In vivo activation of the constitutive androstane receptor β (CARβ) by treatment with dehydroepiandrosterone (DHEA) or DHEA sulfate (DHEA-S)

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Abstract We investigated whether dehydroepiandrosterone (DHEA) or DHEA-sulfate (S) affected the activities of nuclear receptors, with special reference to constitutive androstane receptor β (CARβ). Administration of DHEA or DHEA-S enhanced the DNA binding of hepatic nuclear extracts to responsive elements for the retinoic acid receptor, the retinoic acid receptor β 2 and the peroxisome proliferator activated receptor. The bound complexes were shown to be the CARβ-RXR heterodimer by antibody-supershift assays. The expression of a target gene of CARβ, Cyp2b10, was increased in liver by DHEA or DHEA-S treatment, suggesting that DHEA or DHEA-S actually activated CARβ in vivo. It was suggested that the metabolic conversion of DHEA, DHEA-S to CARβ ligands could occur in vivo and the metabolites could regulate the expression of CARβ target gene expression. Our results provide new insights into the in vivo relationship between DHEA/DHEA-S and CARβ activation.

Key words: Dehydroepiandrosterone; Dehydroepiandrosterone-sulfate; Constitutive androstane receptor β; Retinoid X receptor; Cyp2b10

1. Introduction

Dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEA-S) are adrenal androgens and DHEA-S is the most abundant adrenal steroid in humans. Serum levels of DHEA and DHEA-S exhibit a characteristic change with aging, rising during adolescence, peaking in the second decade of life and gradual falling thereafter, whereas serum levels of other adrenal steroids, such as cortisol and aldosterone, do not change with age. Since the falling levels of DHEA and DHEA-S parallel aging-related pathophysiologies, many investigators have been interested in the effects of administration of DHEA and DHEA-S in elderly people [1].

DHEA is a free steroid with weak androgenic activities and DHEA-S is regarded to be an inactive conjugate that exerts its effects through the release of DHEA by the steroid sulfatase. It has been postulated that DHEA, like other steroid hormones, may have its own nuclear receptor, however, no specific receptor has yet been identified and the molecular mechanism(s) of its action remains unknown. On the other hand, several reports have indicated that DHEA-S per se might have direct and specific actions. For example, DHEA-S blocked gamma-amino-butyric acid (GABA)-induced ion currents in neurons as an allosteric antagonist of the $GABA_A$ receptor in vitro [2], and in vivo treatment with DHEA-S induced a peroxisome proliferating activity in mice [3].

The observation that the effects of DHEA-S appear to be mediated by a nuclear peroxisome proliferator activated receptor (PPAR) α, though indirectly [3], prompted us to investigate the in vivo effects of DHEA and DHEA-S on other nuclear receptor functions. We were particularly interested in analyzing constitutive androstane receptor β (CARβ), since its ligands, androstanol and androstenol, are 17-deoxy-steroids and are thought to have a close relationship with 17-oxy-steroids such as DHEA and DHEA-S.

2. Materials and methods

2.1. Animals and diets

To study the effect of DHEA and DHEA-S, we used C57 ksj *+m/+m* mice, because they are the parental strain of *db/db* mice in which the anti-diabetic action and modulation of hepatic glucose-6-phosphatase activity by DHEA was demonstrated [4,5]. Male C57Bl ksj *+m/+m* mice were purchased from Clea Japan (Tokyo, Japan). Three mice were placed in one cage and given food and water ad libitum throughout the study. A total of 24 mice, aged 9 weeks, were maintained on standard pellet food for 15 days (Oriental MF; Oriental, Tokyo, Japan). The mice were then divided into three equal groups for the administration of DHEA-containing, DHEA-S-containing and standard pellet food, respectively, for 15 days. DHEA or DHEA-S, obtained from Sigma Chemical Co. (St. Louis, MO, USA), was mixed with the standard powder food and pelleted by Oriental Co. Ltd. (Tokyo, Japan) at a final concentration of 0.4% (w/w). On day 16, mice were sacrificed by cervical dislocation. Liver and adipose tissues were removed and weighed. All animal studies were performed after obtaining the approval of the Osaka Medical College Animal Care and Use Committee.

