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Research Article

Phytanic acid impairs mitochondrial respiration through protonophoric action

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Abstract. Refsum disease is a rare, inherited neurodegenerative disorder characterized by accumulation of the dietary branched-chain fatty acid phytanic acid in plasma and tissues caused by a defect in the alpha-oxidation pathway. The accumulation of phytanic acid is believed to be the main pathophysiological cause of the disease. However, the exact mechanism(s) by which phytanic acid exerts its toxicity have not been resolved. In this study, the effect of phytanic acid on mitochondrial respiration was investigated. The results show that in digitonin-permeabilized fibroblasts, phytanic acid decreases ATP synthesis, whereas substrate oxidation *per se* is not affected. Importantly, studies in intact fibroblasts revealed that phytanic acid decreases both the mitochondrial membrane potential and NAD(P)H autofluorescence. Taken together, the results described here show that unesterified phytanic acid exerts its toxic effect mainly through its protonophoric action, at least in human skin fibroblasts.

Keywords. Phytanic acid, Refsum disease, mitochondria, membrane potential, oxidative phosphorylation.

Patients with the rare, inherited neurological disorder Refsum disease (heredopathia atactica polyneuritiformis, MIM #266500) present with markedly increased levels of phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) in their plasma and tissues. The precursor of this branched-chain fatty acid, the chlorophyll side chain phytol (3,7,11,15-tetramethylhexadec-trans-2-en-l-ol), is produced by bacteria in the stomach of ruminants [1–3]. Hence, the main sources of phytanic acid for humans are dairy products and meats of these animals, although some seafood has also been found to contain large amounts of phytol and phytanic acid [4, 5].

In healthy individuals, phytanic acid undergoes oxidative decarboxylation by a process called alpha-oxidation, resulting in the formation of formyl-CoA and pristanic acid (2,6,10,14-tetramethylpentadecanoic acid). Pristanic acid is subsequently degraded by peroxisomal beta-oxidation [reviewed in ref. 6]. In Refsum disease, the alpha-oxidation pathway is deficient due to mutations in either the *PHYH* gene, which encodes phytanoyl-CoA hydroxylase (PHYH), the first enzyme of the alpha-oxidation pathway [7, 8],

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or the *PEX7* gene, which encodes peroxin 7, a protein required for correctly targeting PHYH to peroxisomes [9]. Consequently, patients with Refsum disease accumulate significant amounts of phytanic acid in plasma and tissues, which are thought to be the major cause of the pathology of the disease. Multiple clinical symptoms are observed, the most pronounced being retinitis pigmentosa, which leads to progressive visual loss and ultimately to blindness. Additional symptoms include anosmia, peripheral neuropathy, ataxia, cardiac arrhythmias and ichthyosis [10, 11]. Although a diet low in phytanic acid slows down the progression of the disease, little is known about the mechanism(s) linking the clinical manifestations to the increased accumulation of phytanic acid [reviewed in ref. 12].

Recent studies employing rat hippocampal astrocytes revealed that phytanic acid can cause massive cell death within a few hours of exposure [13–15]. Furthermore, these studies showed that cell death was preceded by an increased release of Ca^{2+} from internal stores, leading to a rise in the cytosolic free Ca^{2+} concentration, increased generation of reactive oxygen species (ROS) and a marked depolarization of the mitochondrial membrane potential. Currently, research on the cytotoxic mechanism of phytanic acid is focused predominantly on mitochondria, which are among the most vulnerable organelles in the cell. Mitochondrial dysfunction impairs cellular and tissue integrity and has been implicated in a wide range of disorders including age-related neurological diseases, cardiovascular disease, cancer and diabetes [reviewed in refs. 16–18]. Mitochondria are central not only to cellular energy and Ca^{2+} homeostasis but also to cell death, and work of the past years has shown that fatty acids can affect each of these functions [reviewed in ref. 19]. As far as phytanic acid is concerned, studies in intact rat brain mitochondria revealed that it can decrease the rate of phosphorylating respiration, decrease the oxidative production of ATP and decrease the activity of adenine nucleotide translocase (ANT) [14, 15, 20, 21], suggesting that phytanic acid primarily acts through inhibition of ADP/ATP exchange. In addition, these studies showed that phytanic acid can increase the rate of non-phosphorylating respiration, indicating an additional effect at the level of the oxidative phosphorylation (OXPHOS) system. Recent work in permeabilized mitochondria revealed that phytanic acid can decrease the activity of NADH:ubiquinone oxidoreductase (complex I), suggesting that phytanic acid can also act by directly inhibiting the fuelling of the OXPHOS system [21]. Finally, studies in intact mitochondria showed that phytanic acid can decrease the NAD(P)H level under non-respiring conditions but that, unexpectedly, this effect of phytanic acid was reversed by the complex I inhibitor rotenone, suggesting that phytanic acid can also act as a protonophore [21]. The latter conclusion, however, contrasts with the finding that phytanic acid can decrease carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP)-stimulated respiration [21; but see also ref. 20].

To gain insight into the mechanism underlying the inhibitory effect of phytanic acid on phosphorylating respiration, we assayed its effect on the production of ATP in digitonin-permeabilized human skin fibroblasts respiring on substrates specific for either complex I or complex II (succinate:ubiquinone oxidoreductase). Importantly, parallel measurement of the consumption of these substrates allowed us to assess the fuelling state of the OXPHOS system. For comparison, we included the complex-specific inhibitors rotenone (complex I) and malonate (complex II), which are expected to decrease ATP production and substrate consumption with the same potency, and the protonophore 2,4-dinitrophenol (DNP), expected to decrease the production of ATP much more potently than the consumption of the substrate. In addition, we performed life cell analysis to study the effects of phytanic acid on mitochondrial shape and membrane potential and intracellular NAD(P)H levels using digital imaging microscopy. Finally, the specificity of the effect of phytanic acid was evaluated using phytol, phytanic acid methyl ester, pristanic acid and palmitic acid. Collectively, the results indicate that phytanic acid decreases mitochondrial respiration primarily through its protonophoric action and that both the carboxyl group and the methyl groups are required for this activity.

