Hepatic zonation of the induction of cytochrome P450 IVA, peroxisomal lipid β-oxidation enzymes and peroxisome proliferation in rats treated with dehydroepiandrosterone (DHEA). Evidence of distinct zonal and sex-specific differences

Konstantin Beier1,3, Alfred Völk1, Christel Metzger2, Doris Mayer1, Peter Bannasch1 and H. Dariush Fahimi1
1Institut für Anatomie und Zellbiologie II, Im Neuenheimer Feld 307, 69120 Heidelberg and 2Deutsches Krebsforschungszentrum, Abteilung Cytopathologie, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany
3To whom correspondence should be addressed

Dehydroepiandrosterone (DHEA) is an intermediate product in the synthesis of male and female sex hormones in the adrenal cortex of man. In livers of rats and mice DHEA increases the levels of cytochrome P450 IVA and peroxisomal β-oxidation enzymes associated with peroxisome proliferation. Prolonged treatment of rats with DHEA induces liver tumors that are more frequent in females arising mainly in the perportal regions of the liver lobule (Metzger et al., Toxicol. Pathol. 21, 591–605, 1995). Because of paucity of information on hepatic zonation of peroxisomal response to DHEA and controversial reports on gender-specific differences of its effects the present study was undertaken using qualitative immunohistochemical and quantitative immunoelectron microscopical techniques in addition to Western blottings. Rats were treated for 24 weeks with 0.6% DHEA supplied with diet. Immunoblot analysis revealed marked induction of peroxisomal β-oxidation enzymes, which by quantitative analysis was equally strong in male and female animals, whilst catalase and urate-oxidase were not increased. Cytochrome P450 IVA, in contrast, was induced significantly stronger in male than in female rats. Immunohistochemistry confirmed the induction of cytochrome P450 IVA showing a marked lobular gradient in female animals with strong induction in pericentral and almost no induction in periportal regions of the liver lobule. In male animals cytochrome P450 IVA was expressed more uniformly across the liver lobule. A similar sex specific zone-dependent response was observed for peroxisomes. DHEA induced in females a significant zonal gradient with marked peroxisome proliferation and a strong induction of peroxisomal hydratase/dehydrogenase in pericentral hepatocytes and a much smaller response in periportal regions. Livers of male animals, in contrast, showed a uniform peroxisomal proliferation to DHEA with only slight zonal differences. The striking homologies of the induction patterns of cytochrome P450 IVA showing a marked lobular and zonal gradient with marked peroxisome proliferation associated with staged peroxisomal gene (22), which indicates the involvement of this receptor in the induction process.

In the present study we have re-investigated the effects of DHEA on peroxisomes and cytochrome P450 IVA in rat liver with particular attention to two important aspects: the liver architecture and the sex specificity. It is well known, that various exogenous and endogenous factors induce a heterogeneous peroxisomal response in the different zones of the liver acinus thus establishing zonal gradients (23–26), and recently it has been shown that DHEA-induced tumor pre-stages are localized almost exclusively in periportal regions of the liver lobule (10). In most previous investigations of DHEA effects on peroxisomes and cytochrome P450 IVA, however, the heterogeneity of the liver architecture has received little attention.

Moreover, a variety of peroxisome proliferating agents have been reported to act more vigorously in male than in female rats (27–30). Investigations of the gender-specific effects of DHEA on peroxisomal parameters, however, have led to controversial results ranging from stronger effects in females (31), or alternatively in males (15), all the way to the statement that it is equally potent in both sexes and that the peroxisome proliferative effect of DHEA is not influenced by sex hormones (32). On the other hand, the incidence of tumor pre-stages induced by DHEA treatment was shown recently to be significantly greater in livers of females than of male rats (10). Since the tumor-inducing effect of many peroxisome proliferating agents has been related to the induction of peroxisomal...
β-oxidation and the associated increase of oxidative stress (33–35), investigations of gender specific differences of DHEA on peroxisomal parameters should be of great interest for the further elucidation of the correlation between tumor induction and peroxisome proliferation.