2.2. Histological analysis

Resected livers were fixed in 10% formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin (HE) for light microscopy. For electron microscopy, formalin-fixed specimens were refixed in 2% osmic acid.

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2.3. Preparation of nuclear extracts

Nuclear extracts were prepared as previously described [6]. Briefly, freshly excised liver specimens were homogenized in the homogenizing buffer (0.35 M sucrose, 10 mM HEPES–KOH, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 14 μg/ml aprotinin, 0.15 mM spermine, 0.5 mM spermidine). Crude nuclear fraction was prepared from the homogenates and extracted with the extraction buffer (20 mM HEPES–KOH, pH 7.9, 0.33 M NaCl, 1.5 mM $MgCl_2$, 10% glycerol, 14 μg/ml aprotinin, 0.5 mM DTT, 0.5 mM PMSF). Protein concentration of nuclear extract was measured by BCA Protein Assay Kit (Pierce, USA).

2.4. Electrophoretic mobility shift assay (EMSA)

In vitro DNA binding activities of nuclear receptors were analyzed by EMSA using liver nuclear extracts. Nuclear extracts containing 5 μg of protein were preincubated in the binding buffer (25 mM HEPES, pH 7.9, 0.2 mM EDTA, 100 mM KCl, 3 mM $MgCl_2$, 2 μg poly(dI-dC), 10% glycerol) on ice for 15 min, then, incubated with ^{32}P-labeled double stranded oligonucleotide probe (50 000 cpm) for 60 min on ice. In antibody supershift experiments, incubation was continued for another 60 min on ice after the addition of each polyclonal antibody (anti-CARβ, anti-RXRα, anti-RAR, anti-PPARα, anti-PPARγ and anti-PXR, Santa Cruz, CA, USA). Complexes containing DNA probes were resolved by electrophoresis on 5% polyacrylamide gels using 0.5×TBE buffer (100 V at room temperature), and analyzed by autoradiography as described [6].

2.5. Oligonucleotides

Double-stranded oligonucleotides corresponding to the vitamin D receptor responsive element (VDRE) [7], the steroid and xenobiotic receptor responsive element (SXRE) [8], the retinoic acid receptor responsive element (RARE) [9], the retinoic acid receptor β2 responsive element (βRARE) [10] and the promoter region of the enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase gene as the PPAR responsive element (HD-PPRE) [11] were end-labeled with the Klenow fragment of DNA polymerase I and [α-^{32}P]dATP for EMSA [6].

The corresponding sequences were: VDRE: 5′-AGCTTGCTCGGGTAGGGTTCCACGAGGTTCACTCGACTCGT-3′, SXRE: 5′-GATCTTCTGAACTCAAAGGAGGTCAGGAA-3′, RARE: 5′-AGCTTCAGGTCACCAGGAGGTCAGAGAGCT-3′, βRARE: 5′-AAGGGTTCACCGAAAGTTCACTCGCATTAAGGGTTCACCGAAAGTTCACTCGCATA-3′, HD-PPRE: 5′-CCTCTCCTTTGACCTATTGAAACTATTACCTACATTTGA-3′.

2.6. Western blot analysis

Liver nuclear extracts containing 20 μg of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (12% polyacrylamide gel) and then electroblotted onto Imobilon P (Millipore, Bedford, MA, USA). Membranes were incubated with a polyclonal antibody against CARβ (1:750 dilution) or RXRα (1:1500 dilution) (Santa Cruz, CA, USA) and processed with chemiluminescence detection system (Amersham-Pharmacia Biotech, UK). Chemiluminescence signals were analyzed densitometrically using the NIH image software.