Materials and methods

Chemicals. Phytanic acid and pristanic acid were purchased from the VU University Medical Center Metabolic Laboratory (Dr. H. J. ten Brink, Amsterdam, The Netherlands). Phytol and palmitic acid were from Merck (Darmstadt, Germany) and phytanic acid methyl ester was from Ultra Scientific (North Kingstown, R.I.). DMSO was obtained from Sigma-Aldrich (St. Louis, Mo.). Cell culture material, media and tetramethyl rhodamine methyl ester (TMRM) for life cell imaging were from Invitrogen (Breda, The Netherlands). All other chemicals and enzymes were of the highest quality available.

Cell culture for enzymatic assays. Human skin fibroblasts from control individuals were cultured at 37 °C (5 % CO_2) in nutrient mixture Ham's F-10 with L-glutamine and 25 mM HEPES supplemented with

10% (v/v) fetal calf serum, 100 IU/ml penicillin, 100 IU/ml streptomycin and 250 ng/ml amphotericin B.

Cell culture for life cell imaging. Fibroblasts were obtained from a healthy individual (#5120) according to the relevant Institutional Review Boards and cultured in medium 199 with Earle's salt supplemented with 10% (v/v) fetal calf serum, 100 IU/ml penicillin and 100 IU/ml streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. For microscopy analysis, cells were seeded on glass coverslips (24 mm diameter) and cultured to ~70% confluence.

ATP synthesis assay in digitonin-permeabilized fibroblasts. The ATP synthesis assay was performed essentially as described by Wanders et al. [22] with slight modifications in the protocol. In brief, human skin fibroblasts were grown to confluency followed by detachment from the culture flask by trypsinization as described before [23]. The protein content of the fibroblast suspension was estimated by light scattering ($OD_{600} = 0.14$ corresponds to ~0.05 mg/ml protein). Subsequently, the suspension was centrifuged ($500 \times g_{av}$) and cells were taken up in fresh medium. From this suspension, aliquots (30 μg of protein) were taken and seeded into the wells of a 24-well plate. The next day, cells were washed twice with PBS followed by addition of the reaction medium [containing 150 mM KCl, 25 mM Tris-HCl, 2 mM EDTA, 10 mM potassium phosphate, 1 mM ADP, 0.1% (w/v) bovine serum albumin, 40 μg/ml digitonin and either 10 mM malate (plus 10 mM glutamate), or 10 mM succinate (plus 50 μM rotenone)]. Phytanic acid, 2,4-dinitrophenol, rotenone, malonate and the phytanic acid analogues were all added to the reaction mixture from DMSO stock solutions. Reactions were allowed to proceed for 30 min at room temperature. When samples were prepared for aspartate measurements, the cells were harvested from culture flasks and incubations were performed with the cells in solution (0.5 mg/ml in 100 μl). Reactions were terminated with perchloric acid, protein was removed by centrifugation and the acidic protein-free supernatants were neutralized [22]. Subsequently, ATP was measured fluorimetrically as described previously [24] using a Cobas Fara centrifugal analyzer. Afterwards, the same samples were used for aspartate or malate measurements.

Aspartate measurements. Aspartate was measured using the end-point method described in Bergmeyer et al. [25]. This method uses the subsequent conversion of aspartate to malate via oxaloacetate in a reaction mixture containing glutamate-oxaloacetate-transaminase, malate dehydrogenase, NADH and α-ketoglutarate. The decrease in NADH as a consequence of the reduction of oxaloacetate to malate by malate dehydrogenase was measured fluorimetrically with a Cobas Fara centrifugal analyser. Quantification was done by means of a standard curve.

Malate measurements. Malate was determined fluorimetrically (excitation at 365 nm, emission at 450 nm) on a Cobas Fara centrifugal analyser by the end-point measurement of the reduction of the NAD^+ analogue 3-acetylpyridine adenine dinucleotide (APAD) to APADH by malate dehydrogenase. The reaction was performed in a 100 mM Tris/HCl buffer at pH 9 containing 0.1% Triton X-100 and 1 mM APAD. Malate was quantified by means of a standard curve.

Quantification of mitochondrial shape and membrane potential. Fibroblasts were cultured to ~70% confluence on glass coverslips, and were incubated with 100 nM TMRM for 30 min at 37 °C. After loading, the cells were washed and incubated for another 30 min at 37 °C in medium containing either the vehicle (DMSO) or the compound of interest. After incubation, the medium was replaced by a HEPES-Tris-buffered medium (132 mM NaCl, 4.2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5.5 mM D-glucose, 10 mM HEPES, pH 7.4) containing either DMSO or the compound of interest. Next, the coverslips were mounted in a temperature-controlled (37 °C) incubation chamber attached to the stage of an inverted microscope (Axiovert 200 M; Carl Zeiss, Jena, Germany) equipped with a ×63, 1.25 NA Plan NeoFluar objective. TMRM was excited at 540 nm using a monochromator (Polychrome IV; TILL Photonics, Gräfelfing, Germany) and fluorescence emission light was directed by a 560DRLP dichroic mirror (Omega Optical, Brattleboro, Vt.) through a 565ALP emission filter (Omega Optical) onto a CoolSNAP HQ monochrome CCD-camera (Roper Scientific Photometrics, Vianen, The Netherlands). Routinely, 25 fields of view were analysed per coverslip using an image capturing time of 100 ms. Mitochondrial shape and membrane potential were quantified using an automated image-processing algorithm described in detail previously [26–28]. Briefly, after correction for background fluorescence, images were subjected to linear contrast stretching followed by top-hat filtering, median filtering and thresholding. This procedure yields binary images showing white mitochondria against a black background. Next, these binary images were used for quantification of mitochondrial area, number and formfactor. The latter is a combined measure of mitochondrial length and degree of branching. In addition, these images were used as mitochondrial masks, which, when superimposed onto the original

