Modulation of the peroxisomal gene expression pattern by dehydroepiandrosterone and vitamin D: therapeutic implications

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Abstract

Peroxisomes are ubiquitous organelles required for several metabolic functions. Their dysfunction is responsible for a group of human inherited disorders. In the search for endogenous factors regulating the peroxisomal compartment in normal liver, we treated female rats with dehydroepiandrosterone (DHEA) and 25-hydroxycholecalciferol for 1 and 6 days. Relative transcription levels of 39 selected genes were evaluated by real-time quantitative RT-PCR analysis. Catalase (peroxisomal marker)-specific activity was assayed in total liver homogenate and peroxisomes were visualized by catalase localization. DHEA induced peroxisome proliferation and raised catalase specific activity. Expression levels of 16 (of which 11 were peroxisomal) genes were altered. Pex 11, acyl-CoA oxidase, L- and D-multifunctional enzyme, thiolase 1, phytanoyl-CoA hydroxylase, 70 kDa peroxisomal membrane protein and very long chain acyl-CoA synthetase were upregulated, three others were downregulated. Vitamin D caused downregulation of six genes. Administration of vitamin D to peroxisomal disorder patients may be contraindicated. The adrenocortical hormone DHEA is a potential natural regulator of the peroxisomal compartment. Its therapeutic use in X-linked adrenoleukodystrophy, some other β-oxidation defects and classical Refsum should be considered.

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Introduction

Peroxisomes are ubiquitous organelles required for several metabolic functions such as breakdown of very long chain fatty acids (VLCFA), phytanic acid, prostaglandins and polynolamines, and synthesis of cholesterol, bile acids, plasmalogens and the polyunsaturated fatty acid (FA) docosahexaenoic acid. Their importance is illustrated by the existence of a group of human inherited metabolic diseases caused by the impairment of one or more peroxisomal functions. In most of these disorders, the nervous system is affected, as in the Zellweger cerebro-hepato-renal syndrome, infantile and classical Refsum disease, X-linked adrenoleukodystrophy (X-ALD) and rhizomelic chondrodysplasia punctata (Van den Bosch et al. 1992, Wanders 1999).

In early embryonic liver, peroxisomes are very few in number, small, and their matrix content is incomplete (Espeel et al. 1997, Depreter et al. 1998). In the rat, a gradual maturation process over a 9–day interval was noticed whereas, in human liver, peroxisomes reach adult size and number around week 20 of gestation (Espeel et al. 1993).

The genes encoding peroxisomal components such as the peroxin-encoding (PEX) genes have been well studied in recent years (Gould et al. 2001, Moser et al. 2001, Wanders et al. 2001a and references therein), but the regulatory factors controlling these genes during development are unknown. The peroxisomal compartment in eukaryotic cells differs between different cell types (Roels et al. 1970, 1987, 1991, Roels & Goldfischer 1979, Roels 1991), and even between individual cells within a tissue (Farioli-Vecchioli et al. 2000), suggesting a tight regulation.

Induction of peroxisome proliferation in rodent liver by xenobiotic compounds (fibrates, phthalates) has been known for many years (Svoboda & Azarnoff 1966). Upregulation in rodents by fenofibrate of two ATP binding cassette (ABC) half transporters (adrenoleukodystrophy-related protein and the 70 kDa peroxisomal membrane protein (PMP 70)) in the peroxisomal membrane has been reported more recently (Albet et al. 1997). This response, if present in man, may represent a therapeutic approach to X-ALD since these ABC half transporters show partial functional overlap with the adrenoleukodystrophy protein (ALDP) (Braiterman et al. 1998,
One putative natural/endogenous regulator of the peroxisomal compartment is the adrenocortical hormone dehydroepiandrosterone (DHEA). DHEA sulphate (DHEA-S) was shown previously to have peroxisome proliferative capacity (Frenkel et al. 1990, Beier et al. 1997) and to stimulate the activity of some peroxisomal enzymes (Leighton et al. 1987, Wu et al. 1989, Hertz et al. 1991, Prough et al. 1994, Milewich et al. 1995, Peters et al. 1996, Beier et al. 1997). In humans, DHEA-S is produced by the foetal adrenal cortex early in gestation (6–12 weeks) (Stäubli & Hess 1975, Bichet et al. 1990, Blaauboer et al. 1990, for reviews see Bentley et al. 1993, De Craemer et al. 1997). In the foetal period, DHEA-S secretion is high, and decreases considerably after birth (Friess et al. 2000). DHEA/DHEA-S levels rise again during childhood and early adulthood. In elderly persons, blood levels decline (Yen & Laughlin 1998). DHEA is another naturally occurring substrate possibly influencing peroxisome maturation and function. This hormone is important in calcium metabolism and bone formation (skeletal mineralization); in addition, the nuclear receptor for VD (VDR) belongs to the same group as other receptors involved in peroxisome proliferation: the peroxisome proliferator-activated receptor (PPARα), retinoid acid receptor (RAR) and thyroid hormone receptor (THR). These receptors all recognize a two-tandem repeat hexanucleotide sequence in their respective response elements which is common for these group II receptors (Jiang et al. 1995, Chawla et al. 2001), with different spacing in between the two-tandem repeats for each receptor. Their DNA-binding domains consist of two Zn-finger-like structures, responsible for specific recognition of the hormone response element (Umesono et al. 1991). PPARα, THR, RAR and VDR mostly form heterodimers with retinoid X receptor (RXR) (Mangelsdorf & Evans 1995). The ligands for PPARα (Reddy & Hashimoto 2001), RAR (Hertz & Bar-Tana 1992) and THR (Hartl & Just 1987) are known to induce some peroxisomal enzymes such as acyl-CoA oxidase, and variable degrees of peroxisome proliferation.