2.7. Real-time PCR quantification of the Cyp2b10 gene expression

Treated animals were killed and livers were immediately immersed into RNAlater (Ambion, Austin, TX, USA). Total RNA was extracted using Isogen (Nippongene, Japan) and cDNA was produced using RT-PCR Kit (Takara, Japan). The quantitative real-time, one-step PCR was performed using the LightCycler quick system using SYBRGreen I kit (Roche Diagnostics GmbH, Germany) according to the manufacturer's instructions. The quantification data were analyzed with the LightCycler analysis software, as described previously [12,13]. Expression levels of the Cyp2b10 mRNA were normalized by the levels of the endogenous G3PDH mRNA. The primers for mouse Cyp2b10 cDNA [14] were 5′-CGTGAATTCCTTGAAGGTTGGCTCAACGACAG and 5′-CGTGAATTCAACATTGGTTAGACCAGGACCATGG. The primers for mouse G3PDH cDNA as internal control are 5′-GGGTGTGAACCACGAGAAAT and 5′-CTGCTTCACCACCTTCTTGA.

2.8. Measurement of serum levels of DHEA and DHEA-S

Serum levels of DHEA and DHEA-S were measured by ELISA in SRL Co. Ltd. (Tokyo, Japan).

3. Results

3.1. DHEA and DHEA-S induce hepatomegaly and peroxisome proliferation

Administration of DHEA or DHEA-S increased serum concentrations of both DHEA and DHEA-S (Table 1). These treatments also increased liver weight and the liver to whole body weight ratio, but not epididymal adipose tissue weight (Table 1). Microscopic examination revealed that DHEA-S and DHEA induced liver peroxisome proliferation (data not shown) as described previously [15]. Increase in liver weight and peroxisome proliferation were more prominent in DHEA-S-treated mice than in DHEA-treated mice.

3.2. DHEA and DHEA-S enhance RARE- and β-RARE-binding activity containing RXR but not RAR

As shown in Fig. 1, DHEA or DHEA-S treatment markedly enhanced the binding activity of liver nuclear extracts to RARE. On the other hand, there was little difference in the DNA binding activities to VDRE and SXRE among three groups.

To further clarify the components of the RARE-bound complex, another EMSA was performed using βRARE, an element closely related to RARE [10,16]. Just like with RARE, in vivo DHEA and DHEA-S treatment enhanced the DNA binding activity to βRARE (Fig. 1). Bands were supershifted by anti-RXRα but not by anti-RAR antibodies (Fig. 2, lanes 3 and 9). These observations clearly indicate that RXR but not RAR is involved in the enhanced DNA binding activities to RARE and βRARE in DHEA- and DHEA-S-treated mouse liver.

3.3. DHEA and DHEA-S treatment enhance the DNA binding activity of the CARβ-containing complex

Since it was reported that CARβ could bind to the βRARE in heterodimer with RXR [10,16], we investigated the involvement of CARβ in the enhanced DNA binding activity. The components of the βRARE bound complexes were super-