background-corrected images, allow determination of the fluorescence intensity for each mitochondrial pixel. Finally, the mean fluorescence intensity per mitochondrial pixel was calculated as an estimate of the average mitochondrial membrane potential. Image processing and analysis were performed using Image Pro Plus 5.1 (Media Cybernetics, Silver Spring, Md.). In each experiment, the average value obtained with DMSO-treated cells was set at 100 %, to which all other values were related. Numerical results were visualized using Origin Pro 7.5 (Originlabs, Northampton, Mass.) and values from multiple experiments were expressed as means ± SE. Statistical significance (Bonferroni corrected) was assessed using Student's t-test.

Quantification of intracellular NAD(P)H autofluorescence. Intracellular NAD(P)H autofluorescence was measured as described previously [29]. Briefly, fibroblasts, cultured to ~70 % confluence on glass coverslips, were preincubated for 30 min at 37 °C in medium containing either DMSO or the compound of interest. Immediately before imaging, the medium was replaced by HEPES-Tris medium containing either the vehicle or the compound of interest. Coverslips were transferred to the incubation chamber on the stage of the inverted microscope equipped with a Zeiss 40×/1.3 NA Plan NeoFluar objective. The cells were excited at 360 nm using the monochromator, and fluorescence emission light was directed by a 415DCLP dichroic mirror (Omega Optical) through a 510WB40 emission filter (Omega Optical) onto the CCD camera. Routinely, ten fields of view were analysed per coverslip using an image-capturing time of 1 s. The mean fluorescence intensity was determined in an intracellular region of interest and, for the purpose of background correction, in an extracellular region of identical size. Quantitative image analysis was performed with Metamorph 6.0. In each experiment, the average value obtained with vehicle-treated cells was set at 100 %, to which all other values were related. Numerical results were visualized using Origin Pro 7.5 and values from multiple experiments were expressed as means ± SE. Statistical significance (Bonferroni corrected) was assessed using Student's t-test.

Results

Effect of phytanic acid on ATP synthesis via complex I. To examine the effect of phytanic acid on mitochondrial ATP synthesis, we used digitonin-permeabilized fibroblasts [22] rather than isolated mitochondria. Malate (plus glutamate) or succinate (plus rotenone) were used as respiratory chain substrates. Incubations were performed in the absence and presence of either phytanic acid or established mitochondrial inhibitors and uncouplers including rotenone, a well-known inhibitor of complex I [reviewed in ref. 30], malonate, an inhibitor of complex II, and the protonophore DNP.

Figure 1 depicts the effects on ATP synthesis when malate was used as a substrate for the respiratory chain in combination with glutamate. Malate is converted by malate dehydrogenase in the mitochondrial matrix to oxaloacetate with the simultaneous reduction of the cofactor NAD^+ to NADH. Consequently, NADH is readily available for use as a substrate for complex I of the OXPHOS system, while oxaloacetate and the added glutamate are converted by glutamate-oxaloacetate transaminase to α-ketoglutarate and aspartate, thereby eliminating the product inhibition of malate dehydrogenase by oxaloacetate. Phytanic acid gradually decreased ATP synthesis when its concentration was increased up to 100 μM (Fig. 1a). A similar result was obtained with DNP when using concentrations up to 50 μM (Fig. 1b). Rotenone appeared much more potent in decreasing ATP synthesis than either DNP or phytanic acid (Fig. 1c). The complex I inhibitor exerted its inhibitory effect in the nanomolar range, while phytanic acid and the uncoupler were effective in the micromolar range.

Next, we determined the effect of phytanic acid, DNP and rotenone on the formation of aspartate, which is the end-product of malate oxidation, at least when malate oxidation is studied in the presence of glutamate. Aspartate was measured in the same samples used for ATP measurement. Phytanic acid (Fig. 1a) and DNP (Fig. 1b) inhibited aspartate synthesis considerably less potently than ATP synthesis, while, in sharp contrast, rotenone decreased both processes equipotently (Fig. 1c). The inhibition pattern of rotenone is in agreement with a reduction in complex I-mediated NADH oxidation, decreasing both the enzymatic conversion of malate to oxaloacetate and the proton motive force-driven synthesis of ATP. The inhibition pattern of phytanic acid is similar to that of DNP but clearly different from that of rotenone, thus disfavoring the idea that it acts by inhibiting complex I and suggesting that it rather acts as a protonophore.