Further indication for a possible role of VD in the regulation of the peroxisomal compartment is provided by studies that demonstrated peroxisome proliferation in chicken liver (Davis et al. 1990) and gut epithelium (Davis & Jones 1984) in response to VD. The same authors have demonstrated induction by VD of peroxisomal β-oxidation in rat liver (Davis et al. 1989).

Examination of the effect of candidate natural regulators on the expression level of a large series of genes involved in peroxisomal biogenesis and function may not only test the hypothesis that a particular substance indeed has a regulatory role in normal peroxisome expression, but may also reveal new possibilities for therapeutic application of these compounds.

Materials and Methods

Animals

For the DHEA experiment, 8-week-old female OFA Sprague–Dawley rats (Ifa Credo, Brussels, Belgium) on a normal control diet (EF 1/0; Sniff) received DHEA (Sigma D-4000) in their food (0.51–0.88 g/kg body weight per day). A similar dose was used by McCormic et al. (1996). DHEA was dissolved in ethanol; the solution was absorbed by the chow and ethanol was evaporated before feeding. In a short-term experiment, a single dose was given to four rats, which were killed 17.5–18.5 h later. Six other animals were treated for 6 days. Treated rats were compared with vehicle-only controls.

For the VD experiment, 10- to 12-week-old female VD-deficient rats (25-hydroxycholecalciferol below normal value) were obtained by the administration of a VD-restricted diet (EF 1/52; Sniff; containing 1.7% Ca and 0.4% P) to the maternal rats from before pregnancy and to their offspring until the age of 10–12 weeks. The animals were housed in a u.v.-free room. (u.v. transmission of the lampshade covering normal tube light bulbs present in the room was measured by the use of a Cary 500 UV–VIS–NIR spectrophotometer (175–3300 nm) (Varian Inc., Palo Alto, CA, USA). Transmission between 200 and 380 nm was lower than 0.01%. In a second measurement, transmission between 280 and 370 nm was even below 0.001%). In a short-term experiment, VD-deficient rats received either 500 IU 25-hydroxycholecalciferol dissolved in propylene glycol (Dedrogyl) in their food (four treated rats), or the equivalent amount of propylene glycol without VD (four control rats). They were killed 14 h later. For the 6-day experiment, VD-deficient rats received 100 IU 25-hydroxycholecalciferol/day for 6 days (four treated rats) or the equivalent amount of propylene glycol in the same dosage schedule (four controls).

All rats were killed by decapitation. The protocol was in accordance with the FRAME guidelines on experiments involving the use of laboratory animals and was approved by the Ethical Committee for Animal Experiments, University of Ghent (ECP01/17).

Blood analysis

In the VD experiment, blood samples from the tail were taken at the age of 6 weeks after ether anaesthesia. To
evaluate the VD deficiency of the rats receiving a VD-depleted diet, the 25-hydroxyvitamin D blood level was measured by means of a double-antibody radioimmunoassay (Diasorin, Stillwater, MN, USA) after extraction with acetonitrile. Blood was also collected at the moment of decapitation and 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D were measured. The method used for analysis of 1,25-dihydroxyvitamin D was a radioimmunoassay (IDS Gamma–B 1,25-dihydroxyvitamin D; IDS Ltd, Boldon, Tyne and Wear, UK) after delipidation and immune extraction of the sample using a highly purified specific solid-phase monoclonal antibody.