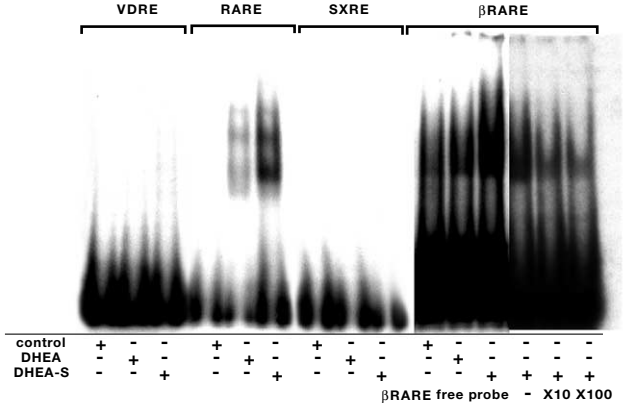

Fig. 1. DHEA- and DHEA-S-treatment enhanced the DNA binding activity of liver nuclear extracts to RARE and βRARE, but neither to VDRE nor SXRE. EMSAs were performed with liver nuclear extracts from control-treated, 0.4% (w/w) DHEA-treated and 0.4% (w/w) DHEA-S-treated mice using ^{32}P-labeled VDRE, SXRE, RARE and βRARE as probes. DHEA treatment and to a much greater extent, DHEA-S treatment enhanced the DNA binding activity of liver nuclear extracts to RARE and βRARE. The bands were diminished by an excess amount of unlabeled βRARE (lanes 13–15) or RARE (data not shown).

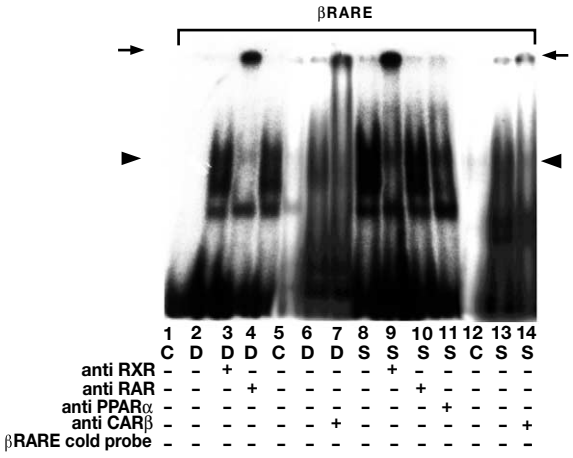

Fig. 2. DHEA- and DHEA-S-treatment enhanced the DNA binding activity of the complex containing RXRα and CARβ to βRARE. EMSAs were performed with liver nuclear extracts from control-treated (C), DHEA-treated (D) and DHEA-S-treated (S) mice. Supershift assays with anti-RXRα, anti-RAR or anti-CARβ antibody. The arrow indicates the band supershifted by anti-RXRα antibody (lanes 3 and 9) and anti-CARβ antibody (lanes 7 and 14) and the arrowhead indicates the disappeared band by supershift, showing the involvement of RXR and CARβ in the βRARE–nuclear protein complex. No supershifted bands were observed with either anti-RAR (lane 10) or anti-PPARα antibodies (lane 11).

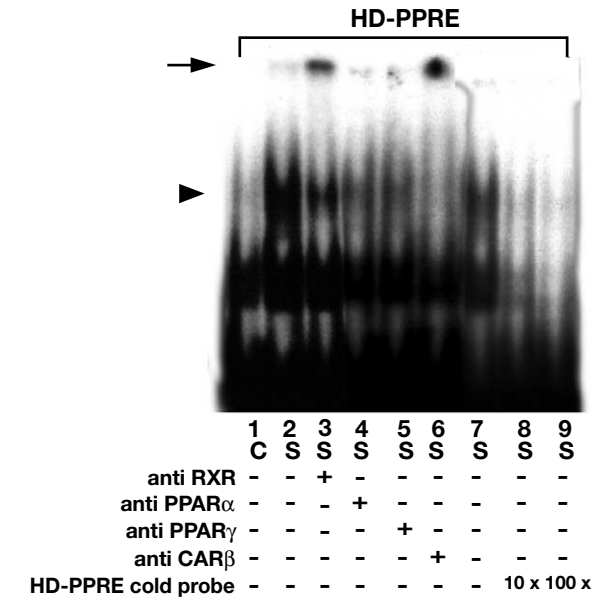

Fig. 3. The DNA binding activity to HD-PPRE is also enhanced DHEA and DHEA-S treatment. EMSAs were performed with liver nuclear extracts prepared from control-treated (C), DHEA-treated (D) and DHEA-S-treated (S) mice, using ^{32}P-labelled HD-PPRE as probes. The arrow indicates band supershifted by anti-RXRα antibody (lane 3) and anti-CARβ antibody (lane 6) and the arrowhead indicates disappeared bands, showing the involvement of RXRα–CARβ in the HD-PPRE nuclear protein complex. These bands disappeared by an excess amount of unlabeled HD-PPRE (lanes 7–9). No supershifted bands were observed with anti-PPARα (lane 4) or anti-PPARγ antibodies (lane 5).