Effect of phytanic acid on ATP synthesis via complex II. To demonstrate that the inhibitory effect of phytanic acid was not restricted to complex I-mediated ATP synthesis, we next determined its effect on ATP synthesis when the complex II substrate succinate was used as electron donor for the respiratory chain. Rotenone was included in the reaction medium

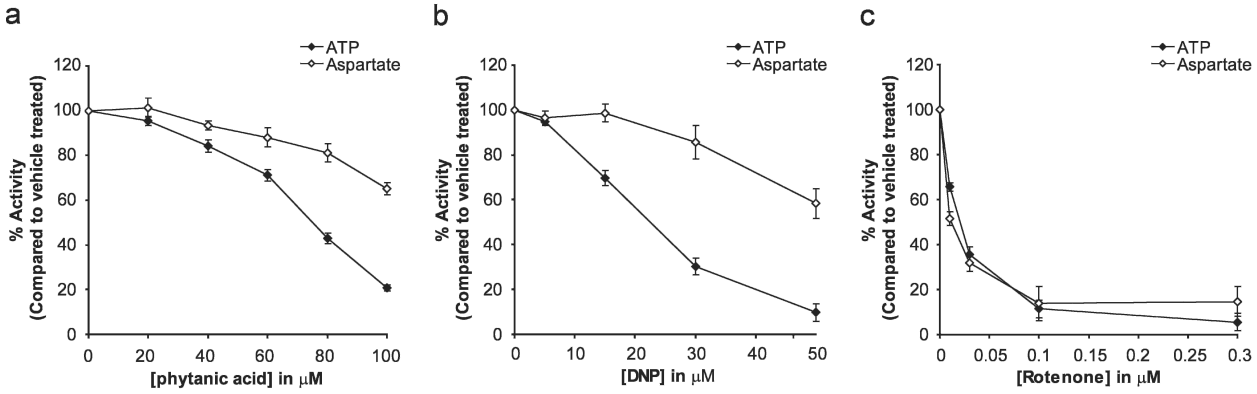

Figure 1. Effect of phytanic acid on ATP and aspartate synthesis in digitonin-permeabilized fibroblasts. Digitonin permeabilized fibroblasts were incubated with different concentrations of phytanic acid (*a*), 2,4-dinitrophenol (DNP) (*b*) and rotenone (*c*) for 30 min in the presence of malate (plus glutamate). Subsequently, the amount of ATP and aspartate was measured as described in Materials and methods. Vehicle (DMSO)-treated cells were set at 100%. Data represent average values of separate measurements depicted as a percentage of vehicle-treated cells ± SE (phytanic acid, n = 6; DNP, n = 8; rotenone, n = 7).

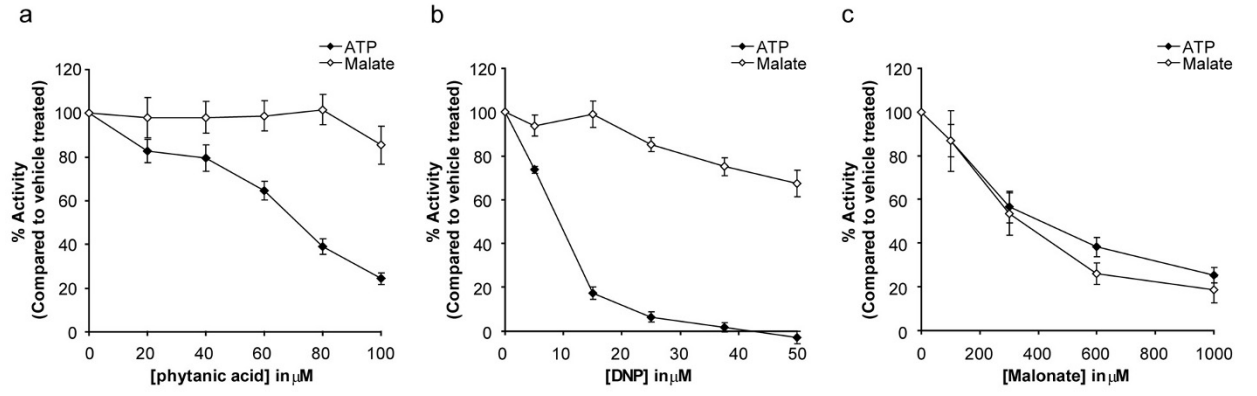

Figure 2. Effect of phytanic acid on ATP and malate synthesis in digitonin-permeabilized fibroblasts. Digitonin-permeabilized fibroblasts were incubated with different concentrations of phytanic acid (*a*), DNP (*b*) and malonate (*c*) in the presence of succinate (plus rotenone) for 30 min. Subsequently, the amount of ATP and malate were measured as described in Materials and methods. Vehicle (DMSO)-treated cells were set at 100%. Data represent average values of separate measurements depicted as a percentage of vehicle-treated cells ± SE (phytanic acid, n = 9; DNP, n = 8; malonate, n = 6).

to inhibit the electron flow coming from complex I. Under these conditions, malate is the end-product of succinate oxidation via the formation of fumarate and its amount was determined in the same samples used for ATP measurement as a measure of complex II activity. Both phytanic acid (Fig. 2a) and DNP (Fig. 2b) inhibited ATP synthesis in a dose-dependent manner, while decreasing the formation of malate much less potently. As observed for complex I, specific inhibition of respiratory chain complex II by malonate decreased both processes equipotently (Fig. 2c). Taken together, the results obtained for complex II-mediated ATP synthesis are in agreement with the notion that phytanic acid inhibits ATP synthesis primarily through its protonophoric action.

To assess the specificity of the inhibitory effect of phytanic acid, we subsequently determined the effect of different analogues of phytanic acid on complex I-mediated ATP synthesis. Of the analogues tested, pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) inhibited ATP synthesis as equipotently as phytanic acid (Fig. 3). Neither the methyl-ester of phytanic acid nor its precursor phytol (3,7,11,15-tetramethylhexadec-trans-2-en-l-ol) influenced malate-dependent ATP synthesis, suggesting that the carboxyl group is important for the inhibitory effect of phytanic acid. Furthermore, the straight-chain fatty acid palmitic acid (hexadecanoic acid) inhibited ATP synthesis, but much less efficiently than phytanic acid and pristanic acid. This suggests that in addition to the carboxyl group, methyl branches contribute to the capacity of phytanic acid and pristanic acid to decrease ATP synthesis.

