UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS - BIOQUÍMICA

EFEITOS *IN VITRO* DOS ÁCIDOS FITÂNICO E PRISTÂNICO SOBRE VÁRIOS PARÂMETROS DO METABOLISMO ENERGÉTICO EM CÓRTEX CEREBRAL DE RATOS JOVENS

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Dissertação de mestrado apresentada ao Programa de Pós-Graduação em Ciências Biológicas - Bioquímica da Universidade Federal do Rio Grande do Sul como requisito parcial à obtenção do grau de Mestre em Bioquímica.

Porto Alegre, 2011

AGRADECIMENTOS

A Universidade Federal do Rio Grande do Sul por me proporcionar ensino gratuito e de qualidade.

Ao meu orientador, Professor Moacir Wajner, por todo ensinamento, pelo carinho e pela paciência.

Aos amigos do laboratório 38, por toda amizade e pela colaboração.

Aos professores Patrícia e Gustavo, por todos os ensinamentos bioquímicos, por toda ajuda e pela amizade.

As amigas Carolina e Anelise, cuja ajuda foi essencial para o trabalho, mas também por serem pessoas incríveis que admiro muito e amigas especiais.

As minhas grandes amigas Ângela e Alana, por serem exatamente como são, por todo o carinho, compreensão, risadas e amizade.

Aos colegas Carolina, Bianca, César, Alexandre e Guilhian, por colaborarem com este trabalho e por sua amizade.

Aos queridos bolsistas, principalmente o Mateus, por estarem sempre prontos a ajudar e por toda a amizade.

Ao Caio, por me mostrar que o amor pode superar tudo.

Aos meus pais, os melhores do mundo, por todo apoio, compreensão, incentivo e amor.

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PARTE I

Introdução e Objetivos

RESUMO

Os ácidos fitânico (Fit) e pristânico (Prist) são ácidos graxos saturados de cadeia lateral ramificada, cujas concentrações estão aumentadas em diversas doenças peroxissomais. Os pacientes afetados por essas desordens apresentam manifestações clínicas heterogêneas com envolvimento neurológico importante. O aumento nas concentrações do Fit e Prist, que podem chegar a 5000 μM e 300 μM no plasma, respectivamente, parece estar correlacionado com a severidade das doenças, indicando que estes ácidos graxos possam ser neurotóxicos. Considerando que a fisiopatologia dos sintomas neurológicos dessas doenças ainda não está bem estabelecida e tendo em vista a importância do metabolismo energético para o sistema nervoso central, o presente trabalho se propôs a investigar os efeitos *in vitro* dos ácidos Fit e Prist sobre vários parâmetros do metabolismo energético. Inicialmente, observamos que o Fit não diminuiu a produção de CO_2 a partir de D-[U-^{14}C] glicose e ácido [1-^{14}C] acético, sugerindo que não houve comprometimento da via glicolítica ou do ciclo do ácido cítrico (CAC). Esse último resultado foi confirmado pela determinação da atividade das enzimas do CAC, onde não foram observadas alterações. Por outro lado, o Fit diminuiu significativamente a atividade dos complexos I, I-III, II, II-III e IV da cadeia respiratória, indicando que o fluxo de elétrons por essa cadeia está prejudicado na presença desse ácido graxo. Também verificamos que a atividade da enzima creatina quinase não foi alterada pelo Fit. Finalmente, medimos a atividade da enzima Na^+,K^+-ATPase na presença de Fit e observamos que esse ácido graxo diminuiu de maneira acentuada essa atividade, indicando que a neurotransmissão está prejudicada por esse metabólito. Por outro lado, investigamos a ação do Prist sobre a produção de CO_2 a partir de ácido [1-^{14}C] acético e observamos que esse metabólito diminuiu esse parâmetro, indicando o comprometimento do CAC. Avaliamos se esse efeito deletério pudesse ter sido causado pela diminuição de coenzima A devido a uma possível competição entre o Prist e acetato por essa coenzima. Observamos que os efeitos inibitórios sobre essa atividade não foram devidos a uma depleção de coenzima A. Em seguida, investigamos o efeito do Prist sobre as atividades dos complexos da cadeia respiratória e observamos que esse ácido graxo diminuiu a atividade dos complexos I, II e II-III sem interferir com a atividade do complexo IV, o que indica claramente que esse ácido graxo interfere no fluxo dos elétrons pela cadeia respiratória, podendo potencialmente comprometer a geração de ATP. Também observamos que a atividade da enzima creatina quinase não foi alterada pelo Prist. Entretanto, foi observada uma diminuição acentuada na atividade da enzima Na^+,K^+-ATPase causada pelo Prist, o que indica que pode haver um comprometimento na manutenção do potencial da membrana necessário para o funcionamento da neurotransmissão. Nossos resultados sugerem que os ácidos graxos Fit e Prist acumulados em algumas doenças peroxissomais comprometem o metabolismo energético e possivelmente a neurotransmissão e que esses mecanismos podem estar envolvidos no dano neurológico apresentado pelos pacientes afetados por essas desordens.