shifted by not only anti-RXRα but also anti-CARβ (Fig. 2, lanes 7 and 14). As Kassam and colleagues [11] have shown that the CARβ-RXR heterodimer also binds to HD-PPRE, another EMSA was performed using HD-PPRE as a probe. DHEA and DHEA-S treatment also enhanced the DNA binding activity to HD-PPRE (Fig. 3). And they disappeared with an excess amount of unlabeled HD-PPRE (Fig. 3, lanes 7–9). To examine the involvement of CARβ in the enhanced DNA binding activity by DHEA/DHEA-S, supershift assays were performed with anti-CARβ antibody (Fig. 3). As shown in Fig. 3, anti-CARβ antibody supershifted the DHEA/DHEA-S-enhanced binding to HD-PPRE (Fig. 3, lane 6), as to βRARE. From these observations, it was concluded that in vivo DHEA/DHEA-S treatment enhanced the DNA binding activity of a complex containing the RXR-CARβ heterodimer in mouse liver.

3.4. *DHEA and DHEA-S increase the quantity of nuclear RXR protein, but not of CARβ protein*

We next examined whether treatment with DHEA or DHEA-S alters the quantity of RXR and CARβ proteins in mouse liver. As shown in Fig. 4A, larger amounts of RXRα protein were present in liver nuclear extracts from DHEA- and DHEA-S-treated mice compared with those from control mice. Neither DHEA nor DHEA-S affected the quantity of CARβ protein in mouse liver nuclear extracts (Fig. 4B).

3.5. *DHEA and DHEA-S induce the expression of a target gene of RXR-CARβ*

We investigated whether DHEA and DHEA-S altered the expression of the target gene of the CARβ/RXR heterodimer. As shown in Fig. 5, both DHEA and DHEA-S induced the expression of the mouse Cyp2b10 gene, a target gene of CARβ/RXR. There is a correlation between the expression level of Cyp2b10 gene and serum concentration of DHEA-S in DHEA-treated mice (Fig. 6), and an inverse correlation in DHEA-S-treated mice. On the other hand, there is no correlation between the expression level of Cyp2b10 gene and serum DHEA concentration (data not shown).

4. Discussion

In the present study, we investigated the effects of treatment with DHEA and DHEA-S on the activities of nuclear hormone receptors, since several lines of evidence have suggested that actions of DHEA and DHEA-S were mediated through nuclear hormone receptors. For example, PPARα had been regarded as a candidate receptor for DHEA and DHEA-S because of the absence of the peroxisome proliferative effect

Table 1
Whole body weight, liver weight, epididymal fat weight, serum concentrations of DHEA and serum concentrations of DHEA-S in control, DHEA-treated and DHEA-S-treated mice (mean ± S.D.)

	Whole body weight (g)	Fat (g)	Liver (g)	Ratio of liver/whole body weight (%)	DHEA (ng/ml)	DHEA-S (ng/ml)
Control	22.27 ± 1.61	0.37 ± 0.21	1.17 ± 0.11	5.26 ± 0.39	0.867 ± 0.186	69.667 ± 0.210
0.4% DHEA	20.33 ± 0.55	0.30 ± 0.10	1.47 ± 0.08	7.23 ± 0.21	198.800 ± 120.238	281.900 ± 91.599
0.4% DHEA-S	23.80 ± 1.48	0.34 ± 0.05	2.27 ± 0.18	9.52 ± 0.38	43.167 ± 5.879	434.167 ± 123.935

