Dehydroepiandrosterone sulfate and cytochrome P450 inducers alleviate fatty liver in male rats fed an orotic acid-supplemented diet

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ABSTRACT — The effects of the peroxisome proliferator, dehydroepiandrosterone sulfate (DHEAS), and the typical cytochrome P450 (CYP) inducers phenobarbital (PB) and 3-methylcholanthrene (3-MC) on fatty liver were examined in rats. Treating rats with orotic acid caused marked accumulation of lipid droplets in the liver. This effect of orotic acid was almost eradicated by co-treatment with DHEAS and PB. While DHEAS or PB alone also alleviated fatty liver, treatment with 3-MC caused little effect on a reduction in lipid droplets. Histopathological examinations revealed numerous peroxisomes in the liver of rats treated with DHEAS. In addition, a significant increase in the expression on hepatic CYPs was observed in rats the fatty liver of which was attenuated. Regarding other enzymes associated with hepatic fatty acid oxidation, the expression levels of sirtuin 1, sirtuin 6, and carnitine palmitoyltransferase 1 were also upregulated most markedly by treatment with DHEAS alone. Thus, the attenuation in fatty liver observed in the present study is likely due to peroxisome proliferation and the induction of fatty acid-metabolizing enzymes by DHEAS and typical CYP inducers.

Key words: Fatty liver, DHEAS, Phenobarbital, PPARα, Constitutive androstane receptor, Cytochrome P450

INTRODUCTION

In recent years, the number of patients suffering from non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH) has increased (Browning et al., 2004; Kojima et al., 2003). NAFL represents one form of metabolic syndrome in the liver (Ekstedt et al., 2006; Lonardo et al., 2013), but this has long been considered to be reversible because of its seemingly non-serious symptoms. However, accumulating evidence does not support this view, and it has come to light that NAFL easily deteriorates into NASH in combination with other factors such as oxidative stress and iron excess (Kowdley et al., 2012; Tilg and Moschen, 2010). In spite of this knowledge, there are few drugs which are currently available for treating NAFL and NASH (Chalasani et al., 2012).

The hepatic level of triglyceride (TG) is determined by a balance between supply from food and adipose tissue (Redgrave, 1970) and loss from the tissue because of the consumption of fatty acids (Antony and Landau, 1968) and their release into the bloodstream (Wetterau and Zilversmit, 1984). Fatty acids in the liver are usually metabolized by β-oxidation in the mitochondrion and peroxisome, and they also undergo ω-hydroxylation by cytochrome P450 (CYP) localized in the endoplasmic reticulum (Lu and Coon, 1968). However, fatty acid-metabolizing enzymes in these organelles seem to be unable to work on the excess amounts of fatty acids accumulated in the fatty liver.

A previous study aiming to identify ways to alleviate fatty liver has demonstrated that dehydroepiandrosterone (DHEA) and its co-administration with phenobarbital...
(PB), which are a peroxisome proliferator and a typical CYP2B inducer, respectively, prevent the accumulation of lipid droplet in the fatty liver induced by feeding male rats with diets containing orotic acid and sucrose at a high level (Goto et al., 1998a). In this context, it was suggested that the reduced form of dehydroepiandrosterone sulfate (DHEAS) in the serum is a useful biomarker for the diagnosis of non-alcoholic fatty liver disease (NAFLD) (Tokushige et al., 2013). Another CYP2B inducer, 1,4-bis-[2-(3,5,-dichloropyridyloxy)] benzene (TCPOBOP), also attenuates steatohepatitis through the activation of the constitutive androstane receptor (CAR) in mice with fatty liver induced by a methionine/choline-deficient (MCD) diet (Baskin-Bey et al., 2007). However, it is not yet fully understood whether DHEAS or a combination of this drug and CYP2B inducers increase the expression of fatty acid-metabolizing enzymes, and whether these treatments improve the toxic markers and pathology of the fatty liver. A transcription factor, sirtuin (SIRT) 1, is thought to play a role in the regulation of proteins serving in fatty acid β-oxidation. Conceivably, DHEAS and CYP inducers may alter the level of SIRT1 to exert their protective effects on steatosis in the liver although this possibility remains unproven. To address these issues, we examined the effects of DHEAS, PB and their co-administration on the expression of β-oxidation- and α-oxidation-related genes and the pathological change in the fatty liver induced with orotic acid and sucrose. The effect of 3-methylcholanthrene (3-MC), an inducer of the CYP1A subfamily, was also examined.