Catalase activity

Catalase activity in total liver homogenate from the rats in the 6-day experiment was determined according to the method of Baudhuin et al. (1964). Protein concentration was measured by the method of Bradford (1976).

Microscopy

 Peroxisomes were visualized for light and electron microscopy by staining for catalase activity (Roels et al. 1995).

RNA isolation and cDNA synthesis

Immediately after decapitation, fragments of liver were snap frozen in liquid nitrogen and stored at –80 °C. Tissue blocks (<250 mg) were homogenized in RLT lysis buffer (Qiagen, Uden, The Netherlands) for 45–60 s using an Ultra-Turrax T25 (IKA-Werke, Staufen, Germany). The homogenates were stored at –80 °C in lysis buffer until further use. Total RNA was isolated from all individual liver samples using the column-based RNeasy Midi isolation kit from Qiagen, Uden, The Netherlands. Total RNA was quantitated with a TD-360 Mini-Fluorometer (Turner Designs, Sunny Vale, CA, USA) using RiboGreen (Molecular Probes, Leiden, The Netherlands) as a detection method. To evaluate the quality of isolated total RNA, electrophoresis in RNAse-free conditions was performed. Residual genomic DNA was removed by an RQ1 DNase treatment (Promega, Madison, WI, USA). Samples were purified and desalted by transferring them to a Microcon-100 column (Amicon no. 42413, Millipore Corp., Bedford, MA, USA). The resulting DNAse-free RNA was reverse-transcribed to cDNA by use of Superscript II reverse transcriptase (Invitrogen, Ghent, Belgium) and random hexamer primers (Amersham Biosciences, Bucks, UK).

Real-time quantitative PCR

The single-stranded cDNA was then used in real-time quantitative PCR for evaluation of relative expression levels of the 39 genes of interest. Selected genes and primers are shown in Tables 1 and 2, and Fig. 1.

Real-time quantitative PCR was performed according to Vandesompele et al. (2002a). In short, using Primer Express 1·0 software (Applied Biosystems, The Perkin–Elmer Corp., Foster City, CA, USA) and the default TaqMan parameters, with modified minimum amplification length requirements (75 bp), specific primers were selected and purchased (Invitrogen) (Fig. 1). All samples were examined in duplicate. The PCR gene-specific amplification was performed in the Applied Biosystems (ABI5700) real-time PCR machine with the GeneAmp 5700 Sequence detection system software. The qPCR Mastermix for Sybr Green I kit (Eurogentec, Seraing, Belgium) was used for quantification. Controls without template were always included. Each well contained 2 µl template cDNA (approximate final concentration 0·8 ng/µl), 0·8–1·25 µl specific forward and reverse primers (maximal final concentration 250 nM) and 0·75 µl Sybr Green I stock solution (final dilution 1/66 000; Eurogentec), 12·5 µl 2 × reaction buffer (containing dNTPs including dUTP, hot goldstar DNA polymerase (Eurogentec), uracil-N-glycosylase, MgCl₂ (final concentration 5 mM), Carboxy-X-Rhodamine (ROX) passive reference), and RNAse-free water (Sigma). Total reaction volume was 25 µl.

The reactions were run in 96-well plates at 60 °C for 2 min to activate the uracil N‘-glycosylase and then for 10 min at 95 °C to inactivate the uracil N‘-glycosylase and activate the hot goldstar polymerase. The reactions were performed for 40 cycles of 15 s at 95 °C and 60 s at 60 °C. A dissociation thermal protocol with a start temperature at 60 °C was added for each experiment, to analyse the melting peaks of the PCR products generated.

Relative expression levels were determined with the comparative threshold cycle (Ct) method, with amplification efficiency set at 97%. Relative expression levels of the target genes were normalized to the geometric mean of the three most stable of six tested internal control genes (Hprt, Ywhaz, Hmbs). The expression stability of the control genes was determined by the geNorm algorithm described by Vandesompele et al. (2002b).

Statistics

Significance of differences was analysed by the Mann–Whitney U non-parametric test, with P<0·05 as the level of significance.