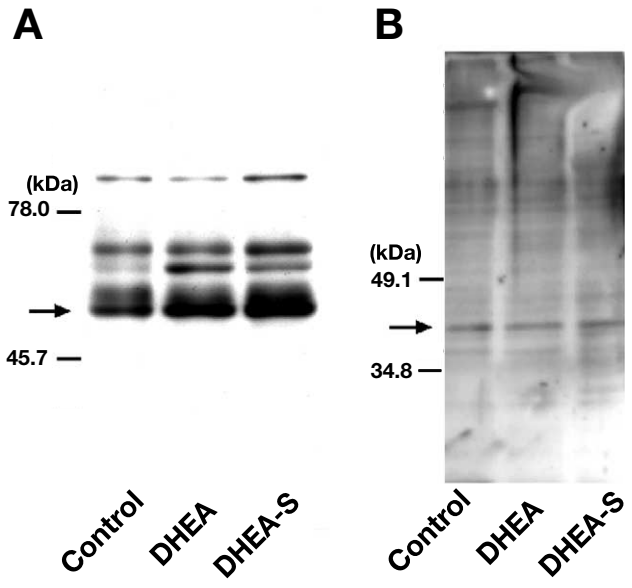

Fig. 4. DHEA and DHEA-S treatment increased nuclear RXR protein but not nuclear CARβ protein. Western blot analyses were performed with liver nuclear extracts from control, DHEA-treated and DHEA-S-treated mice using anti-RXR polyclonal antibody (1:1500 dilution) (A) or anti-CARβ polyclonal antibody (1:750 dilution) (B). Arrows indicate RXR and CARβ.

of DHEA-S in PPARα knockout mice, although it was demonstrated later that neither DHEA nor DHEA-S activated PPARα directly [3]. We demonstrated that in vivo treatment with DHEA and DHEA-S not only specifically enhanced the DNA binding activity of the CARβ-RXR heterodimer but also induced the expression of the CARβ target gene.

The increased target DNA binding in DHEA- or DHEA-S-treated mice could be due to the receptor activation by increased ligand binding in vivo. In vivo conversion of DHEA and DHEA-S to androstanol and androstenol, CARβ ligands, could occur and they could affect CARβ activity, since both androstanol and androstenol are 17-deoxy-steroids and have a close relationship with 17-oxy-steroids such as DHEA and DHEA-S [17]. In fact, there are two reports suggesting the production of androstenol from DHEA and DHEA-S in human [18,19]. Mason and Schneider reported the detection of a musk-smelling androst-16-en-3α-ol in the urine of two female patients with adrenal cortical neoplasm, which were supposed to secrete excessive amounts of glucocorticoids and adrenal androgens including DHEA and DHEA-S. This musk-smelling steroid was assumed to be androstenol, 5α-androst-16-en-3α-ol, since 5β-androst-16-en-3α-ol is odorless [20]. They proposed that androstenol was a product of the adrenal cortical neoplasm or a product of the metabolism of some precursor produced by the neoplasm. Thus, DHEA or DHEA-S could be converted to CARβ ligands in vivo and enhance the DNA binding activity of the CARβ-RXR heterodimer.

The finding that DHEA and DHEA-S treatment increased the amount of RXR in liver nuclear extracts suggests that increase in the amount of the CARβ-RXR heterodimer is another possible mechanism involved in the enhancement of target DNA binding. The increase of RXR could be a result of an increase in RXR gene transcription, a result of elongation of the half-life of RXR mRNA or protein, or a result of an increase of nuclear localization of RXR. However, it seems that the increase of RXR protein is not the only mechanism of the enhanced DNA binding of the CARβ-RXR heterodimer because we could not detect the effect of DHEA/DHEA-S on DNA binding activity with various nuclear hormone responsive elements targeted by heterodimers between RXR and other heterodimer partners than CARβ (Fig. 1). Other possible mechanisms include the post-translational modifications of CARβ-RXR and co-factors, by DHEA or DHEA-S treatment, to gain the enhanced target DNA binding ability. All these above mentioned mechanisms are not mutually exclusive and could work synergistically. For example, the modification of RXR might increase its half-life and nuclear localization, both of which could lead to the increased amount of nuclear RXR.