**MATERIALS AND METHODS**

**Materials**

DHEAS sodium hydrate, orotic acid 1-hydrate, sodium PB and 3-MC were purchased from Wako Pure Chemical Industries (Osaka, Japan). Other reagents were of the highest purity commercially available.

**Animals and treatments**

Male Sprague-Dawley (SD) rats at the age of 4 weeks were purchased from Charles River Japan, Inc. (Shiga, Japan). The rats were bred in stainless steel wire-mesh cages, fed a powdered diet (CRF-1, Oriental Yeast, Tokyo, Japan) and water was available ad libitum under controlled environmental conditions (temperature, 23 ± 2°C; humidity, 60% ± 10%; and 12-hr light/12-hr dark cycle). Rats were divided into 6 groups (n = 5 per group) so as to obtain comparable mean body weight among the groups. Fig. 1 shows the schedules of animal treatment used in the study. To induce fatty liver, rats were fed a diet containing 50% sucrose and 1% orotic acid for 4 weeks. Some groups were fed the above diet containing 0.5% DHEAS (sufficient to induce peroxisome proliferation in rat liver, unpublished data) for weeks 3 and 4, and one group was co-treated with PB (80 mg/kg/day, i.p., once a day) as well as DHEAS. The other rats suffering from fatty liver were treated only with PB under the same schedule as above, or with 3-MC (25 mg/kg/day, i.p., once a day). These doses of PB and 3-MC are sufficient to induce hepatic CYP2B1/2B2 and CYP1A1/1A2 in rats (Correia, 1995). Control rats (the intact group) were fed the powdered form of CRF-1 (Oriental Yeast) throughout the experiment (2 weeks + 2 weeks). Body weight and food consumption were measured once a week. The animals were sacrificed on the 29th day after treatment, and their livers were removed. The final body weight was obtained after fasting for 20-22 hr to calculate the relative liver weight (liver/body weight ratio). The experimental protocols and procedures were pre-approved by the Animal Ethical Committee of Otsuka Pharmaceutical Co., Ltd.

**Blood test and quantification of liver triglyceride content**

The blood samples were obtained from the cervical vein on the day of necropsy. The plasma was prepared...
by centrifuging blood gathered in a heparinized tube at 4 °C and 1,800 x g for 20 min. The following blood components were assayed using an automatic analyzer (Model 7180, Hitachi, Tokyo, Japan): triglyceride (TG), phospholipid (PL), total cholesterol (CHO), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP). The liver TG content was determined according to the method of Maglich et al. (2009).

Histopathological examination
For light microscopic analysis, the liver was fixed in 10% neutral formalin, embedded in paraffin, sectioned into a 3 μm preparation, and stained with hematoxylin and eosin (H&E). In a separate experiment, the liver was subjected to transmission electron microscopic examination. The tissue was pre-fixed in a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde for 2 hr, and post-fixed in 1% osmium tetroxide for 2 hr. The fixed tissue was stained en bloc with uranyl acetate, dehydrated with increasing concentrations of acetone, and embedded in epoxy resin. Then, the centrilobular zone of the livers which exhibited abnormal changes upon histopathological examination was sectioned ultra-thinly using an ultramicrotome, stained with lead citrate, and observed using a JEM-1200EX transmission electron microscope (Japan Electron Optics Laboratory, Tokyo, Japan). The centrilobular zone was defined as a 3-4-cell radius from the central vein towards its periphery. The peripheral zone was defined as a 3-4-cell radius from portal tracts towards the central vein. The intermediate zone was defined as the zone located between the centrilobular and peripheral zones.

Assay for the hepatic contents of CYP and its redox partners
The contents of total CYP and cytochrome b were determined according to the methods of Omura and Sato (1964a, 1964b). NADPH-CYP reductase activity was determined by the method of Phillips and Langdon (1962).

Western blot analysis
Protein concentration was determined by a kit (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as a standard. Liver microsomal proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% gel), transferred to a polyvinylidene difluoride (PVDF) membrane, and then probed with antibodies against rat CYP1A1/1A2, 2B1/2B2, 2C6, 2C11, 2C13, 2E1, 3A2 and 4A1 (Nosan Corporation, Yokohama, Japan). For the loading control, the PVDF membrane after transfer was also blotted using antibody against a housekeeping protein, ribosomal protein, large, P0 (RPLP0, ProteinTech Group, Inc, Chicago, IL, USA). The liver homogenate was processed in a similar fashion, and probed with antibodies against rat SIRT1, carnitine palmitoyltransferase 1-liver (CPT1-L, Santa Cruz Biotechnology, Dallas, TX, USA), and SIRT6 (Abnova Corporation, Taipei, Taiwan). After incubation with secondary antibodies conjugated to horseradish peroxidase (ECL prime detection system, GE Healthcare, Piscataway, NJ, USA), the bands associated with the antibody were visualized using an IVIS imaging system (PerkinElmer, Waltham, MA, USA). The expression of the target protein was normalized to RPLP0.