Results

Blood analysis of VD-treated rats

By giving a VD-depleted diet to the maternal rats from before pregnancy and during weaning and thereafter
to their progeny, rats with VD deficiency were obtained. After 6 weeks, their 25-hydroxyvitamin D blood levels were significantly lower (\(<12.5\) nmol/l) than in age- and sex-matched rats that received a control diet (38.8 ± 5.2 nmol/l) (\(P<0.001\)). 25-Hydroxyvitamin D blood levels were significantly higher in the rats that received a 6-day or single dose VD treatment versus the rats that received the vehicle (propylene glycol) only for 6 days or in a single dose (\(P<0.05\)) (6-day experiment: 193.9 ± 24.6 nmol/l versus 10.1 ± 5.4 nmol/l; short-term experiment: 159.3 ± 47.2 nmol/l versus 10.6 ± 3.7 nmol/l (means ± s.d.)). 25-Hydroxyvitamin D blood levels of control rats given vehicle only and the VD-deficient rats before treatment did not differ.

1,25-Dihydroxyvitamin D blood levels were significantly higher in VD-treated animals than in the vehicle-only controls after a single-dose VD treatment (\(P<0.05\) (6-day experiment: 193.9 ± 24.6 nmol/l versus 10.1 ± 5.4 nmol/l; short-term experiment: 159.3 ± 47.2 nmol/l versus 10.6 ± 3.7 nmol/l (means ± s.d.))). 25-Hydroxyvitamin D blood levels of control rats given vehicle only and the VD-deficient rats before treatment did not differ.

**Table 1** Effect on transcription level of genes encoding

<table>
<thead>
<tr>
<th>*Proteins involved in peroxisome biogenesis disorders: Assembly deficiencies in man: Peroxins:</th>
<th>DHEA effect</th>
<th>VD effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroxin 2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Peroxin 3</td>
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<tr>
<td>Peroxin 6</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Peroxin 12</td>
<td>—</td>
<td>(\downarrow) (both)^†</td>
</tr>
</tbody>
</table>

| *Protein involved in multiple peroxisomal enzyme deficiencies in man: Peroxin 7 | — | — |

<table>
<thead>
<tr>
<th>*Protein that is deficient in or related with a single peroxisomal enzyme/protein deficiency in man:</th>
<th>DHEA effect</th>
<th>VD effect</th>
</tr>
</thead>
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<tr>
<td>VLC acyl-CoA synthetase</td>
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<tr>
<td>Adrenoleukodystrophy-related protein</td>
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<tr>
<td>PMP 70</td>
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<td>—</td>
</tr>
<tr>
<td>Acyl-CoA oxidase</td>
<td>↑ (both)</td>
<td>—</td>
</tr>
<tr>
<td>Pristanoyl-CoA oxidase</td>
<td>—</td>
<td>(\downarrow) (6 days)</td>
</tr>
<tr>
<td>Trihydroxycoprostanoyl-CoA oxidase</td>
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<td>—</td>
</tr>
<tr>
<td>(\delta) bifunctional enzyme</td>
<td>↑ (both)</td>
<td>—</td>
</tr>
<tr>
<td>(\lambda) bifunctional enzyme</td>
<td>↑ (both)</td>
<td>—</td>
</tr>
<tr>
<td>Thiolase 1</td>
<td>↑ (both)</td>
<td>—</td>
</tr>
<tr>
<td>Thiolase 2</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Acyl-CoA: dihydroxy acetone phosphate acyltransferase</td>
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<td>(\downarrow) (6 days)</td>
</tr>
<tr>
<td>Alkyl-dihydroxyacetonephosphate synthase</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Phytanoyl-CoA hydroxylase</td>
<td>↑ (both)</td>
<td>—</td>
</tr>
<tr>
<td>Catalase</td>
<td>↑ (6 days)</td>
<td>—</td>
</tr>
<tr>
<td>Serine:pyruvate/alanine:glyoxylate aminotransferase</td>
<td>↑ (6 days)</td>
<td>—</td>
</tr>
<tr>
<td>Racemase</td>
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<td>ND</td>
</tr>
<tr>
<td>Mevalonate kinase</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Glutaryl-CoA oxidase</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

—, no significant difference; ^†both short-term and 6-day experiments; ND, not done.

*Modified from Gould et al. (2001); Wanders (1999) and Raymond & Moser (1997).