Effect of phytanic acid on mitochondrial shape and membrane potential. The results described above

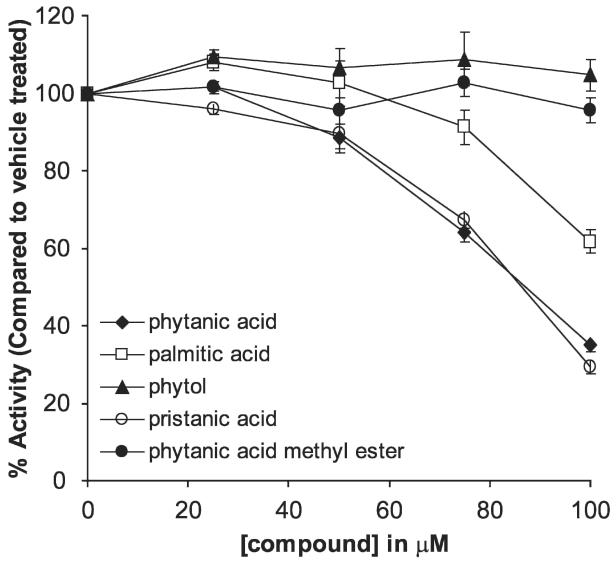

Figure 3. Effect of analogues of phytanic acid on ATP synthesis in digitonin-permeabilized fibroblasts. Digitonin-permeabilized fibroblasts were incubated with different concentrations of phytanic acid (◆), palmitic acid (□), phytol (), pristanic acid (○) and phytanic acid methyl ester (●) using malate as a substrate for the respiratory chain (plus glutamate) for 30 min. ATP formation was measured as described in Materials and methods. Vehicle (DMSO)-treated cells were set at 100%. Data represent average values of separate measurements depicted as a percentage of vehicle-treated cells ± SE (all compounds, n = 4).

were obtained in experiments performed in an artificial cell system in which mitochondrial oxidative phosphorylation was studied after the plasma membrane had been disrupted by digitonin. To investigate the action of phytanic acid under more physiological conditions, i.e. in intact cells, we performed experiments with intact living human skin fibroblasts. It has been demonstrated that protonophores such as FCCP can cause marked alterations in mitochondrial shape [31]. Therefore, to substantiate the notion that phytanic acid decreases mitochondrial ATP synthesis primarily by acting as a protonophore, we next investigated the effect of phytanic acid on mitochondrial shape and membrane potential ($\Delta\psi$). To this end, fibroblasts were loaded with the lipophilic fluorescent cation TMRM for 30 min and subsequently incubated with increasing concentrations of phytanic acid for another 30 min. The accumulation of TMRM in the mitochondrial matrix follows the Nernst equation and, therefore, its fluorescence intensity is a sensitive readout of $\Delta\psi$ [32]. As reported previously, the automated image-processing algorithm for quantitative analysis of mitochondrial membrane potential can also be used for quantification of mitochondrial shape and number [26–28]. Figure 4 shows that phytanic acid altered both the shape of the mitochondria (Fig. 4a, middle panel) and the amount of fluorescence originating from these organelles (Fig. 4a, lower panel). Subsequent quantification of mitochondrial shape revealed a decrease in mitochondrial area (Fig. 4b), an increase in mitochondrial number (Fig. 4c) and a decrease in mitochondrial formfactor (Fig. 4d). Together, these data indicate that phytanic acid causes a decrease in mitochondrial length and degree of branching thus reducing mitochondrial area. In addition, the increase in mitochondrial number shows that phytanic acid can also induce mitochondrial fragmentation. Quantification of the mean mitochondrial pixel intensity per cell revealed a dose-dependent decrease with increasing concentrations of phytanic acid (Fig. 5a). This result is compatible with a depolarizing effect of phytanic acid on the mitochondrial membrane potential. To obtain information on the relative magnitude of the decrease in $\Delta\psi$, TMRM-loaded fibroblasts were treated with 100 nM rotenone for 30 min. This treatment significantly decreased the mitochondrial fluorescence intensity to $90.5 \pm 0.96\%$ of the value obtained with untreated control cells ($n = 82$ cells for both the untreated and rotenone-treated condition; $p < 0.001$). This value was slightly lower than that obtained with 100 μM phytanic acid.

Next, we investigated the effect of the different phytanic acid analogues. Based on the phytanic acid dose-inhibition curve (Fig. 5a), each analogue was tested at a concentration of 30 μM. At this concentration, all analogues caused a decrease in mitochondrial TMRM staining (Fig. 5b). However, in agreement with the findings in the permeabilized cell system (Fig. 3), the effect was largest for phytanic acid and pristanic acid and lowest for phytanic acid methyl ester and phytol. These results demonstrate that phytanic acid can also alter the driving force for mitochondrial ATP production when added to the intact cell.

Effect of phytanic acid, DNP and rotenone treatment on NAD(P)H autofluorescence in healthy fibroblasts. A depolarized $\Delta\psi$ can result from uncoupling and/or inhibition of the electron transport chain. Therefore, it is not possible to differentiate between these mechanisms by measuring the mitochondrial TMRM fluorescence intensity. We recently demonstrated that mitochondrial uncoupling by FCCP decreased cellular NAD(P)H levels in living fibroblasts [29]. Similarly, it has been shown that DNP treatment reduces NAD(P)H levels in living rat ventricular myocytes [33]. Conversely, we observed that specific inhibition of complex I by rotenone increased intracellular NAD(P)H levels [29].