Statistical analysis
Statistical differences were estimated by ANOVA using Tukey’s post-hoc test. When the P value was below 0.05, the difference was judged to be statistically significant.

RESULTS
Orotic acid/sucrose-induced fatty liver and its alleviation
The rats fed a diet supplemented with orotic acid and a high level sucrose for 4 weeks had reduced body weight and experienced hepatomegaly (Table 1). Neither co-treatment with DHEAS, PB nor 3-MC could reverse these symptoms. In addition, liver hepatomegaly deteriorated in the case of 3-MC treatment (Table 1). Similarly, double treatment with DHEAS and PB aggravated rather than recovered hepatomegaly (Table 1). However, as
far as the outward appearance is viewed, a liver-specific reddish color was observed not only in the control but also in the DHEAS- and PB-treated rats, while a whitish colored fatty liver was observed only in rats treated with orotic acid/sucrose in the presence and absence of 3-MC (Supplementary Fig. 1). In this context, histopathological examination revealed that numerous lipid droplets were accumulated in the liver of rats treated with orotic acid/sucrose (Fig. 2A, B). Fatty liver was either recovered or alleviated by treating rats with DHEAS, PB or their combination (Fig. 2C-E), whereas an apparent improvement was not observed in the 3-MC treatment group (Fig. 2F). The simultaneous administration of DHEAS and PB was the most effective, resulting in almost complete eradication of lipid droplets over the whole liver lobules (Table 2).

Electron microscopy analysis showed that in the steatosis model produced by orotic acid/sucrose, lipid droplets accumulated in the cytosol and within the dilated vesicles of the rough endoplasmic reticulum (rER) (Supplementary Fig. 2; see arrows for a change in the pathology of the rER). In the DHEAS-, PB- and DHEAS/PB-treated rats, the number of vacuolated vesicles seen in fatty liver rats was markedly reduced (Fig. 3C-E). By contrast, little reduction in the number of lipid droplets was observed in 3-MC-treated group (Fig. 3F). Electron microscopy analysis also suggested an increase of the smooth ER (sER) in the PB- and DHEAS/PB-treated groups (Fig. 3D, E), and a marked increase of the peroxisomes in the DHEAS- and DHEAS/PB-treated groups (Fig. 3C, D). In the DHEAS + PB group, atypical peroxisomes (Fig. 3D, indicated by open arrow) and an intramitochondrial tubular structure (Fig. 3D, indicated by closed arrow) were observed.

**Blood biochemistry and liver TG content in rats carrying fatty liver**

In contrast to the liver lipid droplets, the plasma TG level was decreased by orotic acid/sucrose treatment (Fig. 4A). On the other hand, the plasma TG value in rats treated with PB alone and DHEAS + PB was significantly higher than that in the fatty liver rats that were not medicated (Fig. 4A). The plasma levels of PL and CHO were significantly increased in rats treated with DHEAS + PB, PB and 3-MC compared with the control and fatty liver rats (Fig. 4B, C). The activity of AST and ALT, both markers for liver injury, were significantly increased following treatment with orotic acid and sucrose. ALP exhibited a lower value only in the DHEAS + PB group compared with the control and fatty liver groups (Fig. 4D-F).

![Image](image_url)
A significant increase in the hepatic TG was observed in the orotic acid/sucrose treatment group (5.1-fold, Fig. 5). This elevated level of TG was attenuated in rats treated with DHEAS, DHEAS + PB and PB to a comparable level as the control.

