Refsum disease diagnostic marker phytanic acid alters the physical state of membrane proteins of liver mitochondria

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Abstract Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid), a branched chain fatty acid accumulating in Refsum disease to high levels throughout the body, induces uncoupling of rat liver mitochondria similar to non-branched fatty acids (e.g. palmitic acid), but the contribution of the ADP/ATP carrier or the aspartate/glutamate carrier in phytanic acid-induced uncoupling is of minor importance. Possible deleterious effects of phytanic acid on membrane-linked energy coupling processes were studied by ESR spectroscopy using rat liver mitochondria and a membrane preparation labeled with the lipid-specific spin probe 5-doxylstearic acid (5-DSA) or the protein-specific spin probe MAL-TEMPO (4-maleimido-2,2,6,6-tetramethyl-piperidine-1-oxyl). The effects of phytanic acid on phospholipid molecular dynamics and on the physical state of membrane proteins were quantified by estimation of the order parameter or the ratio of the amplitudes of the weakly to strongly immobilized MAL-TEMPO binding sites (*W/S* ratio), respectively. It was found, that phytanic acid (1) increased the mobility of phospholipid molecules (indicated by a decrease in the order parameter) and (2) altered the conformational state and/or the segmental mobility of membrane proteins (indicated by a drastic decrease in the *W/S* ratio). Unsaturated fatty acids with multiple cis-double bonds (e.g. linolenic or arachidonic acid), but not non-branched FFA (ranging from chain length C10:0 to C18:0), also decrease the *W/S* ratio. It is hypothesized that the interaction of phytanic acid with transmembrane proteins might stimulate the proton permeability through the mitochondrial inner membrane according to a mechanism, different to a protein-supported fatty acid cycling.

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Key words: Phytanic acid; ESR spectroscopy; Liver mitochondria

1. Introduction

Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is a characteristic diagnostic marker of Refsum disease, which accumulates to highly elevated levels in blood and tissue due to a defective α-oxidation step in phytanoyl-CoA degradation (for review see [1,2]). Being an amphipathic compound, phytanic acid is assumed to accumulate in membranes. Protonophoric activity of phytanic acid was observed in studies with phospholipid bilayer membranes [3,4] and mitochondria [5,6] and, therefore, phytanic acid is substantial to depolarize energized membranes which probably caused a depletion of the cellular ATP level. Skulachev was the first to postulate that the protonophoric activity of long-chain FFA is the result of a spontaneous transbilayer passage of the undissociated FFA from the external side of the inner membrane to the matrix side and that the ADP/ATP carrier partly mediates the reverse transport of the fatty acid anion (fatty acid cycling model) [7]. It has been discussed that other mitochondrial anionic carrier systems as the aspartate/glutamate carrier and, to a lesser extent the dicarboxylate carrier contribute to the protonophoric effect of FFA as well [8–10].

In studies with liver mitochondria, we found that phytanic acid undergoes in its protonated form a fast flip-flop movement within the membrane and, in addition, interacts strongly with the ADP/ATP carrier. However, its protonophoric activity was only weekly sensitive to carboxyatractyloside [5,6,11]. Moreover, there are indications that incorporation of phytanic acid into phospholipid model membranes alters membrane properties quite different compared to the straight chain analogue palmitic acid. Thus, phytanic acid decreased the phase transition temperature, whereas palmitic acid elevated it [12]. In addition, from NMR spectroscopic studies it was reported that phytanic acid induced a conformational change of the head groups in phosphatidylcholine bilayers [13]. All these findings point to a further membrane-perturbating action of phytanic acid. To get more insight into the interaction of phytanic acid with membrane constituents, we studied its action on phospholipid and membrane protein molecular dynamics in membranes of rat liver mitochondria by ESR spectroscopy. Membranes were labeled with 5-doxylstearic acid as lipid-specific spin probe or 4-maleimido-2,2,6,6-tetramethylpiperidine-1-oxyl as protein-specific spin probe.

