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Letter

Comment concerning the article: 'Phytanic acid impairs mitochondrial respiration through protonophoric action' by Komen et al.: Branched chain phytanic acid inhibits the activity of the mitochondrial respiratory chain

P. Schönfeld[a] and G. Reiser[b,*]

[a]Institut für Biochemie, Otto-von-Guericke-Universität Magdeburg, Leipziger Str. 44, Medizinische Fakultät, 39120 Magdeburg (Germany)
[b]Institut für Neurobiochemie, Otto-von-Guericke-Universität Magdeburg, Leipziger Str. 44, Medizinische Fakultät, 39120 Magdeburg (Germany), Fax: + 49 391 6713097, e-mail: georg.reiser@ med.ovgu.de

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The inherited neurodegenerative Refsum disease is associated with phytanic acid (Phyt) accumulation. The mechanism of Phyt toxicity in brain is still a matter of debate. A major site of cellular toxicity seems to be mitochondrial inhibition. Recently in Cell. Mol. Life Sci., a paper [1] was published, titled 'Phytanic acid impairs mitochondrial respiration through protonophoric action' (2007, 64 3271 – 3281), where the authors used experiments on fibroblasts and confirmed that mitochondria are mainly involved in the cellular toxicity of Phyt. The authors proposed that Phyt-related decline of mitochondrial ATP production is mainly due to protonophoric activity of Phyt. Here, we comment on this conclusion and present a comprehensive analysis of the scenario of mitochondrial toxicity of Phyt. We summarise the evidence on an additional effect of Phyt, i.e. inhibition of electron transport (ET) by Phyt in mitochondria. The latter effect was largely ruled out in the report by Komen et al. Therefore, we performed additional experiments to evaluate this discrepancy.

Refsum disease is a neurological syndrome characterized by adult-onset retinitis pigmentosa, anosmia, sensory neuropathy and phytanic acidaemia. Defective gene products prevent the initiation of the peroxisomal degradation of the branched-chain Phyt (3,7,11,15-tetramethylhexadecanoic acid) [2]. Clinical signs of Refsum disease are mostly attributed to high tissue levels of Phyt.

Mitochondria were recognized as a main target of detrimental activities of non-esterified Phyt in cells [3 – 5]. Thus, depending on the functional state, the respiration of isolated mitochondria is possibly affected by two activities of Phyt (see Fig. 1): (1) depolarization of the inner mitochondrial membrane (IMM) in resting mitochondria by protonophoric action, and/or (2) inhibition of the ET within the respiratory chain (RC) in phosphorylating or uncoupler-stimulated actively respiring mitochondria [3, 5, 6]. Depolarization is best explained by an increased H^+ conductance of the IMM due to the combined operation of protonophoric activity and enhanced H^+ leakage. Remarkably, both activities of Phyt, (1) uncoupling of resting mitochondria and (2) inhibition of the oxygen consumption by RC-activated mitochondria, were found to operate in the same concentration range of Phyt [5, 6].

In the recent study [1] investigating the mechanism of toxicity of Phyt, the protonophoric action of Phyt was

* Corresponding author.