DHEA treatment resulted in a strong upregulation of Pex 11 (15- to 18-fold) and of several \(\beta\)-oxidation enzymes: acyl-CoA oxidase, both \(\lambda\) and \(\delta\) forms of multifunctional enzyme (L-BP and D-BP), 3-ketoacyl-CoA thiolase (thiolase 1) (straight chain FA), but not pristanoyl-CoA oxidase, trihydroxycoprostanoyl-CoA oxidase and sterol carrier protein-x (SCPx) (thiolase 2) (branched chain FA). Phytanoyl-CoA hydroxylase mRNA was increased by a factor of 2. Transcription levels of the 70 kDa peroxisomal membrane protein and very long chain (VLC) acyl-CoA synthetase were increased after DHEA treatment, whereas adrenoleukodystrophy-related protein and 22 kDa peroxisomal membrane protein expression levels remained constant. PPAR\(\alpha\) was upregulated in both the

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6-day and the short-term experiments. A small effect was seen on mRNA of VD 3–25-hydroxylase that was induced only after short-term DHEA treatment. Catalase, serine-pyruvate aminotransferase and the bile salt export pump were moderately upregulated only in the 6-day DHEA experiment. In contrast, HMG-CoA reductase, fructose-1, 6-biphosphatase and p21 were downregulated. Expression levels of three out of four mitochondrial marker enzymes did not change.

| Table 2 Effect on transcription level of genes encoding peroxisomal proteins and functions after VD treatment (Tables 1 and 2, and Fig. 2) |
|-------------------------------------------------|-------------------------------------------------|
| **Peroxisomal proteins**                        | **DHEA effect**                                 |
| δ amino acid oxidase                            | —                                               |
| Uricase                                        | —                                               |
| PMP 22                                         | —                                               |
| HMG-CoA reductase                               | ↓ (6 days)                                      |
| Isocitrate dehydrogenase (NADP)                | —                                               |
| **Proteins related to peroxisome biogenesis or proliferation** | **VD effect**                                   |
| Peroxin encoded by Pex 11                       | ↑ (both)†                                       |
| Peroxin encoded by Pex 14                       | —                                               |
| PPARα                                          | —                                               |
| RXRα                                          | ↓ (short term)                                  |
| **Mitochondrial markers**                       | **DHEA effect**                                 |
| Isocitrate dehydrogenase (NAD)                  | —                                               |
| Superoxide dismutase 2 (mitochondrial)          | —                                               |
| Cytochrome c oxidase subunit Vlc                | —                                               |
| Vitamin D3 25-hydroxylase                      | ↑ (short term)                                  |
| **Other**                                       | **VD effect**                                   |
| Multidrug resistance associated protein 3      | —                                               |
| Bile salt export pump                          | ↑ (6 days)                                      |
| Fructose 1,6-biphosphatase                     | ↓ (6 days)                                      |
| p21                                            | ↓ (6 days)                                      |
| 1,25-dihydroxy vitamin D3 receptor             | —                                               |
| —, no significant difference; †both short-term and 6-day experiments.

Quantitative PCR analysis of relative gene expression levels after VD treatment (Tables 1 and 2, and Fig. 2)

Six days of VD treatment caused downregulation of δ amino acid oxidase (DAAOX), dihydroxyacetonephosphate acyltransferase (DHAPAT) and pristanoyl-CoA oxidase (which accepts both pristanoyl-CoA and straight chain acyl-CoA as substrates). Pex 12 was downregulated both after short-term and 6-day treatment. VD 3–25-hydroxylase and PPARα were downregulated in the short-term experiment.

For eight genes, expression levels of both VD-treated and vehicle-treated control groups were significantly altered in the 6-day experiment compared with the short-term experiment. For 31 other genes, the expression levels were not significantly changed. The eight genes were: DAAOX (higher expression level in 6-day experiment), trihydroxycoprostanoyl-CoA oxidase, enoyl-CoA: hydratase-δ-3-hydroxyacyl-CoA dehydrogenase (D-BP), NADP-dependent isocitrate dehydrogenase, Pex 11, Pex 12, PMP 70 and p21 (higher expression level in the short-term experiment).

Catalase activity

The small increase in expression level of catalase mRNA (× 1.25) (P < 0.05) after DHEA treatment corresponded to the 1.3-fold increase in catalase specific activity (Units Baudhuin/mg protein) (P = 0.01) after 6 days of treatment. After 6 days of VD treatment, catalase activity was the same for control and treated groups.