In accordance with previous results, we show here that DNP, when added at a concentration of 30 μM for

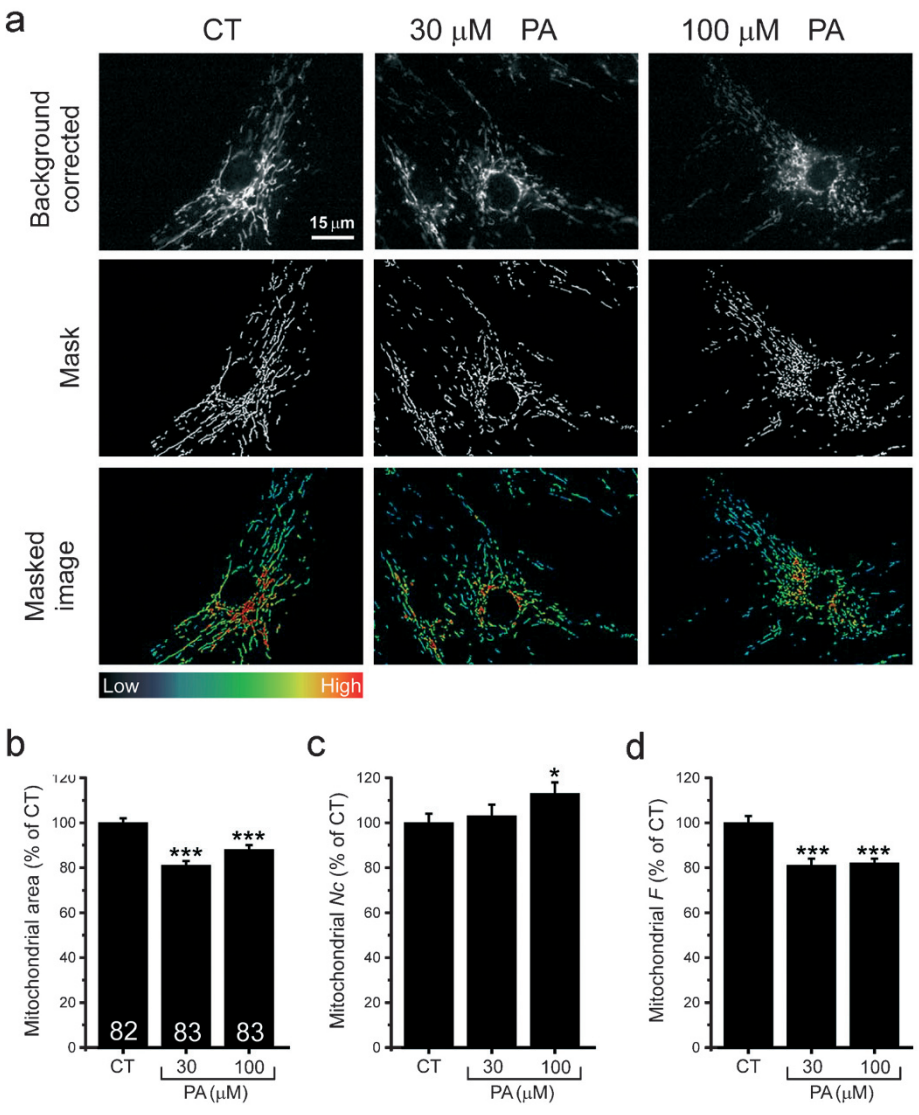

Figure 4. Effect of phytanic acid on mitochondrial shape and membrane potential. (*a*) Fibroblasts were incubated with the cationic probe TMRM for 30 min and subsequently with phytanic acid (PA) for another 30 min. After incubation, cells were subjected to video-imaging microscopy. Images were first background-corrected (upper panel) and then binarized to generate a mask (middle panel). Note that phytanic acid induces a more punctate mitochondrial appearance. Next, this mask was applied to the original background-corrected image (lower panel), and the TMRM fluorescence intensities were visualized by the gradation of color values: blue, low intensity; red, high intensity. Note that phytanic acid markedly decreases the number of high-intensity pixels. (*b–d*) In addition, the mitochondrial mask was used for quantitative analysis of mitochondrial area per cell (*b*), number of mitochondria per cell (*Nc*) (*c*) and mitochondrial formfactor (*F*) (*d*), which is a combined measure of mitochondrial length and degree of branching. In each experiment, the average value obtained with DMSO-treated control fibroblasts (CT) was set at 100 %, to which all other values were related. *$p < 0.05$, ***$p < 0.001$ relative to vehicle-treated control. (*b*) Numerals represent the number of individual cells analysed.

30 min, significantly reduced NAD(P)H autofluorescence, whereas, in sharp contrast, 100 nM rotenone, when present for the same period of time, markedly increased the NAD(P)H signal (Fig. 6). Treatment of the fibroblasts with either 30 or 100 μM phytanic acid for 30 min decreased NAD(P)H autofluorescence to the same extent as DNP. These findings, furthermore, substantiate the idea that phytanic acid acts as a protonophore to decrease the proton motive force and thus the driving force for mitochondrial ATP production.