We found that phytanic acid increased the mobility of phospholipid molecules similar to palmitic acid. However, contrary to unbranched FFA, phytanic acid changes the conformational and/or segmental mobility of membrane proteins. A possible consequence of this finding in respect to the phytanic acid uncoupling of mitochondria is discussed.

2. Materials and methods

2.1. Mitochondria and membranes

Rat liver mitochondria with respiratory control ratios routinely greater than 5 were prepared from adult female Wistar rats (mean weight 150–180 g) according to our standard protocol [5]. The mitochondrial pellet was resuspended in 250 mM sucrose solution. A membrane preparation from the mitochondrial suspension was obtained essentially as described in [14]. Briefly, mitochondrial pellets were suspended in 30 ml ice-cold lysing buffer containing 10 mM HEPES, 2 mM EGTA, 2 mM EDTA, at pH 7.4 and the resulting suspension was then spun down at $30\,000 \times g$ for 10 min. This procedure was repeated two more times to remove any residual protein.

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Abbreviations: FFA, non-esterified fatty acids; Pal, palmitic acid; Phyt, phytanic acid; 5-DSA, 5-doxylstearic acid; MAL-TEMPO, 4-maleimido-2,2,6,6-tetramethyl-piperidine-1-oxyl; $T'_{\parallel}$, parallel hyperfine splitting parameter; $T'_{\perp}$, perpendicular hyperfine splitting parameter; CAT, carboxyatractyloside; GLU, glutamate

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The resulting pellet was resuspended in lysing buffer. Protein was assayed by a modified Biuret method.

2.2. Spin labeling and ESR measurements

2.2.1. 5-DSA spin labeling. Mitochondria (2 mg protein/ml) suspended in the 'respiration medium' (see below) were supplemented with the lipid-specific anisotropic spin probe 5-doxylstearic acid (as ethanolic solution) at the probe to mitochondrial phospolipid molecular ratio of about 100. The concentration of phytanic or palmitic acid was 70 nmol FFA/mg protein. The ESR spectra were recorded at 9.77 GHz on a Bruker ECS 106 ESR spectrometer. Instrumental operating parameters were: incident microwave power = 16 mW, modulation amplitude = 1 G, time constant = 81.92 ms, conversion time = 163.84 ms, field sweep = 100 G. ESR measurements were performed at 22°C with a mitochondrial equivalent of 2 mg protein/ml. The order parameter was calculated from the T-tensor elements $T'_{\parallel}$ and $T'_{\perp}$, according to Gafney [15]. A decrease in the order parameter indicates an increase in membrane fluidity in phospholipid regions.

To examine interactions of phytanic acid with membrane proteins, mitochondrial membranes were covalently labeled with the thiol group-specific 4-maleimido-2,2,6,6-tetramethylpiperidine-1-oxyl (MAL-TEMPO).

2.2.2. MAL-TEMPO spin labeling. To an aliquot (5–7 mg protein) of the membrane preparation MAL-TEMPO was added to a final concentration of 20 μg/mg protein in the absence or presence of fatty acids (50 nmol/mg protein). The resulting mixture was shaken by hand and incubated at 4°C for 18–24 h. The samples were then centrifuged and washed repeatedly with the buffer to remove not covalently bound spin label. Finally, the samples were resuspended in 1 ml lysis buffer. Labeling of intact mitochondria suspended in 250 mM sucrose solution was done similarly. Instrumental operating parameters were: incident microwave power = 15.9 mW, modulation amplitude = 1 G, time constant = 1.28 ms, conversion time = 10.24 ms, field sweep = 100 G.

2.3. Other methods

Oxygen consumption of mitochondria was measured polarographically using a Clark-type electrode in a thermostated chamber maintained at 25°C. The 'respiration medium' was composed of 110 mM mannitol, 60 mM KCl, 60 mM Tris-HCl, 10 mM K_2HPO_4, 0.5 mM Na_2EDTA, 5 mM succinate and 2 μM rotenone as respiratory substrate (pH 7.4). Mitochondria were partly uncoupled by addition of 25 nmol FFA/mg protein. The involvement of the ADP/ATP carrier or the aspartate/glutamate carrier in the protonophoric effect was estimated from the depression of FFA-stimulated respiration after addition of carboxyatractyloside or glutamate, respectively.