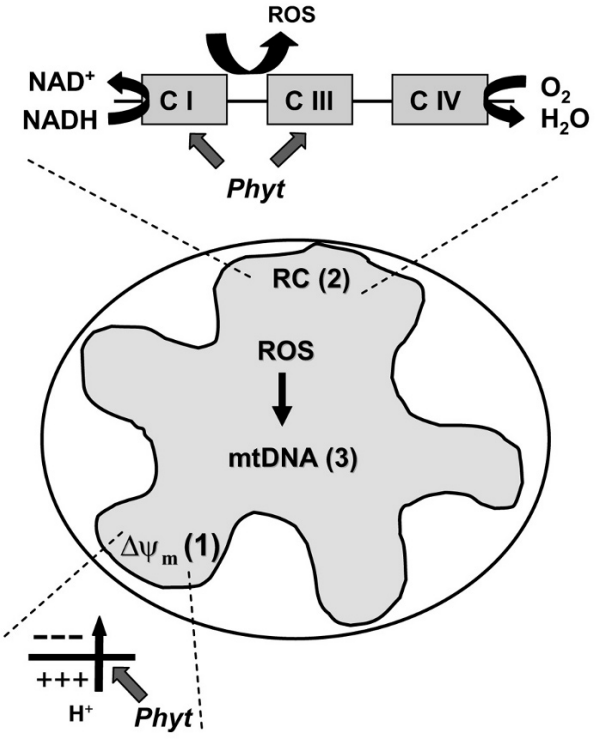

Figure 1. Effects of phytanic acid (Phyt) on the H^+ conductance of the inner mitochondrial membrane (IMM) and on electron transport along the respiratory chain (RC). Phyt increased the H^+ conductance of the IMM, thereby decreasing the membrane potential ($\Delta\psi_m$) and oxidative ATP generation (pathway 1). Decreased oxidative ATP generation is compensated by glycolytic ATP generation (not shown). Interference of Phyt with complexes I and III (CI, CIII) of the RC stimulates RC-linked ROS production (pathway 2). ROS released into the matrix compartment of mitochondria damages mitochondrial DNA (mtDNA) (pathway 3).

derived from experiments using digitonin-permeabilized fibroblasts, where *'in situ'* mitochondria were fed either with malate plus glutamate or succinate in the presence of the complex I inhibitor rotenone. The authors concluded that a Phyt-related decline of mitochondrial ATP production is exclusively due to the protonophoric activity of Phyt [1]. Arguments in favour of this conclusion were that inhibition of electron transport in the RC by Phyt was not seen in digitonin-permeabilized fibroblasts and that the autofluorescence of NAD(P)H in mitochondria-rich regions was decreased. For quantification of NADH oxidation by complex I of the RC, the authors determined aspartate formation. Aspartate results from oxidation of malate, which is followed by conversion of the formed oxaloacetate into aspartate. To quantify $FADH_2$ oxidation by complex III of the RC, they determined malate formation, derived from complex II-mediated succinate oxidation. Based on the observation that in digitonin-permeabilized fibroblasts neither aspartate nor malate levels declined in the presence of Phyt, the authors observed that, in contrast with previously reported data obtained with mitochondria of various rat tissues [5–7], Phyt does not exert an inhibitory effect on the electron transport activity in fibroblasts. Komen et al. additionally support this view by showing that Phyt slightly decreased NAD(P)H fluorescence, similar to the effect of the protonophoric uncoupler 2,4-dinitrophenol. However, we must consider that on the cellular level inhibition of mitochondrial ATP generation by fatty acids enhances glycolytic ATP generation, either by uncoupling or by inhibition of the respiratory chain. Therefore, the observed change in the NAD(P)H level comprises cytosolic processes which could also affect the fluorescence signals obtained in mitochondria-rich regions.

To extend the results from the experiments using permeabilized fibroblasts [1], we measured the effect of increasing concentrations of Phyt on the uncoupled respiration as well as the corresponding NADH oxidation of intact isolated mitochondria. For that purpose, rat liver mitochondria (RLM) were fed with the complex I substrate β-hydroxybutyrate (βOHB), and ET in the RC was stimulated with the uncoupler FCCP. βOHB is taken up by RLM in a carrier-mediated manner, most likely by the combined activity of the monocarboxylate and dicarboxylate transporters [8]. Inside the mitochondria, βOHB is oxidized to acetoacetate by the βOHB-dehydrogenase at the matrix side of the IMM [9]. Since this is a simple oxidation reaction, we used βOHB as substrate. The formation of the oxidation product acetoacetate corresponds to NADH formation. Under our experimental conditions this is the only source of NADH that can be oxidized by the RC. The situation is more complex when glutamate plus malate are used as respiratory substrates, as done by Komen et al. [1]. In that case, both substrates can serve as NADH sources. (1) Malate dehydrogenation, where the formed oxaloacetate undergoes transamination to aspartate, and (2) glutamate oxidation by the glutamate dehydrogenase are possible pathways.