Discussion

Previous studies in isolated rat brain mitochondria indicated that phytanic acid can affect the function of mitochondria in multiple ways. Depending on the experimental conditions, evidence was provided that phytanic acid can act as a protonophore, as an inhibitor of complex I and as an inhibitor of ANT [20, 21]. Using digitonin-permeabilized human skin fibroblasts, we show here that phytanic acid can effectively inhibit the production of ATP in mitochondria selectively respiring on either the complex I substrate malate (plus glutamate) or the complex II substrate succinate (plus rotenone). This finding indicates that inhibition of complex I alone cannot be the sole explanation for the inhibitory effect of phytanic acid on mitochondrial ATP synthesis. Parallel measurement of either the complex I-mediated production of aspartate or the complex II-mediated production of malate revealed that phytanic acid hardly affected the activities of these two complexes. In addition, this result excludes the possibility that phytanic acid acts solely by inhibition of ANT. Exactly

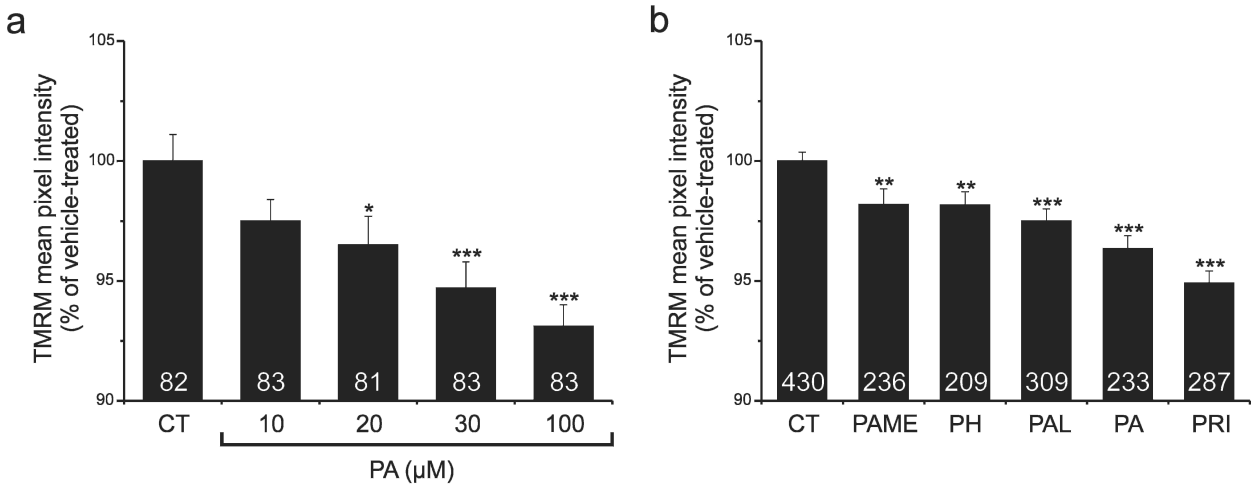

Figure 5. Dose-dependence of the phytanic acid effect, and the effect of phytanic acid analogues on mitochondrial membrane potential. (*a*) Fibroblasts were incubated with TMRM for 30 min and subsequently with the indicated concentrations of phytanic acid for another 30 min. Images were collected by video-imaging microscopy and processed to the masked images as depicted in the lower panel of Figure 4a. Next, these images were used to calculate the mean TMRM fluorescence intensity per mitochondrial pixel as an estimate of the mitochondrial membrane potential. In each experiment, the average value obtained with DMSO-treated control fibroblasts (CT) was set at 100 %, to which all other values were related. (*b*) Effect of phytanic acid and its analogues on the mean TMRM fluorescence intensity. Cells were loaded with TMRM for 30 min and subsequently incubated with 30 μM of the indicated compound for another 30 min. After calculation of the mean TMRM fluorescence intensity per mitochondrial pixel, the average value obtained with DMSO-treated control fibroblasts (CT) was set at 100 %, to which all other values were related. Numerals in (*a*) and (*b*) represent the number of individual cells analysed. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, significantly different from vehicle-treated control. PA, phytanic acid; PAME, phytanic acid methyl ester; PH, phytol; PAL, palmitic acid; PRI, pristanic acid.

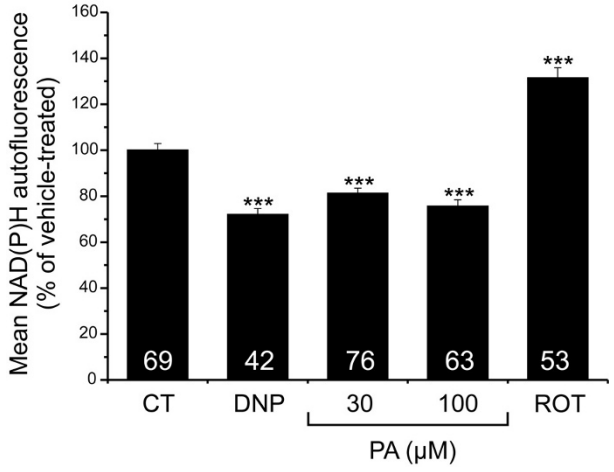

Figure 6. Phytanic acid treatment decreases NAD(P)H autofluorescence in living human fibroblasts. Cells were treated with either rotenone (100 nM), DNP (30 μM) or phytanic acid (PA; 30 and 100 μM) for 30 min and subsequently monitored for NAD(P)H autofluorescence by video-imaging microscopy. For each cell, the average fluorescence intensity per pixel was calculated for a mitochondrial-rich region as a measure of the local NAD(P)H level. The average value obtained with vehicle-treated control cells was set at 100 %, to which all values were related. Numerals represent the number of individual cells analysed. ***$p < 0.001$; significantly different from vehicle-treated control cells.

the same pattern of inhibition was obtained with the protonophore DNP, whereas, in sharp contrast, both the complex I inhibitor rotenone and the complex II inhibitor malonate decreased the production of ATP and either aspartate (rotenone) or malate (malonate) equipotently. Only at higher concentrations phytanic acid caused some reduction in either aspartate or malate production, but it should be noted that the same degree of reduction was observed at DNP concentrations that were equally effective in decreasing the production of ATP. Finally, phytanic acid inhibited complex I- and complex II-mediated ATP production with the same potency, demonstrating the absence of any additional inhibitory effect on complex I alone. Collectively, these results demonstrate that phytanic acid does not significantly inhibit the activity of either complex I or complex II in respiring mitochondria. Moreover, they suggest that under these conditions, phytanic acid inhibits mitochondrial ATP production primarily by acting as a protonophore. This protonophoric action of phytanic acid may be caused by distortion of the packing of the lipids in the inner mitochondrial membrane [described previously in ref. 34].