2.4. Chemicals

Fatty acids, glutamate, succinate, carboxyatractyloside and 5-DSA were from Sigma. MAL-TEMPO was from Aldrich. Fatty acids were dissolved in ethanol up to 50 mM.

3. Results

3.1. Susceptibility to recoupling

Palmitic and phytanic acid added in equal concentrations to incubations of rat liver mitochondria (supplemented with succinate plus rotenone as respiratory substrate) exert a similar protonophoric effect. Respiration in State 4 was stimulated to 40 ± 4 and 35.5 ± 4 nmol O_2/min/mg protein with 25 nmol of either FFA per mg protein ($n = 5$), respectively. The recoupling of phytanic acid-stimulated respiration by carboxyatractyloside, glutamate or the combination of both was smaller than that seen when the same effectors were added to mitochondria uncoupled by palmitic acid (Fig. 1). This indicates that the ADP/ATP carrier and the aspartate/glutamate carrier are of minor importance in mediating the protonophoric effect of phytanic acid. Thus, some other unidentified proteins could be involved in the phytanic acid cycling and/or that phytanic acid exerts a membrane-perturbating effect allowing an increased proton flux across the inner membrane. We consid-

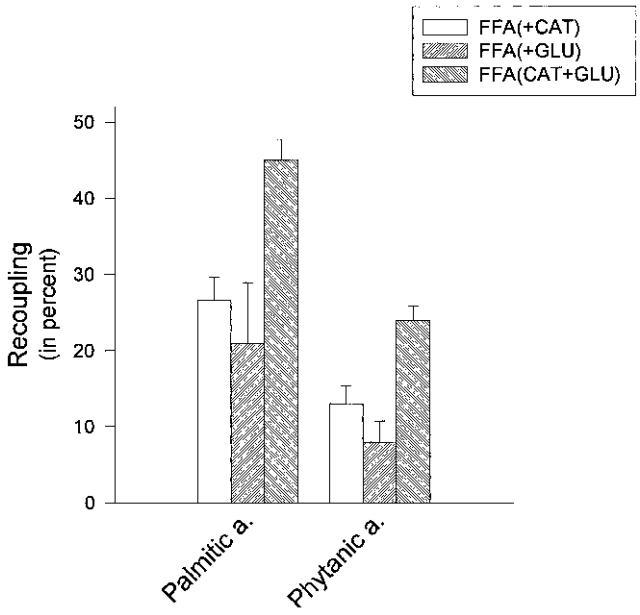

Fig. 1. Recoupling of palmitate- and phytanate-stimulated State 4 respiration by carboxyatractyloside, glutamate or a combination of both. Mitochondria (2 mg/ml) were incubated in 2 ml medium as described in Section 2. State 4 respiration (15 ± 2 nmol O_2/min/mg protein) was stimulated by addition of 25 nmol palmitic or phytanic acid per mg protein to 40 ± 4 nmol O_2/min/mg protein (palmitate) and 35.5 ± 4 nmol O_2/min/mg protein (phytanate). Respiration was decreased by addition of carboxyatractyloside (5 μM) and/or glutamate (5 mM). The data represent mean values ± S.D. of five separate preparations. The recoupling was calculated from the effector-induced decrease in FFA-stimulated respiration and the net stimulation of State 4 respiration by FFA.

ered the possibility that the incorporation of the branched chain phytanic acid into the phospholipid bilayer regions weakens the interaction between phospholipid molecules and, therefore, increased their mobility.