Materials and methods

Treatment of animals
Male and female Sprague–Dawley rats, purchased from the Zentralinstitut für Versuchstierzucht (Hannover, Germany) were maintained in compliance with the guidelines for humane care and use of laboratory animals. They were held under standardized conditions (22°C, 12 h 7 a.m.–7 p.m. light–dark cycle) and received a laboratory diet and tap water ad libitum. The treatment was started at the age of 18 weeks. The animals were fed a standard nitisinone-poor pellet diet (Altromin, Lage/Lippe, Germany) containing 0.6% (w/w) DHEA (Scherering, Berlin, Germany). Control animals received the same standard diet without DHEA. After 24 weeks of treatment the animals were killed under ether anesthesia and processed for histochemical and morphological studies.

Tissue processing

Livers from six controls and six DHEA-treated animals from each sex were removed, rinsed with 0.9% saline and quickly frozen on dry ice for homogenization and immunoblot analysis. Another part of the tissue was fixed for immunohistochemistry of peroxisomal proteins in Carnoy’s fixative (36) and embedded in paraffin (Paraplast plus, Sherwood Medical Co., St Louis, MO). For morphometry and electron microscopic immunocytochemistry livers from two controls and two DHEA-treated animals from each sex were fixed by perfusion through the portal vein (37) with a fixative containing 0.25% glutaraldehyde and 2% sucrose in 0.1 M Pipes buffer, pH 7.4. The tissue was cut into 70 µm sections with a DSK-Microslicer (Dosaka EM Co., Kyoto, Japan). One part of the microsliced sections was embedded directly in LR-white (London Resin Co. Ltd, Hampshire, UK) for immunocytochemistry. The other sections were embedded in alkaline dianisobenzene (DAB) medium for cytochemical visualization of catalase (38), followed by osmication and embedding in Epon 812. In addition, for assessment of very long-term effects of DHEA and comparison with the animals treated for 24 weeks, a few slices of liver from rats treated for 78 weeks were fixed in Carnoy’s fixative and processed for light microscopic immunohistochemistry as described (36).

Preparation of antibodies

Polyclonal antibodies to catalase (Cat), urate-oxidase (UOX) and two enzymes of the peroxisomal β-oxidation chain: acyl-CoA oxidase (AOX) and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (PH) were prepared and their specificity was assessed as described previously (40). The antibody to cytochrome P450 IVA was obtained from Alexis Deutschland GmbH (Grüningen, Germany) and its mono-specificity was confirmed by Western blotting.

Analysis of peroxisomal proteins by immunoblotting

Liver tissue from controls and six animals of each sex that were treated with DHEA, were homogenized in 5 vol. (w/v) of homogenization buffer containing 250 mM sucrose, 1 mM EDTA, 0.1% ethanol, 5 mM morpholinopropane sulfonic acid (MOPS) pH 7.4, and as inhibitors 0.2 mM dithiothreitol, 0.2 mM phenylmethyl-sulfonylfluoride, 1 mM 6-aminopropionic acid and 5 mM benzamidine–HCl. For SDS-PAGE, aliquots of tissue extracts were boiled for 4 min in 4 vol. of sample buffer (50 mM Tris, 1% SDS, 10% glycerol and 5% mercaptoethanol), and equal amounts of protein were loaded per lane. Polyacrylamide were resolved in 12% polyacrylamide slab gels containing 0.1% SDS and were subsequently transferred to nitrocellulose (41). Blots were soaked in 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS)-TWEEN buffer, pH 7.4 for 1 h at 37°C to block unspecific binding sites, and further incubated overnight at 4°C with primary antibodies diluted in PBS-TWEEN containing 0.1% BSA. Concentrations of antibodies were 10 µg/ml. After repeated washing, a peroxidase-conjugated goat anti-rabbit antibody (1:10 000, Sigma, München, Germany) was added for 1 h at room temperature, and enhanced chemiluminescence (ECL, Amersham International, Amersham, England) was used to visualize the immune complexes. The signals obtained were quantitated using an image analysis system (Quantimet 500+, Leica, Bensheim, Germany).

Light microscopic immunohistochemistry

Paraffin sections (5 µm) from the embedded livers were incubated for immunohistochemistry using either an antibody against multifunctional enzyme (PH) or one against cytochrome P450 IVA. The bound antibody was visualized by a biotinylated secondary antibody followed by incubation with an avidin-peroxidase conjugate (ExtrAvidin Peroxidase Staining Kit, Sigma, Munich, Germany) and aminoethylcarbazol as substrate (36).