Microscopy (Figs 3 and 4)

Light microscopic examination of sections by use of a code not identifiable by the observer unambiguously demonstrated an increase of peroxisome number in all DHEA-treated animals, which were easily distinguished from all control samples. Counts of peroxisomes per area on light micrographs of DHEA-treated livers showed an increase of 1.6 times (P < 0.01). In addition, peroxisomal size in treated animals became very heterogeneous, with the appearance of both very small and strongly enlarged organelles. In DHEA-treated samples, an alignment of peroxisomes in rows was frequently observed.
<table>
<thead>
<tr>
<th>Symbol</th>
<th>protein</th>
<th>REVERSE PRIMER</th>
<th>FORWARD PRIMER</th>
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<td>Pex6</td>
<td>5'GGGTTTCCAGCCTGCTG3'</td>
<td>5'GAGATCTGCTGCTGCTG3'</td>
</tr>
</tbody>
</table>

**Putative rat homologues for human and mouse pex 5 and pex 7 were identified by BLAST analysis (Basic Logical alignment search tool, http://www.ncbi.nlm.nih.gov/BLAST) of the rat EST database (Primer expressed sequence tags, http://www.ncbi.nlm.nih.gov/dbEST).**

*Unigene cluster (http://www.ncbi.nlm.nih.gov/UniGene) Rn.12543 was 87% identical to human and mouse pex 7. Rn.1435 was 96.2% and 92.4% identical to mouse and human pex 5 respectively. ** Pex 11/afii9825 as described by Passreiter et al. (1998). *** This primer pair was used for further analysis.

Primers were chosen in a domain that is common for the A and B gene as described by Bodnar & Rachubinski (1990).

Primers were chosen downstream of the two promoter regions as described by Sugiyama et al. (2001) and, as such, both peroxisomal and mitochondrial ACT transcript variants are amplified.

Figure 1: Putative boundaries for human and mouse pex 5 and pex 7. Primers were chosen to be able to amplify a fragment of the pex5 and pex7 promoters. A primers were chosen to be able to amplify a fragment of the pex5 and pex7 promoters. A primers were chosen to be able to amplify a fragment of the pex5 and pex7 promoters. A primers were chosen to be able to amplify a fragment of the pex5 and pex7 promoters.
Electron microscopy confirmed the increases of peroxisomal number and size (Fig. 3). It also showed clusters of nearly touching organelles (Fig. 4). Peroxisomal diameter was increased to 1·0 µm in treated animals, versus 0·83 µm as the largest diameter in the controls.

There was no difference between the treated and the control group in the VD experiment either by light microscopic examination or in light micrographs.

Discussion

In this study, we investigated the possible role of DHEA and VD as natural regulators of peroxisome expression.

Our results with DHEA are in agreement with earlier reports (Leighton et al. 1987, Wu et al. 1989, Frenkel et al. 1990, Hertz et al. 1991, Prough et al. 1994, Peters et al. 1996, Beier et al. 1997). Light and electron microscopy after localization of catalase activity unambiguously demonstrated proliferation of peroxisomes after DHEA. The clusters of nearly touching organelles as seen by electron microscopy strongly suggest a fission or budding process and are comparable with the image seen in liver after treatment with the peroxisome proliferator nafenopin for 17 days (see Fig. 9 in Roels 1991). This is consistent with the strong induction of Pex 11α. Pex 11α and β are involved in peroxisome proliferation (Abe et al. 1998, Passreiter et al. 1998, Schrader et al. 1998). The enlarged size of part of the peroxisomes might possibly result from a disequilibrium between a very moderate increase in number of peroxisomes (1·6 times) and a strong induction of several peroxisomal enzymes. mRNA increased up to 23·5 times (23·5-fold 1-bifunctional enzyme induction; 7·5-fold thiolase 1 induction, 4·2-fold acyl-CoA oxidase 1 induction). At the dose of DHEA that our rats consumed, induction levels may be expected to be maximal (Yamada et al. 1991). However, Yamada et al. (1991) showed that even a dose of 0·05 g/kg per day for 14 days can induce total peroxisomal β-oxidation activity.

In our study, in addition to its effects on the peroxisome population, the natural hormone DHEA caused a down-regulation of p21. Interestingly, very recently, a relationship has been suggested between high p21 expression and hepatocarcinogenesis in cirrhosis after hepatitis C virus infection (Wagayama et al. 2002).

DHEA provoked a small induction of the peroxisomal marker enzyme catalase but more important positive effects on several other peroxisomal membrane and matrix components. A particularly strong increase (15–18 times) of Pex 11α was seen. Induction of its gene product in rat liver was described after treatment with thyroid hormone (Passreiter et al. 1998) and clofibrate (Abe et al. 1998). In yeast, disruption of Pex 11β caused oleate-induced giant peroxisomes (Erdmann & Blobel 1995) while overexpression resulted in peroxisome proliferation (Marshall et al. 1995). In a recent study in yeast, expression of Pex 11 was found to be co-regulated with the β-oxidation machinery and it was proposed that Pex 11p might be a transporter for medium chain fatty acids or for essential cofactors involved in β-oxidation (Van Roermund et al. 2000).