In the experiments shown in Figure 2, respiration was stimulated to 68 or 96% of the State 3 respiration by applying two different concentrations of FCCP. Materials and methods used are briefly described in the legend to Figure 2. When the capacity of the RC was partially activated (68%), the addition of 25 μM Phyt significantly increased both the respiration and the formation of acetoacetate (Fig. 2A). Higher concentrations of Phyt decreased the FCCP-stimulated respiration and the formation of acetoacetate. In contrast, when the RC of mitochondria was stimulated by FCCP to a rate corresponding to full-level State 3

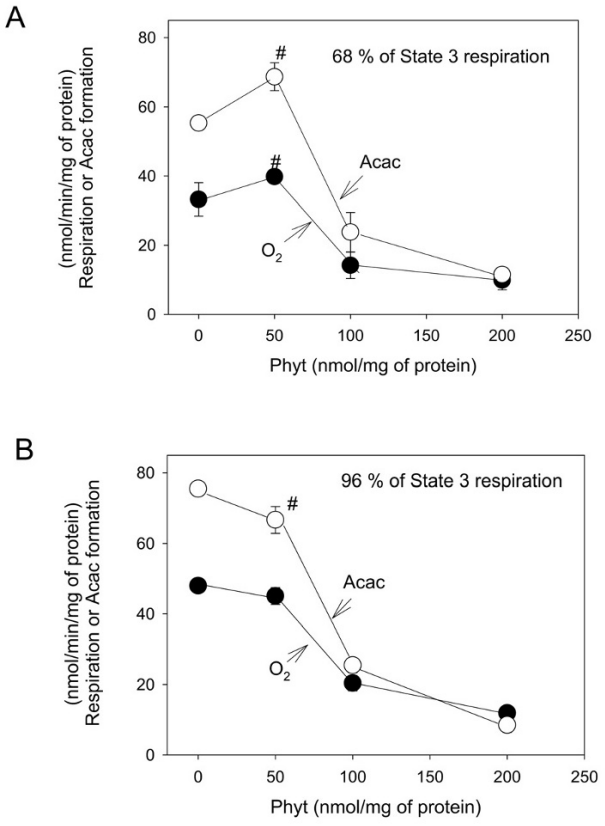

Figure 2. Inhibition of uncoupled mitochondrial respiration and related NADH oxidation in mitochondria. Rat liver mitochondria (RLM) (1 mg of protein) were suspendend in 2 ml of incubation medium containing 5 mM βOHB. Respiration was stimulated with 15 (*A*) or 22.5 nM (*B*) FCCP. The concentration of added Phyt was 25, 50 and 100 μM, corresponding to 50, 100 and 200 nmol/mg of protein. Formation of acetoacetate (Acac) from oxidation of βOHB was measured enzymatically. Data shown are mean values from four experiments. Rates of oxygen uptake and Acac formation are expressed as nmol (O_2; Acac)/min/mg of protein. Methods: RLM were suspendend in incubation medium (110 mM mannitol, 60 mM KCl, 60 mM Tris-HCl, 10 mM KH_2PO_4, 0.5 mM EDTA, 5 mM βOHB; pH 7.2, 37°C). Respiration was stimulated with FCCP. Respiration was measured using an oxygraph (Oroboros Oxygraph^R, Bioenergetics and Biomedical Instruments, Innsbruck, Austria). Formation of acetoacetate from oxidation of βOHB was measured enzymatically [17].

respiration, even the low concentration of 25 μM Phyt clearly decreased the respiration and the associated formation of acetoacetate (Fig. 2B).

The results in Figure 2 convincingly show that the inhibitory effect of Phyt on ET is overwhelming in fully RC-activated mitochondria (Fig. 2B). In a state of intermediate stimulation of the RC, the effect of Phyt on ET reflects its dual activity, the protonophoric activity at low concentration and the inhibitory effect on the RC at high concentrations (Fig. 2A). We suppose that, in the experiment reported by Komen et al. [1] the digitonin treatment, which must be mild to not disrupt mitochondrial membranes, leads to incomplete permeabilization of fibroblasts. Under these conditions, ADP, which is supplemented in the incubation medium, cannot access all mitochondria. Phyt in contrast has access to all mitochondria due its lipophilicity. This could explain why no inhibitory activity of Phyt on ADP-stimulated NADH oxidation by digitonin-permeabilized fibroblasts can be seen at relatively low concentrations of Phyt, since the RC capacity was not in all mitochondria fully stimulated. In addition, in the experiment where permeabilized fibroblasts were supplied with succinate in the presence of rotenone, a possible inhibitory effect of Phyt on complex I, as suggested in [6], cannot affect the succinate oxidation (see [1]). Moreover, such inhibition is *per se* abolished due to the presence of the complex I inhibitor rotenone.