3.2. Effect on phospholipid mobility

The effect of phytanic acid and palmitic acid on the order parameter of membrane phospholipids was examined with 5-DSA as a spin probe. Due to the rigid connection between the doxylradical moiety and the hydrocarbon tail, intercalated 5-DSA senses the motional freedom of phospholipid hydrocarbon tails. Fig. 2 shows typical ESR spectra of 5-DSA incorporated in mitochondrial membranes in the absence and in the presence of phytanic acid. The spectra reflect the superposition of the ESR signals of free (indicated by arrows) and immobilized 5-DSA. Addition of phytanic acid added to mitochondrial membranes (approximately 20 mol % of total membrane lipids) supports further incorporation of free 5-DSA into the mitochondrial membrane. In comparison to the control, a significant decrease in the order parameter was found in palmitic or phytanic acid-treated mitochondria (Fig. 3). This observation indicates that both fatty acids increase similarly the phospholipid mobility in the microenvironment of 5-DSA.

3.3. Effect on the physical state of membrane proteins

The well-known affinity of FFA to proteins suggests that membrane-incorporated FFA might change the physical state of membrane proteins, namely their conformational or seg-

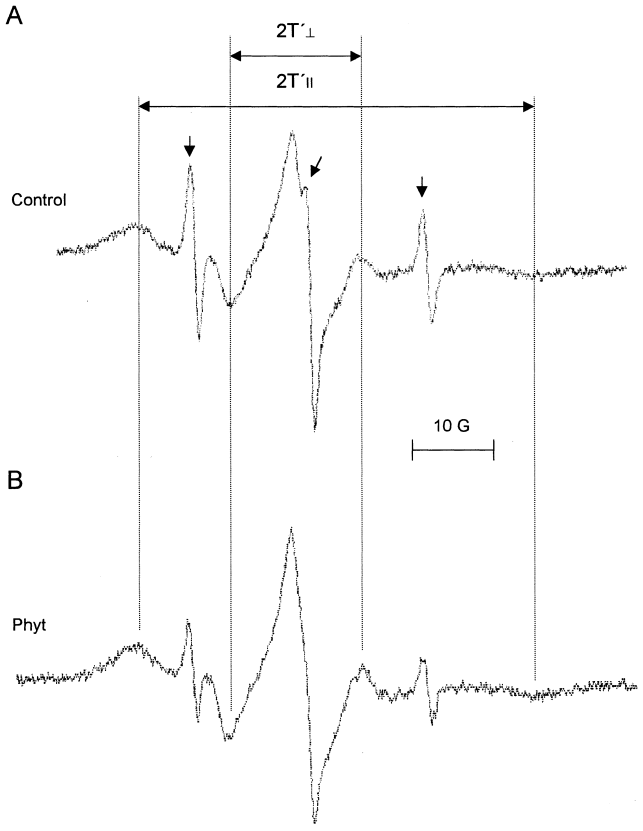

Fig. 2. ESR spectra of 5-doxyl stearic acid immobilized to rat liver mitochondria. A: Spectrum in the absence of phytanic acid. The arrows indicate the resonance lines arising from the spin label free in solution. The parallel ($T'_{\parallel}$) and perpendicular ($T'_{\perp}$) tensor components of the hyperfine splittings were measured. The order parameter was calculated from these values. B: Spectrum in the presence of phytanic acid (50 nmol/mg protein). The spectra represent the mean of 10 scans.

mental protein mobility. To investigate such an effect of phytanic acid, mitochondrial membranes were labeled with the protein-specific spin probe MAL-TEMPO. MAL-TEMPO is immobilized at two types of sulfhydryl group binding sites of

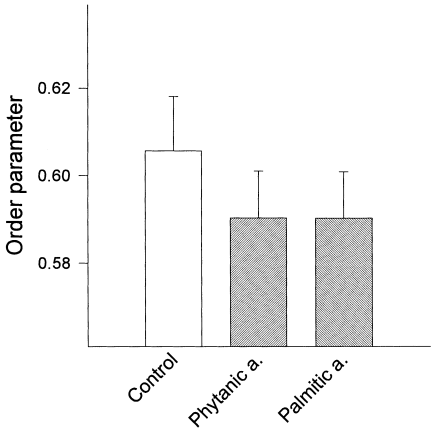

Fig. 3. Order parameter changes in mitochondrial membranes following addition of palmitic or phytanic acid. The data represent mean values ± S.D. obtained from six mitochondrial preparations. The difference between the order parameter of palmitate- or phytanate-treated mitochondria and untreated was found to be significant ($P < 0.001$; paired Student's t-test).