In previous work, rat brain mitochondria were freeze-thawed in order to make complex I accessible for externally added NADH [21]. However, this procedure will also increase the availability of phytanic acid, which is normally not taken up by mitochondria. Artificially adding phytanic acid to disrupted mitochondria may, therefore, not be representative for the *in vivo* situation. In our experiments, we used digitonin to selectively per-

meabilize the plasma membrane [see ref. 22]. This procedure leaves the mitochondria intact, which may explain the lack of effect of phytanic acid on the activity of complex I in this study.

The present work demonstrates that phytanic acid can cause marked changes in mitochondrial shape and number. A similar effect on mitochondrial shape has been reported previously for the protonophore FCCP [31]. It was concluded by the authors of this study that FCCP exerted its effect primarily by depolarizing the mitochondrial membrane potential. It remains to be established whether, and if so how, the observed changes in mitochondrial shape contribute to the toxic effect of phytanic acid. In agreement with the above explanation for the FCCP-induced changes in mitochondrial shape, we show here, using a mitochondrial mask to specifically determine the amount of TMRM fluorescence from this compartment, that phytanic acid can readily depolarize the inner mitochondrial membrane in human skin fibroblasts [see also refs. 13–15]. The decrease in TMRM fluorescence obtained with 100 μM phytanic acid, the maximal concentration used in this study, was small but significant and only slightly less than that obtained with 100 nM rotenone. Previous work in rat neurons revealed that rotenone, when added at a 10-fold higher concentration of 1 μM, depolarized the inner mitochondrial membrane by only ~17 mV [35]. However, the phosphorylation state of the cytosolic ATP pool has been demonstrated to be very sensitive to small changes in mitochondrial membrane potential [36], and, indeed, rotenone was found to reduce dramatically the cellular ATP level when administered in a chronic fashion [37]. Therefore, the apparently minor depolarization of the mitochondrial membrane potential evoked by phytanic acid might have severe consequences for long-term energy homeostasis in Refsum patients.

In addition to its effect on the mitochondrial membrane potential, phytanic acid readily decreased the steady-state NAD(P)H level in living fibroblasts. The same observation was obtained with the protonophore DNP, whereas, in sharp contrast, the complex I inhibitor rotenone markedly increased the steady-state NAD(P)H level [see also ref. 29]. These findings are in agreement with our conclusion that phytanic acid acts primarily as a protonophore, also stimulating the rate of NADH consumption in mitochondria respiring under phosphorylating conditions. In agreement with the present findings, previous work showed that phytanic acid readily decreased the NAD(P)H level in isolated rat brain mitochondria respiring under non-phosphorylating conditions and that the complex I inhibitor rotenone reversed this effect [21]. On the other hand, our conclusion that the protonophoric action of phytanic acid is much stronger than its alleged action as an inhibitor of complex I is clearly at variance with previous work showing that phytanic acid can decrease FCCP-stimulated respiration in isolated rat brain mitochondria incubated under phosphorylating conditions (21; but see also ref. 20]. At present we have no explanation for these contradictory findings other than possible differences between the test system: isolated rat brain mitochondria [20, 21] versus digitonin-permeabilized and intact human skin fibroblasts (this study).

ATP synthesis was most potently inhibited by pristanic acid and phytanic acid. Palmitic acid was considerably less potent, whereas phytol and phytanic acid methyl ester had no inhibitory effect at all. Similarly, when added at a concentration of 30 μM, pristanic acid and phytanic acid decreased the mitochondrial membrane potential more effectively than palmitic acid, whereas phytol and phytanic acid methyl ester were the least effective. These findings suggest that the carboxyl group of pristanic acid and phytanic acid is important for their protonophoric action. Indeed, mild uncoupling by free fatty acids is known to occur due to import of protons via their carboxyl group from the acidic cytosol to the alkaline matrix [19]. The lower potency of palmitic acid, which has the same carbon backbone as phytanic acid but lacks the methyl side chains, suggests that these latter side chains contribute significantly to the protonophoric action of pristanic acid and phytanic acid.

The present finding that pristanic acid and phytanic acid are equally effective in decreasing the mitochondrial membrane potential and inhibiting the oxidative production of ATP is readily explained by their great similarity, i.e. the carbon backbone of pristanic acid is only one C atom shorter. Importantly, pristanic acid is known to accumulate in a number of inherited disorders of metabolism including D-bifunctional protein deficiency (MIM# 261515), α-methylacyl-CoA racemase deficiency (MIM# 604489) and the recently identified sterol carrier protein X deficiency (MIM# 184755) [reviewed in ref. 38]. Although pristanic acid is not the only compound accumulating in these disorders, it may contribute to the pathology of these diseases.

Previous work in isolated mitochondria revealed that phytanic acid can readily promote the formation of superoxide, increase the oxidation of the glutathione pool and decrease the activity of aconitase, suggesting that chronic elevation of phytanic acid levels may cause oxidative damage [14, 15, 21]. In addition, these studies showed that phytanic acid can cause opening of the permeability transition pore [20] and induce the release of cytochrome c [14, 15], key events in the initiation of apoptotic cell death. In view of the present conclusion that phytanic acid acts primarily as

a protonophore, decreasing the oxidative production of ATP, it remains to be established if and how this property is related to its stimulatory effect on mitochondrial superoxide production.

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