One could speculate that the modulation of the expression levels of two peroxisomal enzymes by DHEA might represent feedback mechanisms: (1) HMG-CoA reductase, a peroxisomal enzyme involved in the biosynthesis of cholesterol, a precursor of DHEA, was downregulated, and (2) On the other hand, 17β-hydroxysteroid dehydrogenase type IV, which is identical to β-bifunctional enzyme (Dieuaide-Noubhani et al. 1996) was upregulated. According to Prough et al. (1994), it appears that DHEA is metabolized by this enzyme. A severe peroxisomal disorder is due to deficiency of the latter enzyme (β-bifunctional enzyme deficiency) (Van Grunsven et al. 1999).

Several other genes deficient in peroxisomal disorders were upregulated by DHEA: acyl-CoA oxidase 1 (isolated defect described as ‘pseudo–NALD’; Poll-The et al. 1988), thiolase 1 (pseudo-Zellweger syndrome; Goldfischer et al. 1986, Schram et al. 1987), phytanoyl-CoA hydroxylase (defective in classical Refsum disease; Wanders et al. 2001b) and VLC acyl-CoA synthetase (functionally impaired in X-ALD, but the mutation is in ALDP, i.e. a different gene; Moser et al. 2001). PMP 70, a peroxisomal membrane protein that can partially complement the VLCFA β-oxidation defect in cultured X-ALD fibroblasts (Braiterman et al. 1998), was upregulated as well. In contrast, 16 peroxisomal genes were not changed in expression level. This underlines the fact that distinct genes, although related to the same organelle or function, are controlled by separate factors. Oxidases of the straight chain FA, and of the branched chain compounds (bile acids and pristanic acid) are differentially regulated.

A similar differential regulation of peroxisomal oxidases is seen in response to the classical xenobiotic peroxisome proliferators such as clofibrate (Reddy & Hashimoto 2001). Ostlund Farrants et al. (1990), however, described induction of the branched-chain trihydroxycoprostanoyl-CoA oxidase by fatty diets in rodents. Phytanoyl-CoA hydroxylase activity was induced up to four times in cell lines grown in the presence of phytic acid (Zomer et al. 2000). In mouse liver, phytic acid caused a differential induction of palmitate peroxisomal β-oxidizing capacity (5-fold), of total catalase activity (only 1·5-fold) and urate or glycolate oxidase activities (no change). Moreover, peroxisome proliferation was induced in liver and duodenal epithelium, but not in the kidney (Van den Branden et al. 1986).

Differential regulation of peroxisomal proteins was also observed during normal development (Depreter et al. 1998). In primary cultures of hepatocytes, microenvironmental factors control liver-specific features, such as albumin secretion, glycogen deposition and biotransformation activities, but without preserving peroxisomal catalase and...
Observation of the peroxisome marker enzyme catalase or of any other single enzyme will give an incomplete view of peroxisomal functional capacity. This will be particularly important if treatment of a patient with a peroxisomal disorder is proposed. For example, the strongest mRNA increase after DHEA was observed for L-BP, but no impairment of this enzyme has yet been discovered in human disease. DHEA increased transcription of D-BP; in a recent review D-BP was still considered to be non-inducible (Reddy & Hashimoto 2001).

A drug should be selected that upregulates specific components belonging to the metabolic function that is impaired. In this respect, our results suggest that DHEA is a candidate drug for the treatment of X-ALD. Indeed, X-ALD is a peroxisomal disease typically affecting the adrenal glands and the central nervous system (CNS). In the normal foetus, the adrenal cortex secretes high levels of DHEA. In the adrenal cortex of an X-ALD foetus, pathologic features (polarizing inclusions and acetone-insoluble fat) are present (Depreter et al. 2002b). Also in 26 X-ALD patients, DHEA levels were lower than in control subjects (Assies et al. 1998). Low levels of the hormone
might contribute to inflammatory brain disease (Rook et al. 1994). CNS involvement in ALD is characterized mainly by an inflammatory demyelination of the cerebral and cerebellar white matter, which can start from early childhood to adulthood. These data in the literature are in favour of the hypothesis that DHEA might be an important endogenous mediator in establishing a normally functioning peroxisome population, and that this mediator is defective in X-ALD.

In X-ALD, a mutation was identified in the ALDP gene. However, it is VLC acyl-CoA synthetase that is functionally deficient. Since VLC acyl-CoA synthetase is upregulated by DHEA, the latter might improve the function of this enzyme impaired in X-ALD. There is around 20% residual β-oxidation capacity in X-ALD (Singh et al. 1984a,b, 1998), suggesting that upregulation should be possible. Residual activity is also present in acyl-CoA oxidase–1 deficiency (Poll-The et al. 1988); upregulation by DHEA might be tried.