ABSTRACT

Phytanic acid (Phyt) and pristanic acid (Prist) are branched-chain saturated fatty acids whose concentrations are elevated in various peroxisomal disorders. Patients affected by these disorders present heterogeneous clinical manifestations with predominant neurological involvement. The elevation of plasma, Phyt and Prist concentrations, that can reach up to 5000 μM and 300 μM, respectively, seems to be correlated with the severity of the symptoms, indicating that these fatty acids may be neurototoxic. Considering that the pathophysiology of the neurological symptoms of these diseases are not well established and the importance of the energy metabolism to the central nervous system, the present work proposed to investigate the *in vitro* effects of Phyt and Prist on various parameters of energy metabolism. Initially, we observed that Phyt did not reduce CO_2 production from labeled glucose and acetate, suggesting that this acid did not alter glycolysis and citric acid cycle (CAC) activity. CAC enzyme activities were also not modified by Fit reinforcing the view that the CAC is not disturbed by Fit. On the other hand, Phyt diminished the activities of complexes I, I-III, II, II-III and IV of the respiratory chain, indicating that the electron flow through this chain is impaired by this fatty acid that could lead toa disturbance of ATP generation. We also verified that the activity of creatine kinase was not altered by this metabolite. Finally, we measured the activity of Na^+,K^+-ATPase in the presence of Phyt and observed that this fatty acid drastically reduced this activity, indicating that the maintenance of membrane potential and consequently neurotransmission is probably compromised by this metabolite. On the other hand, we investigated the effect of Prist on CO_2 production from labeled acetate and observed that this metabolite decrease this parameter, indicating an impairment of CAC functioning. We also evaluated if this effect could be due to a decrease of coenzyme A due to a possible competition between Prist and acetate for this coenzyme. We observed that the inhibitory effects on this activity was not caused by depletion of coenzyme A. Next, we investigated the effect of Prist on the activities of the respiratory chain complexes and observed that this fatty acid reduced the activity of complexes I, II and II-III without interfering with complex IV, which clearly indicates that this fatty acid compromises the electron flow through the respiratory chain that could potentially reduce ATP generation. We also verified that the activity of creatine kinase was not altered by Prist. However, we observed a large decrease on the activity of Na^+,K^+-ATPase caused by Prist, which indicates that there can be a compromise on the maintenance of membrane potential necessary to a normal neurotransmission Our results suggest that the fatty acids Phyt and Prist accumulated in some peroxisomal diseases compromise energy metabolism and possibly the neurotransmission and that these mechanisms may be involved with the neurological damage presented by patients affected by these disorders.