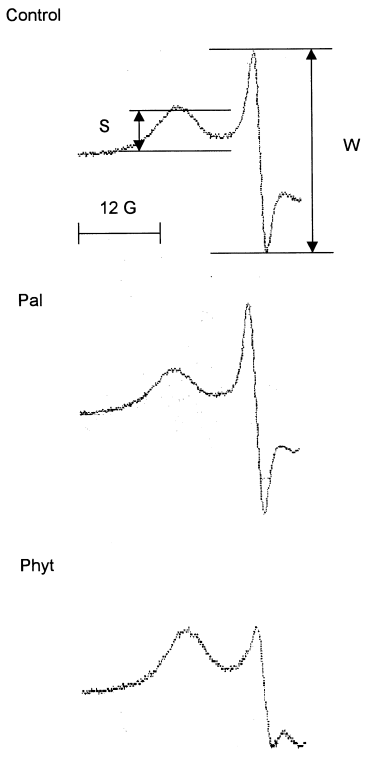

Fig. 4. Typical low-field EPR spectra of MAL-TEMPO-labeled rat liver mitochondria: untreated mitochondria (control); mitochondria treated with palmitic acid (50 nmol/mg protein); mitochondria treated with phytanic acid (50 nmol/mg protein). The spectra represent the mean of 20 scans.

membrane proteins which differ by their mobility (Fig. 4). Attachment of MAL-TEMPO to more rigid sulfhydryl groups (e.g. those at protein pockets) resulted in a broad resonance signal (S) at the low-field site of the low-field ESR spectrum (strong immobilization). Covalent binding of MAL-TEMPO to sulfhydryl groups closer to the protein-water interface resulted in a sharp resonance signal (W) at the high-field site of the low-field ESR spectrum (weak immobilization). The ESR spectra of labeled proteins of phytanic acid-treated mitochondria showed that the ratio of weakly immobilized to strongly immobilized SH groups (W/S ratio) was decreased with respect to untreated mitochondria (control), but not when mitochondria were treated with palmitic acid (Fig. 4). The W/S ratio calculated from the signal amplitudes of weakly bound and strongly bound MAL-TEMPO is considered to be a sensitive indicator of the segmental motion of spin label sites on proteins. Fig. 5 collects the W/S ratios obtained from different membrane preparations. In contrast to the drastic reduction of the W/S ratio by phytanic acid ($W/S = 1.6 \pm 0.2$), palmitic acid increased the W/S ratio slightly (from $W/S = 3.70 \pm 0.7$ to 4.05 ± 1.0) with respect to the untreated membranes. Moreover, when MAL-TEMPO was applied to intact mitochondria the W/S ratios were similarly changed by fatty acids.

Moreover, the effect of a variety of FFA on W/S ratio was studied. Straight chain FFA, such as palmitic acid and other saturated fatty acids with chain length ranging from 10 to 18 carbon atoms did not significantly affect the W/S ratio compared with the FFA-free control (results not shown). However, a decrease in the W/S ratio was also seen when mitochondria were pre-treated with unsaturated FFA, such as oleic, linoleic, linolenic or arachidonic acid (results not

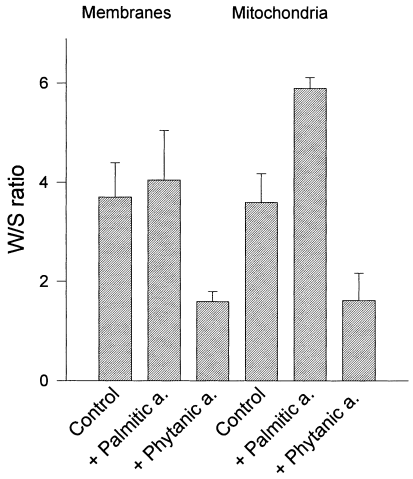

Fig. 5. Effect of palmitate or phytanate on *W/S* ratio. Mitochondria or inner membranes were treated with 50 nmol/mg protein of palmitate or phytanate prior to labeling with MAL-TEMPO. The data represent mean values ± S.D. obtained from six (membrane fraction) and four (mitochondria) preparations.

shown). Their decreasing effect on the *W/S* ratio increases with the increasing number of double bonds.