We showed peroxisome proliferation and hepatomegaly by DHEA or DHEA-S treatment, as previously reported. Interestingly, HD-PPRE, which is the upstream sequence of the peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase gene, contains both PPARα-responsive and CARβ-responsive elements [11]. Because the knockout mice study showed that both DHEA and DHEA-S could induce

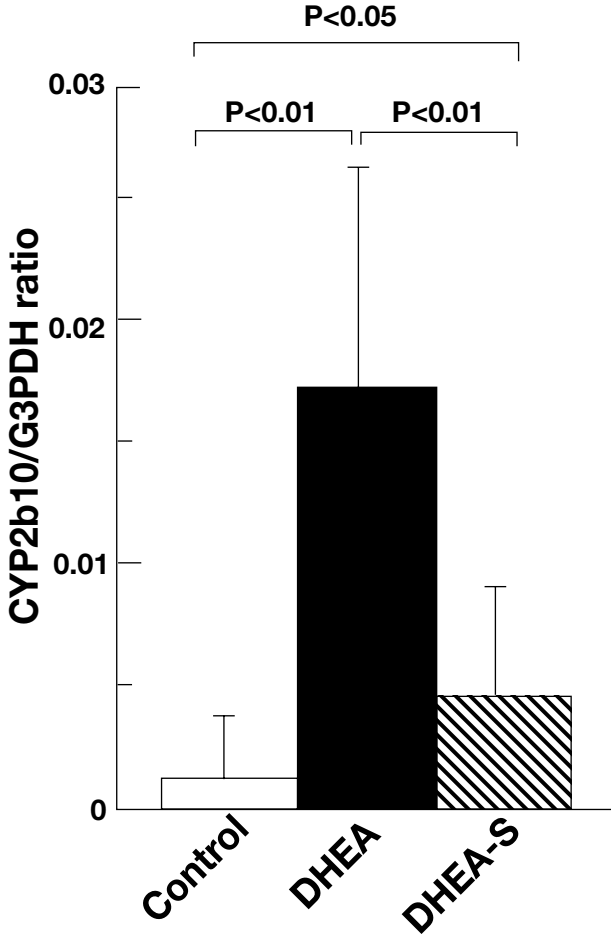

Fig. 5. DHEA or DHEA-S treatment induced the expression of mouse Cyp2b10 mRNA. The expression levels of mouse Cyp2b10 and G3PDH gene were quantified by real-time RT-PCR. The levels of Cyp2b10 were normalized by the G3PDH mRNA levels and expressed as relative arbitrary unit. Data are mean ± S.D. Open bar: control-treated, closed bar: DHEA-treated, hatched bar: DHEA-S-treated mice. Statistical analysis was performed by the Cochran–Cox's test.