**Effects of DHEAS, PB and 3-MC on the expression of fatty acid-metabolizing enzymes**

To investigate the mechanisms underlying the effects of DHEAS, PB and 3-MC on fatty liver, the expression of fatty acid-metabolizing enzymes was analyzed by western blotting. The data for CYPs and enzymes/regulators involved in β-oxidation are shown in Figs. 6 and 7, respectively. In general, there was a trend that the expression of CYPs examined in this study is reduced in rats given fatty liver: the most prominent case (CYP2C6) was a reduction to the one-third the level of the control. In accordance with this trend, the spectrophotometrically-determined content of hepatic CYP tended to be reduced in fatty liver (Supplementary Fig. 3A). Also, cytochrome b₅ and NADPH-CYP reductase tended to be reduced in rats with fatty liver (Supplementary Fig. 3B, C). PB and 3-MC are well known inducers for the CYP2B and CYP1A subfamilies, respectively. As shown in Fig. 6, in rats with fatty liver, the expression of CYP2B1/2 and CYP1A1/2 was markedly increased following PB and 3-MC treatment, respectively. The expression of CYP2C6 (6.4-fold), 2C11 (2.5-fold) and 2C13 (1.9-fold) was also increased by PB, and CYP2C13 (1.9-fold) was increased by 3-MC. In rats with fatty liver, CYP2E1 exhibited little change even after treatment with CYP inducers and DHEAS. An increase in CYP3A2 expression was observed in the DHEAS + PB, and PB groups. The expression of CYP4A1, an enzyme capable of ω-hydroxylating fatty acids, was clearly increased by treating steatotic rats with DHEAS. In terms of the enzymes/regulators involved in β-oxidation (Fig. 7), the expression of SIRT1 (1.9-fold) and SIRT6 (2.9-fold) were increased most markedly in the DHEAS + PB group.

**Table 2.** Histopathological findings of livers in orotic acid-treated rats by treatment with a peroxisome proliferator and CYP inducers.

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<th>Control (n = 5)</th>
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<th>+ DHEAS (n = 5)</th>
<th>+ DHEAS &amp; PB (n = 5)</th>
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Histopathological features were graded using the following scores: ± = very slight, + = slight, ++ = moderate, and +++ = severe.
The expression of CPT1 was increased in all drug-administered groups, while the maximal effect was attained by treatment with DHEAS alone.

**DISCUSSION**

A previous study has already revealed that unconjugated DHEA, PB or a combination of the two prevents orotic acid-induced fatty liver in male rats (Goto et al., 1998a). The preventive effect of DHEA acetate has also been reported (Goto et al., 1998b). In the present study, we demonstrated the protective effect of DHEAS and typical CYP inducers on orotic acid-induced fatty liver in male rats. It has long been known that orotic acid, a precursor for pyrimidine nucleotide biosynthesis, serves as an inducer of fatty liver in rats (Jatlow et al., 1965). In addition, the TG accumulation induced by orotic acid is facilitated by a high-sucrose diet (Novikoff et al., 1974). Our early studies reproduced the marked accumulation of lipid droplets in hepatocytes using a combination of treatments with orotic acid and sucrose (Goto et al., 1998a, 1998b). These observations were also supported by those from the present study. Fatty liver was confirmed to be produced in this rat model based on observations of hepatomegaly, discoloration of the liver, increase in hepatic TG content, and elevation in plasma ALT and AST activities.

In general, the plasma activity of ALP varies according to changes in food consumption. The food consumption tended to decrease in DHEAS and DHEAS + PB groups, and the magnitude of the alteration was comparable between the groups (data not shown). As shown in Fig. 4, although the plasma TG level in rats treated with orotic acid and sucrose tended to decrease compared with the control, the liver TG level was significantly elevated after the treatment (Fig. 5). Based on the significant increase in the plasma TG level in the PB-treated groups (DHEAS + PB, PB), PB is suggested to discharge accumulated fat from the liver into the bloodstream. Additionally, the plasma concentrations of CHO and PL were increased in the PB- and 3-MC-treated groups. Since the same was not observed in the DHEAS group, it is likely that the drug targets were different between DHEAS and PB/3-MC. One of the ATP-binding cassette transporters, ABCA1, plays an essential role in transporting CHO and PL from the liver to the bloodstream (Yokoyama, 2006). In addition, Wetterau and Zilversmit (1984) revealed that
microsomal triglyceride transfer protein (MTP) transports liver TG, CHO and PL to the bloodstream. Therefore, it is conceivable that PB and 3-MC upregulate or activate these transporters although this still requires further investigation.

The proliferation of hepatic peroxisomes in DHEAS-treated rats observed by electron microscopy suggested that an increase by DHEAS in the peroxisomal β-oxidation of fatty acid (Yamada et al., 1992) contributes somewhat to attenuate fatty liver. CYP4A, a major CYP isoform involved in fatty acid metabolism, primarily mediates fatty acid ω-hydroxylation (Tamburini et al., 1984). In addition to CYP4A, other CYP isoforms such as CYP1A, 2B, 2C and 3A also play substantial roles in fatty acid ω-hydroxylation (Bylund et al., 1998; CaJacob et al., 1988). In drug-treated rats with attenuated fatty livers, a significant increase in CYP1A1/2, 2B1/2, 2C6, 2C11, 2C13, 3A2 and 4A1 expression was observed (Fig. 6). These changes were virtually the same as those produced by DHEAS, PB and 3-MC in normal animals not suffering from fatty liver (Kohalmy et al., 2007; Madan et al., 2007).

