarly with respect to the hormone- (vitamin D₃) and drug- (PCN) mediated induction of *Sult2A1*. Additionally, our results show that an IRO gene enhancer in the liver chromatin, which directs the vitamin D₃ and PCN induction of *Sult2A1*, is active in recruiting VDR, RXR- α and PXR to almost equal extents in young and old animals in response to the hormone (D₃) or drug (PCN) challenge.

The age effect of the nuclear receptor CAR on the induction of the *Sult2A1* gene was not examined in the current study. Nevertheless, the CAR-regulated hepatocyte proliferation and the induction of a Forkhead Box transcription factor in the liver were not altered during aging (Ledda-Columbano et al, 2004). Thus, it is likely that the regulation of *Sult2A1* by CAR is also unaffected by age. It is important to note, however, that a lack of CAR expression is associated with epileptic seizure in young mice and accelerated aging in mice older than 9 months (Gachon et al, 2006). Thus the aging process appears to affect a selected set of gene networks that are regulated by the CAR-mediated nuclear signaling.

ChIP provides a biochemical means to capture a snapshot of proteins that are associated with specific chromatin regions in intact cells, and its application on tissue samples is useful in gaining insights into the transcriptional regulation of genes in a physiologically relevant setting.

For example, metamorphosis related differential coregulator recruitment to specific genes has been studied by the ChIP analysis of tadpole tissues; zebrafish development has been examined using ChIP on embryonic tissues; ChIP has also been used on the mouse liver chromatin to analyze specific enhancer complexes (Parrizas et al, 2001; Chaya and Zaret, 2003; Havis et al, 2003; Havis et al, 2006). The present report further extends the usefulness of tissue-directed ChIP to study chromatin events during aging.

SULT2A1 is member of the cytosolic sulfotransferase (SULT) family that includes 47 individual SULTs (Blanchard et al, 2004). Bile acid detoxification and clearance in humans is mediated exclusively through SULT2A1-directed sulfonation (Palmer, 1971). Thus, SULT2A1 activity plays an important role in cholesterol and bile acid metabolism. Aging is associated with marked up regulation of the *Sult2A1* gene in the rodent liver – a regulatory process that is explained, in part, by the loss of androgen receptor expression in the aging liver (Echchgadda et al, 2004a). Hormone- and age-regulated changes in SULT2A1 expression may alter cholesterol homeostasis due to altered bile acid clearance; changes in SULT2A1 expression may also have impacts as to how steroids, drugs and xenobiotics are metabolized. Therefore, illuminating the mechanism underlying SULT2A1 expression during aging is important in order to assess the physiological and clinical impacts of the SULT2A1 activity. In view of the exclusive role of SULT2A1 in bile acid clearance; its role for other endobiotic and xenobiotic substrates in first-pass metabolism; its age-dependent differential expression; and its induction by multiple nuclear receptors, a detailed knowledge of the genomic events at the *SULT2A1* chromatin has the potential to provide insightful clues to the effective management of diseases related to aberrant steroid and drug metabolism.

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Abbreviations

PCN	pregnenolone-16 α -carbonitrile
D₃	1 α ,25-dihydroxy vitamin D ₃
IR0	inverted repeat-zero spacer
VDR	vitamin D receptor
PXR	pregnane X receptor
RXRα	retinoid X receptor α
CAR	constitutive androstane receptor
ChIP	chromatin immunoprecipitation
RT	reverse transcription
PCR	polymerase chain reaction

Figure 1. Sult2A1 gene induction in the liver of vitamin D₃-injected mice

1A) Induction of Sult2A1 mRNAs as assayed by semi-quantitative RT-PCR. 4-month-old mice were injected either with vehicle (0.05M propylene glycol) or D₃, using two mice in each treatment group. 1B) Activity of the IR0 enhancer to recruit VDR, RXR-α, RNA polymerase II (pol II), SRC-1, SRC-2 and SRC-3 in the D₃-injected mice. In tissue ChIP assay, IR0-containing liver chromatin fragments of the mouse Sult2A1 promoter were immunoprecipitated with the test antibodies and the specifically immunoprecipitated DNAs were analyzed on an agarose gel subsequent to PCR amplification. The COX-2 antibody served as a negative control.

Figure 2. Vitamin D₃ responsiveness of the Sult2A1 gene in young and old mice

2A) RT-PCR analysis of Sult2A1 mRNAs in the livers of vitamin D₃-injected 4 month and 20 month old mice. The numbers at the top in the D₃-treated lanes indicate fold increase of Sult2A1 mRNAs in the young (3-fold) and old (2-fold) compared to vehicle treatment. The fold increase was calculated from the scanned gel bands as described under Results. 2B) Immunoprecipitation of solubilized liver chromatin fragments to assay for the enrichment of VDR and RXR-α at the IR0 in vitamin D₃-injected 4 month and 20 month old mice. The COX-2 antibody was a negative control.

Figure 3. Sult2A1 gene induction in PCN-injected young and old mice

3A) RT-PCR analysis of the Sult2A1 mRNA induction in the livers of 4-month and 20-month old PCN-injected mice. Numbers at the top indicate the fold induction of Sult2A1 mRNAs after PCN injection compared to the vehicle control. 3B) ChIP analysis of liver chromatin from young and old mice to determine the activity of IR0 to recruit PXR and RXR- α in response to PCN injection.