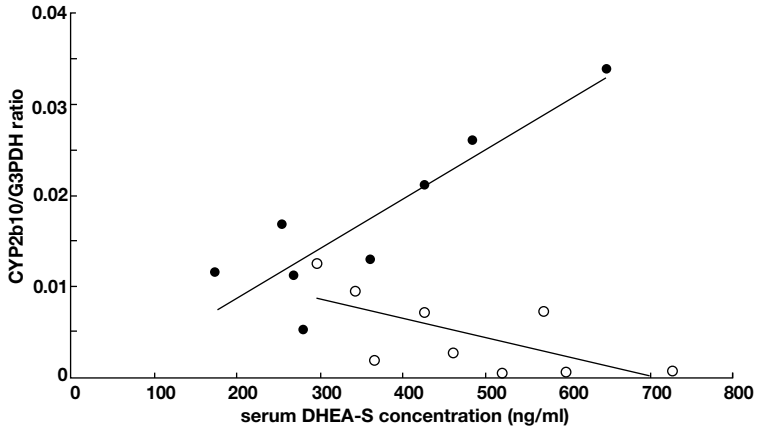

Fig. 6. A correlation between the Cyp2b10 gene expression and serum DHEA-S concentration. There were correlation ($R^2 = 0.78$) between the Cyp2b10 gene expression and serum DHEA-S concentration in DHEA-treated mice (closed circle) and an inverse correlation ($R^2 = 0.43$) between the Cyp2b10 gene expression and serum DHEA-S concentration in DHEA-S-treated mice (open circle). The levels of Cyp2b10 were normalized by the G3PDH mRNA levels and expressed as relative arbitrary unit. Serum concentration of DHEA-S was expressed in ng/ml.

the peroxisome proliferation but could not activate PPARα directly [3], it is a possible new physiological function of CARβ. In next step, we would examine the peroxisome proliferation and the enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase gene expression in DHEA- or DHEA-S-treated mice. Our results may suggest another mechanism of the peroxisome proliferation induced by DHEA/DHEA-S–CARβ pathway.

DHEA and DHEA-S not only enhanced the target DNA binding of CARβ, but also modulated the expression of a target gene of CARβ in mouse liver. These results strongly suggest that DHEA and DHEA-S affect the transcriptional activity of the CARβ-RXR heterodimer in vivo. Because our in vitro experiments suggest DHEA-S can not activate CARβ directly (A. Fujita and D. Furutama, unpublished data), as DHEA can not [10], they would be converted to regulators for CARβ in vivo. Natural ligands of CARβ, androstanol and androstenol, are thought to be negative regulators of CARβ for its target gene transcription because of the direct inhibitory effect of these substances on the interaction between CARβ and SRC1 [10], whereas another ligand of CARβ, phenobarbital, can act as a positive regulator [21]. One of the possible regulators for CARβ, which could be converted from DHEA/DHEA-S in vivo, is estradiol. It is known as a metabolite of DHEA [22] and to activate CARβ and induce the Cyp2b10 gene expression in mouse liver [23]. However, estradiol could elicit nuclear accumulation of CAR in the mouse livers [23], which has not been seen in DHEA- or DHEA-S-treated mice, suggesting that another unknown positive regulator could be produced from DHEA(-S). Androstenol and androstanol could be also converted from DHEA/DHEA-S in vivo. In addition, DHEA can be converted from DHEA-S by steroid sulfatase and DHEA-S can be converted from DHEA by DHEA sulfotransferase. Thus, various regulators, which are in vivo metabolites by preferential conversion from DHEA or DHEA-S, could affect the CARβ activity and the expression of CARβ target genes positively or negatively.

It remains unknown why in vivo effects of DHEA/DHEA-S, including the peroxisome proliferation, the enhancement of DNA binding of CARβ and the induction of its target gene, were more prominent in DHEA-S-treated mice than in DHEA-treated mice. Since differences in these effects between DHEA and DHEA-S are only quantitative throughout our study, the greater effects of DHEA-S could be simply explained by assuming that, for example, DHEA-S is a better substrate than DHEA for the converting enzyme, generating CARβ ligands. We speculate the preferential conversion of DHEA-S to androstenol or androstanol, because the position 17 of DHEA-S is more easily modified than that of DHEA because the position 3 of DHEA-S is blocked by sulfate [24]. Such in vivo preferential conversion, i.e. DHEA-S to CARβ ligands and DHEA to estradiol or other activators, could account for the differential effects of DHEA and DHEA-S on the target gene induction and DNA binding. In addition, it could account for the discrepancy between the target DNA binding activity and the target gene expression level in DHEA(-S)-treated mice. Elucidation of the relationship between CARβ activation and in vivo conversion of DHEA/DHEA-S to CARβ ligands is the necessary next step. We believe that our current data about the effects of DHEA and DHEA-S on CARβ and RXR provide important clues to clarify the physiological functions of DHEA and DHEA-S.

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