Fig. 4. Effects of DHEAS, DHEAS + PB, PB, 3-MC administrations on blood biochemistry in fatty liver rats. (A) TG, triglyceride; (B) PL, phospholipid; (C) CHO, total cholesterol; (D) AST, aspartate aminotransferase; (E) ALT, alanine aminotransferase; (F) ALP, alkaline phosphatase. Each value represents the mean ± S.D. for 5 rats in each group except for the PB group (n = 4, death of one animal). Significantly different from control (*P < 0.05); and from fatty liver group (#P < 0.05).

Fig. 5. Effects of DHEAS, DHEAS + PB, PB, 3-MC administrations on liver TG levels. Each value represents the mean ± S.D. for 3 rats. Significantly different from control (*P < 0.05); and from fatty liver group (#P < 0.05).
2003; Thomas et al., 1983). Taken together, it is likely that under the conditions used in this study, CYP4A and other isoforms induced by DHEAS, PB and 3-MC facilitates fatty acid oxidation in concert with each other. However, since little alleviation in the number of lipid droplets was observed in the 3-MC group, CYP1A1/2 is considered to scarcely contribute to fatty acid oxidation.

An increase in the hepatic expression of CYP2E1 has been reported in NASH model rats generated by feeding a choline-deficient L-amino acid-defined (CDAA) diet (Takeuchi-Yorimoto et al., 2013) as well as in human NASH (Buechler and Weiss, 2011). However, no significant change of CYP2E1 expression was observed in the present study (Fig. 6). A previous report suggested that the increased levels of fatty acids under disparate conditions, such as a high-fat/low-carbohydrate diet, are directly involved in CYP2E1 induction (Robertson et al., 2001). In terms of the size of lipid droplets and the level of accumulated TG in the liver (see Figs. 2 and 5), orotic acid-induced fatty liver seems to be less malignant than human NASH and its corresponding rat model provoked by a CDAA diet.

Among SIRT proteins, SIRT1 and SIRT6 play important roles in the metabolism of fat by regulating the enzymes involved in fatty-acid β-oxidation, while SIRT1 positively regulates the transcription of SIRT6 gene (Kim et al., 2010). CPT1 serves in the rate-limiting step of fatty acid β-oxidation by transporting fatty acids into the inner cavity of the mitochondria where medium chain fatty acids are oxidized (McGarry et al., 1978). A recent study suggested that the enhanced expression of SIRT1 leads to an increase in CPT1 activity (Derdak et al., 2013). The present study showed that the expression of SIRT1, SIRT6 and CPT1 was significantly increased by treating rats with DHEAS alone (Fig. 7). These increases were attenuated by co-administration with PB. Especially in the case of SIRT6 expression, PB returned the level elevated by DHEAS treatment to the control level (Fig. 7). It was reported that the transcription of peroxisome proliferator-activated receptor alpha (PPARα) gov-
erned genes, e.g. CYP4A, SIRT1, SIRT6 and CPT1, is negatively regulated by PB through CAR activation (Maglich et al., 2009; Tamasi et al., 2009). Since CYP4A1 expression was also suppressed by co-treatment with PB and DHEAS (Fig. 6), it may be reasonable to consider that the above observations are caused by inhibitory crosstalk between CAR and PPARα. The involvement of such crosstalk is also suggested by the pathological findings. For instance, the granular eosinophilic cytoplasm of hepatocytes was decreased by treating animals simultaneously with DHEAS and PB compared with DHEAS alone (data not shown). In this context, Close et al. (1992) reported that clofibrate (PPARα agonist)-induced peroxisome proliferation is antagonized by PB treatment in rat liver.

In conclusion, we revealed that several fatty acid-metabolizing enzymes are induced in hepatocytes by treatment with peroxisome proliferator (DHEAS) and/or typical CYP inducers in rats carrying fatty livers induced with orotic acid. The attenuation in fatty liver observed in this study may provide a clue to future therapeutic options for NAFL/NASH, although further studies addressing the functions of fatty acid-metabolizing enzymes in fatty liver and their related mechanisms are needed.

ACKNOWLEDGMENTS

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Conflict of interest---- The authors declare that there is no conflict of interest.

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DHEAS and PB attenuated steatosis in rats carrying fatty liver