Morphometry and quantitative immunoelectron microscopy

Morphometry. The volume density of peroxisomes was determined in 1-µm light microscopic DAB-stained sections using an automatic image analyzer (Quantimet 500+, Leica, Bensheim, Germany). Periportal and pericentral areas within a distance of about three cell layers from a portal tract or a central vein were analyzed separately. The peroxisomes and the total area of hepatocytes, excluding the sinusoids, were detected separately due to their different gray-levels and the volume density of peroxisomes was calculated as described earlier (42,43).

Quantitative immunoelectron microscopy. Ultra-thin sections of LR-white embedded liver selectively obtained either from periportal or from pericentral regions of the liver lobule were incubated with antibodies to catalase, acyl-CoA oxidase and multifunctional protein using the protein A-gold procedure with 12-nm gold particles (44). Electron micrographs were made from peroxisomes within three cell layers surrounding either the portal tracts or the central veins, and the labeling density (number of gold particles per µm² of peroxisomal matrix), which provided information about the modulation of peroxisomal enzyme content (40,43), was determined using an automatic image analyzer (45). For each enzyme 50 peroxisomes were analyzed from each animal in periportal hepatocytes, and the same number in pericentral hepatocytes. For statistical analysis Student’s t-test was used.

Results

Immunoblot analysis

The results of immunoblot analysis of peroxisomal enzymes are shown in Figure 1. Whereas the enzymes of the peroxisomal lipid β-oxidation, AOX and PH, were significantly induced in DHEA-treated animals, catalase and urate oxidase were not elevated. Similar to earlier observations (26,40,46), AOX showed three distinct bands corresponding to the subunits A, B and C with molecular masses of approximately 72, 52 and 20.5 kDa. All three bands exhibited significant augmentation after DHEA-treatment as shown for band B by densitometric analysis (Figure 1). PH was induced more strongly than AOX in DHEA-treated animals (Figure 1), Quantitative analysis of immunoblots did not reveal any significant sex-dependent differences in the extent of the induction of AOX and PH. In contrast to peroxisomal β-oxidation enzymes, catalase and urate-oxidase were not altered by DHEA-treatment.

Western blots of cytochrome P450 IVA revealed significant increases in the livers of DHEA-treated animals. In contrast to peroxisomal enzymes, cytochrome P450 IVA showed a distinct gender specific difference with significantly stronger augmentation in male than in female animals (Figure 2).

Morphological observations

Peroxisome proliferation. In sections of rat livers stained with alkaline DAB for visualization of catalase, the peroxisomes were markedly proliferated both in male and female animals treated with DHEA. The intensity of the proliferative response within the liver acinus, however, differed between the male and female rats (Figures 3–5). Whereas in male animals the peroxisome proliferation was more or less diffusely distributed across the entire liver lobule (Figure 3) it was much more pronounced in pericentral hepatocytes of female rats (Figure 4).

The results of morphometric analysis (Figure 5) confirmed the visual impressions. In male rats the peroxisomal volume density was homogeneously increased with slightly higher values in pericentral areas. In contrast, the extent of peroxisome proliferation in female animals was substantially higher in pericentral than in periportal areas thus establishing a significant zonal gradient.
Effects of DHEA treatment

**Fig. 1.** Western-blots of peroxisomal proteins and their densitometric analysis. Immunoblots of liver homogenates from DHEA-treated animals (D) and controls (C). Blots from three representative animals per group and sex are shown. Blots were incubated with antibodies to catalase (Cat), acyl-CoA oxidase (AOX), multifunctional enzyme (PH) and urate-oxidase (UOX) and immunocomplexes were visualized by the ECL-technique (for details see Materials and methods). The plot shows the densitometric analysis of immunoblots. Male controls were set to 100%. For AOX the band of the B-subunit was used for quantification. The bars represent means ± SEM from six controls and six DHEA-treated animals from each sex (M, male; F, female). *Significant differences to controls (P < 0.05).

**Fig. 2.** Immunoblots of liver homogenates incubated with an antibody to cytochrome P450 IVA from DHEA-treated animals (D) and controls (C); M, male; F, female animals. The bars show means ± SEM of densitometric analysis. Black bars: controls, white bars: DHEA-treated. Different letters mean significant differences between groups (P < 0.05).