4. Discussion

Refsum disease exhibits serious complications, such as blindness, neurodegeneration and cardiac arrhythmia which might be partly attributed to an impaired cellular energy metabolism due to an enrichment of phytanic acid in the mitochondrial inner membrane. Transmembranal proteins, such as the ADP/ATP carrier, the aspartate/glutamate carrier, the dicarboxylate carrier and the uncoupling protein, are involved in the protonophoric activity of FFA in a tissue-dependent manner (for review, see [16,17]). The protonophoric activity of phytanic acid is comparable to that of the unbranched analogue palmitic acid, but its sensitivity to effectors of the ADP/ATP carrier or the aspartate/glutamate carrier is much weaker in liver mitochondria. Both carrier proteins account for only about 25% to the protonophoric effect of phytanic acid (the situation is different in heart and skeletal muscle mitochondria where the ADP/ATP carrier contributes to about 50% to the protonophoric effect of phytanic acid; results not shown). Therefore, it can be supposed that some other mechanisms might contribute to the protonophoric effect of the phytanic acid enriched in mitochondrial membranes. The present study indicates that phytanic acid has two membrane-perturbating effects in liver mitochondria. Firstly, incorporation of phytanic acid increased the mobility of phospholipid molecules which is, however, similar to that produced by palmitic acid. Secondly, contrary to unbranched FFA (ranging from chain length C10:0 to C18:0), phytanic acid modifies the structural organization of the mitochondrial inner membrane by changing the membrane protein conformation.

A more likely explanation of phospholipid bilayer proton permeability is the existence of hydrogen-bonded water wires inside the bilayer along which protons migrate [18,19]. Incorporation of the branched chain phytanic acid in phospholipid regions of the mitochondrial inner membrane decreased the phospholipid packing which might contribute to the formation of water wires [20]. However, comparison of the proton permeability of liposomes (prepared from mitochondrial inner membrane phospholipids) with that of mitochondria demonstrates that the proton permeability of the bulk phospholipid bilayer regions accounts for only a small proportion of mitochondrial proton permeability [21]. Furthermore, no correlation between liposome proton permeability and phospholipid fatty acid composition was found [22]. Therefore, it is tempting to speculate that the interaction of phytanic acid with membrane proteins different to the above mentioned anion carriers, contributes substantially to the protonophoric activity of phytanic acid in liver mitochondria. These proteins might mediate the protonophoric effect by an additional mechanism, different to a protein-supported fatty acid cycling. One possibility could be that phytanic acid stimulates an already existing proton leak pathway across the mitochondrial inner membrane via an alteration of the conformational state of transmembranal proteins. Thus, it has been reported that α-helical hydrophobic polypeptides form proton selective channels when incorporated into lipid bilayers [23].

Surprisingly, a phytanic acid-like effect on the *W/S* ratio was exerted with arachidonic acid and other FFA with multiple cis-double bonds. Conformational computer calculations suggest that arachidonic acid is most stable in a 'benched' conformation when it is bound at the protein interface, whereas unbranched saturated FFA prefer an all-trans conformation of the hydrocarbon tail [24]. Speculating that the phytanic acid exists in a 'benched' conformation at the protein interface [25], the similar effect of phytanic acid and unsaturated FFA on *W/S* ratio suggests that the marked decrease of the *W/S* ratio is due to the interaction of 'benched' hydrocarbon tail with the interface of membrane proteins.

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