Furthermore, Komen et al. [1] also discuss that the inactivation of RC complexes by Phyt could result from better access of Phyt to the RC after permeabilizing mitochondria by freeze-thawing treatment. However, the amphiphilic nature of Phyt alone is sufficient to allow enrichment of Phyt in IMM, independent of permeabilization or energization of IMM.

In the literature, several reports indicate that Phyt inhibits electron flux in the RC: (1) In well-coupled, morphologically intact mitochondria Phyt decreased the respiration of uncoupler-stimulated mitochondria [5, 6]. (2) Phyt dramatically decreased the reduction of MTT to a blue-colored formazan dye by electron-donating sites of the RC [6]. (3) In uncoupled mitochondria, Phyt increased the reduction of matrix NAD(P) in glutamate plus malate-oxidizing mitochondria [6]. (4) With mitochondria permeabilized to NADH (by freeze-thawing treatment) it was shown that Phyt in a concentration-dependent manner inhibits the enzymatic activities of complexes I and III [6, 7]. (5) An inhibitory activity of Phyt on mitochondrial ET is indirectly suggested by the stimulation of RC-associated reactive oxygen species (ROS) production in intact, NADH- and FADH-oxidizing mitochondria [6, 7], which was also seen in palmitate- and arachidonate-treated mitochondria [10]. (6) Phyt released IMM-bound cytochome c from mitochondria [11]. Depletion of cytochrome c from mitochondria interrupts ET between complex III and complex IV, thereby diminishing overall ET in the RC. Phyt-induced inhibition of respiration recovers partly after supplementation with cytochrome c [12]. It should be emphasized that inhibition of the stimulated respiration of isolated mitochondria or inactivation of the enzymatic activity of complex I by non-esterified, unbranched saturated and unsaturated long-chain fatty acids is a well-established fact [10, 13, 14]. Finally, using rat brain astrocytes, it has been

reported that non-esterified fatty acids can strongly increase lactate release from these cells, an observation indicating that the fatty acids either exert an inhibitory effect on mitochondrial ET or uncouple mitochondrial ATP production also in cells [15].

Finally the question arises why it is relevant to attribute the inhibitory activity of Phyt either to protonophoric uncoupling of mitochondria or to inhibition of the RC in mitochondria. The protonophoric activity of Phyt, as seen in our experiments and reported in [1], could cause a decline of ATP production by phosphorylating mitochondria. On the other hand, in the cells the decline of mitochondrial ATP production (see Fig. 1 in [1]) due to Phyt-mediated uncoupling is largely compensated for by enhanced glycolytic ATP production [11]. Therefore, protonophoric activity of Phyt should have no severe pathological consequences.

All the data summarized and presented here show that Phyt can act as a powerful inhibitor of mitochondrial ET. Inhibition of ET by Phyt stimulates RC-associated ROS generation. This event is likely to damage mitochondrial DNA (mtDNA), especially with long-term exposure of tissues to pathologically elevated concentrations of Phyt. We want to draw attention to the fact that the RC seems to be the main site where pathologically enhanced levels of Phyt could initiate ROS production, thereby causing damage to mtDNA (see Fig. 1). Indeed, impaired ET due to inhibitors of mitochondrial ET (e.g. antimycin A) caused oxidative damage of mtDNA, indicated as formation of 8-hydroxydesoxyguanosine [16]. We suggest that the neurodegenerative activity of Phyt is mainly due to ROS formation in Phyt-treated mitochondria associated with inhibition of ET, whereas protonophoric uncoupling activity of Phyt in mitochondria has a minor impact in the pathogenic mechanism.

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