**Fig. 3.** Light micrographs of rat liver from male animals stained with the alkaline DAB method for cytochemical localization of catalase. Peroxisomes appear as dark stained particles. Normal control liver (a, b). Note the homogenous peroxisome proliferation in periportal and pericentral hepatocytes after DHEA treatment (c, d). P, portal vein; C, central vein. Bars are 20 µm.

**Immunohistochemical observations**

**Cytochrome P450 IVA.** Whereas in control animals the immunohistochemical reaction for P450 IVA was almost negligible (not shown), a prominent staining was observed in the liver of DHEA-treated animals (Figure 6a and b). A distinct gender specific pattern was observed with male rats showing a stronger positive stain that occupied most of the liver lobule except for a narrow ridge around the portal tracts (Figure 6a), in contrast with female animals, which exhibited a prominent stain that was confined to 3–4 cell layers directly surrounding the central veins (Figure 6b).

**Peroxisomal multifunctional enzyme (PH).** In sections immunostained for detection of PH-antigen, a gender specific difference was also noted in DHEA-treated animals. Whereas in male rats a uniformly positive staining was noted extending through the entire liver lobule (Figure 6c), a strong lobular gradient with intense centrilobular reaction was found in females (Figure 6d). This pattern was even further pronounced in female rats treated for 78 weeks with DHEA (Figure 6f). The male animals of the same group again exhibited a diffuse strong stain throughout the liver lobule with a slightly more
The quantitative analysis of immunolabeling with gold particles (Figure 8) showed that the labeling density for PH was significantly increased ($P < 0.05$) in both male and female rats after DHEA treatment in zone I (periportal) and in zone III (pericentral) regions of the liver acinus. Whereas in male rats the labeling density for PH was uniformly increased in both perportal and pericentral hepatocytes, it was significantly ($P < 0.05$) more elevated in pericentral cells of female rats (Figures 7 and 8). The labeling densities for Cat and AOX were less affected by DHEA-treatment with a moderate (statistically insignificant) increase of AOX and a decrease of Cat. This reduction of catalase antigen in individual peroxisomes seems to be sufficient to compensate for the proliferating effect of DHEA on peroxisomes, so that the total catalase antigen as revealed by Western blotting remains unchanged (Figure 1).

**Discussion**

The results of this study have revealed that DHEA induced cytochrome P450 IVA, peroxisomal β-oxidation enzymes and peroxisome proliferation in rat liver in a gender specific and zone-dependent manner.

The DHEA-associated effects with induction of cytochrome P450 IVA, peroxisome proliferation with induction of the enzymes of the peroxisomal β-oxidation, are well known (11,12,14–18,47). As stated above, earlier studies on sex-specific differences of peroxisomal response to DHEA have led to conflicting results (15,31,32). In this study quantitative analysis of immunoblots revealed significant inductions of peroxisomal β-oxidation enzymes but did not show any sex-specific differences in the peroxisomal response to DHEA, thus confirming essentially the findings of Rao et al. (32). The Western blots of cytochrome P450 IVA, however, clearly showed a stronger induction of that protein in male compared with female rats.

The consideration of the zonal heterogeneity of the liver lobule by morphometry and immunohistochemistry also revealed clear gender specific differences in the peroxisomal response to DHEA. Whereas in female rat liver, the hepatocytes in pericentral areas responded more vigorously to DHEA than those of the perportal regions, in male animals these zonal differences were much less pronounced. Since normal rats do not synthesize DHEA in the adrenal gland (48), the differences of the peroxisomal response could be because of sex specific differences in the metabolism of administered DHEA. Indeed, significantly higher plasma levels of DHEA were found in female rats than in males after DHEA treatment (49) implying a higher metabolism and degradation capacity in males than in females.

The sex-specific differences in response to DHEA gain additional importance because of the higher incidence of tumors in female animals after long-term treatment with DHEA (10). Similar to DHEA, different peroxisome proliferating agents induce, after long-term administration and in high doses, hepatocellular tumors in rat liver. This effect has been suggested to be related to the induction of peroxisomal β-oxidation and the associated increase of oxidative stress (33–35). However, the almost exclusive localization of DHEA-induced tumors and their pre-stages in periportal regions of female rats (10), where according to data presented here the peroxisome proliferation is relatively weak, indicate that other factors in addition to peroxisome proliferation may be responsible for the hepatocarcinogenic effect of DHEA. This view is also
Effects of DHEA treatment