Classical Refsum disease (CRD) (deficiency of phytanoyl-CoA hydroxylase) is another peroxisomal disorder that might possibly benefit from the inductive capacity of DHEA. In CRD, irreversible prenatal lesions, such as cerebral neuronal migration defects, have not been described (Powers & Moser 1998). In most cases, a pigmentary retinal degeneration with night blindness is one of the first symptoms. Enzyme activity measurements in liver homogenates of liver specimens from CRD patients showed a very low residual enzyme activity (Wanders et al. 2001b). Together with dietary measures (elimination of phytanic acid), induction of phytanoyl-CoA hydroxylase by DHEA might be considered.

It should be investigated first whether, in humans, DHEA has the same effect as in the rat on peroxisomal genes. It has been suggested that DHEA-S (and not DHEA) exerts its function via PPARα (Peters et al. 1996, Poynter & Daynes 1998, Zhou & Wazman 1998, Yen 2001); in humans, this receptor appears not to be functional for the induction of a number of genes that in rodents are peroxisome–proliferator inducible (Lawrence et al. 2001).

However, in cell-based assays, DHEA was not shown to function through PPARα (Waxman 1996, Webb et al. 1996). Many authors have proposed other mechanisms of action: DHEA might activate the androgen receptor (Bocuzzi et al. 1993, Kasperk et al. 1997), but it was reported that DHEA does not bind the androgen receptor (Donohoue et al. 2001). Bruder et al. (1997) suggested that DHEA might stimulate the oestrogen response element. Very recently, it was proposed that DHEA might activate the pregnane X receptor (Singleton et al. 1999, Ripp et al. 2002). A recent clinical study on the treatment of X-ALD patients with DHEA showed a lowering of long chain FAs but not of C24 FAs (J Assies, E B Haverkort, R Lieverse & P Vreken, unpublished observations). The clinical use of DHEA has not been tested in Refsum disease.

In contrast to DHEA, VD showed few effects on genes involved in peroxisome expression. None was upregulated. This was unexpected since Davis et al. (1989) reported an increase in peroxisomal palmitate oxidation in rat liver after VD administration (a single dose of 500 IU). There is, however, no actual contradiction with our mRNA assay; enzyme capacity can be modified by other mechanisms such as mRNA stability or enzyme turnover (Horie & Suga 1989). In our study, pristanoyl-CoA oxidase, DHAPAT and DAAOX were downregulated. A decrease in DAAOX activity following administration of peroxisome proliferators has previously been described (Watanabe et al. 1989, Reubsaet et al. 1991, Stefaniini et al. 1999).

Downregulation by VD of VD 3–25-hydroxylase is in accordance with the findings of Theodoropoulos et al. (2001) in the duodenum of VD-depleted rats. The downregulation of this enzyme which, in the liver, catalyses the first hydroxylation step, necessary for the activation of calciferol, might represent a negative feedback mechanism.

With respect to VDR, no significant changes in expression level were found. Four different primer pairs were designed and tested for expression analysis (Fig. 1). All resulted in the generation of multiple PCR products (instead of one specific VDR amplicon) as evidenced by melting curve analysis and gel electrophoresis. This can be attributed to alternative splicing, or hitherto unknown genes with very high sequence homology to VDR. As a consequence, the level of VDR expression, which is unchanged, is not considered reliable. All rats were included for analysis of the data, although one animal showed elevated 25-hydroxyvitamin D but low 1,25-dihydroxyvitamin D. Its gene expression levels were in the same range as others in its group.

The significantly altered expression level in 6-day versus short-term propylene glycol controls suggests an
effect of the vehicle on gene expression level. We are aware that this may have caused the masking of small VD effects. The difference in expression level is difficult to interpret and might be due to either stimulation at a higher single dose of propylene glycol, or downregulation by a repeated exposure to a lower dose, or vice versa; the total dose administered in both cases being nearly the same. With a single dose of propylene glycol no effect was found on peroxisomal β-oxidation activity, or on malate synthase or isocitrate lyase activities in chick liver homogenates as compared with the control not receiving propylene glycol (Davis et al. 1990). On cytosolic and microsomal enzyme activities also, some effects of propylene glycol were reported by Yamamoto & Adachi (1978).

Our data indicate that propylene glycol should not be used as a vehicle when peroxisomal gene expression is studied. VD should not be given to patients with peroxisomal disorders.

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