Fig. 6. Light micrographs from DHEA-treated rat liver after immunostaining with an antibody to cytochrome P450 IVA (a, b) and PH (c, d, e, f). Male rats (a, c, e) left side, female rats (b, d, f) right side. Treatment time: 24 weeks (a, b, c, d) and 78 weeks (e, f). P, portal tract with periportal area; C, pericentral area. Bars are 100 µm.

supported by observations with a variety of other non-mutagenic hepatocarcinogens that induce peroxisome proliferation, which indicate that the increased mitosis and suppression of apoptosis occur mainly in periportal regions of the liver lobule (50–53) where the peroxisome proliferation is less intense than in pericentral regions (26). For further examination of the role of oxidative stress in DHEA-induced hepatocarcinogenesis, a model using dexamethasone for selective inhibition of cell proliferation without inhibiting peroxisome proliferation as described recently (54), may be helpful.

The transcriptional activation of both cytochrome P450 IVA and peroxisomal β-oxidation enzymes after treatment with various peroxisome proliferating drugs is mediated by nuclear receptors, namely the peroxisome proliferator activated receptors (PPARs) (19,55–61). A strong correlation between the induction of cytochrome P450 IVA and peroxisome prolifera-
tion was also observed in the present study. Besides their common mediation by PPAR, the correlation of induction of cytochrome P450 IVA with peroxisome proliferation and induction of peroxisomal β-oxidation has led to the hypothesis, that both effects may be causally interrelated (62–66). This hypothesis implies that cytochrome P450 IVA ω-hydroxylates the long chain fatty acids that are subsequently oxidized to the corresponding dicarboxylic acids. These are preferentially oxidized further by peroxisomes and it has been suggested that the induced substrate overload may be responsible for peroxisome proliferation (64,65) and induction of peroxisomal β-oxidation by activation of PPAR (66). This latter hypothesis, however, requires further confirmation since dicarboxylic acids do not activate PPAR-α (20,67). Nevertheless, the gender specificity and the strong zonal correlation in the patterns of cytochrome P450 IVA induction and peroxisomal response
study, the use of hepatocytes isolated selectively from either the peripoal or the pericentral regions of the liver lobule may be helpful in elucidating this problem.

Acknowledgements
The excellent technical assistance of L.Fronmer, F.Kurschus, G.Keimer, H.Mohr and A.Weber is gratefully acknowledged. The study was supported by a grant from the ‘Kommission Stiftungen und Preise’ of the University of Heidelberg and by the Deutsche Forschungsgemeinschaft grant Be 1659/2-1.

References
cytochrome P-452 induction and peroxisomal proliferation by hypo-
lipidaemic agents in rat liver. A mechanistic interrelationship. Biochem. 
Pharmacol., 37, 1193–1201.
cytochrome P-452 and the peroxisomal fatty acid beta-oxidation pathway 
in the rat by clofibrate and di-(2-ethylhexyl)phthalate. Biochem. 
Pharmacol., 37, 1203–1206.
mechanisms of induction of hepatic peroxisome proliferation. Annu. Rev. 
P450 IV gene family. Xenobiotica, 19, 1123–1148.
66. Kalkaus,R.M., Chan,W.K., Lysenko,N., Ray,R., de Montellano,P.R.O. and 
Bass,J.M. (1993) Induction of peroxisomal fatty acid β-oxidation and 
liver fatty acid-binding protein by peroxisome proliferators. Mediation via 
the cytochrome P-450IVA1 omega-hydroxylase pathway. J. Biol. Chem., 
268, 9593–9603.
67. Bocos,C., Göttlicher,M., Gearing,K., Banner,C., Enmark,E., Teboul,M., 
Crickmore,A. and Gustafsson,J.A. (1995) Fatty acid activation of 
ultrastructural changes in rat liver induced by the peroxisome proliferator 
proliferation and morphology in rat liver after gemfibrozil treatment. 
cytochrome-P450IVA1 RNA in rat primary hepatocyte culture by 
Zonal distribution of peroxisomal 3-oxoacyl-CoA thiolase messenger RNA 
in liver from rats treated with di-(2-ethylhexyl)phthalate. Biochim. Biophys. 
(1995) Localization of peroxisome proliferator-activated receptor in mouse 
and rat tissues and demonstration of its nuclear translocation in transfected 

Received on January 24, 1997; revised on April 25, 1997; accepted on April 28, 1997