LISTA DE ABREVIATURAS

AMACR - α-metil-acil CoA racemase

ANOVA – análise de variância de uma via

ADP – adenosina difosfato

ATP – adenosina trifosfato

CAC - ciclo do ácido cítrico

CK – creatina quinase

CoA – coenzima A

EIM – erros inatos do metabolismo

F – valor utilizado em análise de variância

Fit – ácido fitânico

MFP 2 – proteína multifuncional peroxissomal 2

OMIM – *Online mendelian inheritance in man*

Prist – ácido pristânico

Pi – fosfato inorgânico

RXR - receptores nucleares retinóide-X

PPAR - receptores ativadores da proliferação de peroxissomos

SCPx – proteína carreadora de esteróis x

SCP-2 - proteína carreadora de esteróis-2

SZ – síndrome de Zellweger

THC – ácido trihidroxicolestanóico

I.1. INTRODUÇÃO

I.1.1. Erros Inatos do Metabolismo

Em 1908, Sir Archibald E. Garrod usou o termo erros inatos do metabolismo (EIM) para designar doenças como a alcaptonúria, em que os indivíduos afetados excretam grandes quantidades de ácido homogentísico na urina. Garrod observou uma maior frequência desta doença em indivíduos de uma mesma família e maior consanguinidade entre os pais dos pacientes. Baseando-se nas leis de Mendel e no fato de que os pais dos indivíduos afetados não apresentavam a doença, Garrod propôs um modelo de herança autossômica recessiva para este distúrbio. Através da observação de que o ácido homogentísico presente em excesso na urina dos pacientes era um metabólito normal da degradação protéica, ele relacionou este acúmulo a um bloqueio na rota de catabolismo da tirosina. Com o surgimento de novos distúrbios relacionados a alterações genéticas e que envolviam o acúmulo de outras substâncias nos líquidos biológicos dos pacientes, postulou-se que estas doenças resultavam da síntese qualitativa ou quantitativamente anormal de uma proteína, enzimática ou não, pertencente ao metabolismo (Scriver et al., 2001). Presumiu-se, então, que em consequência deste bloqueio metabólico pode ocorrer o acúmulo de precursores da reação catalisada pela enzima envolvida, com a formação de rotas metabólicas alternativas e a deficiência de produtos essenciais ao organismo (Bickel 1987).

Até o momento foram descritos mais de 500 EIM, a maioria deles envolvendo processos de síntese, degradação, transporte e armazenamento de moléculas no organismo (Scriver et al., 2001). Embora individualmente raras, essas doenças em seu conjunto afetam aproximadamente 1 a cada 500 a 2.000 recém nascidos vivos (Barić et al., 2001).

I.1.2. Metabolismo dos ácidos fitânico e pristânico

O ácido fitânico (ácido 3,7,11,15-tetrametilhexadecanóico; Fit) é um ácido graxo saturado de cadeia lateral ramificada derivado da clorofila. O Fit não é produzido no intestino humano, mas microorganismos presentes no sistema gastrointestinal de ruminantes podem metabolizar a clorofila, liberando fitol, que é então convertido nesse ácido (Allen et al., 2008). Esse ácido graxo é proveniente da dieta, principalmente de carne e laticínios e, inicialmente, entra no peroxissomo a partir da proteína carreadora de esteróis 2 (SCP2). O metabolismo peroxissomal do Fit ocorre por α-oxidação principalmente nos rins e no fígado, onde atuam quatro enzimas que transformam o Fit em ácido pristânico (ácido 2,6,10,14-tetramethyl pentadecanóico, Prist) (Figura 1). O Prist é posteriormente degradado através de 3 ciclos da β-oxidação peroxissomal, seguido pela β-oxidação mitocondrial (Figura 2). Esse ácido graxo também pode ser proveniente diretamente da dieta de fontes como gordura bovina e alguns tipos de peixes, entrar no peroxissomo e sofrer a β-oxidação (Wanders et al., 2001; Wanders e Komen 2007; Wierzbicki 2007).

Como apenas o isômero com o grupamento 2-metil na configuração S pode ser degradado via β-oxidação peroxissomal, é necessário que o Prist-2R seja convertido em um isômero 2S pela α-metil-acil CoA racemase (AMACR) antes de sua degradação. Essa enzima cataliza a conversão de vários (2R)-metil-acil-CoAs de cadeia ramificada aos seus Isômeros S correspondentes e não está diretamente envolvida com a β-oxidação peroxissomal, mas é importante para que ela ocorra normalmente, além de também estar envolvida na degradação dos sais biliares (Figura 3).

O primeiro passo da β-oxidação peroxissomal é catalizado pela proteína multifuncional 2 (MFP 2), também chamada de proteína D-bifuncional, que exerce atividades de hidratase e desidrogenase. O último passo dessa via de degradação é catalizada por uma tiolase e o produto final dos tres ciclos da β-oxidação é o composto 4,8-dimetilnonanoil-CoA, que é exportado do peroxissomo para a mitocôndria, onde segue a β-oxidação mitocondrial.

(Ferdinandusse et al., 2000; Ferdinandusse et al., 2006; Mclean et al., 2002; Smith et al., 2010; Verhoeven e Jakobs 2001).

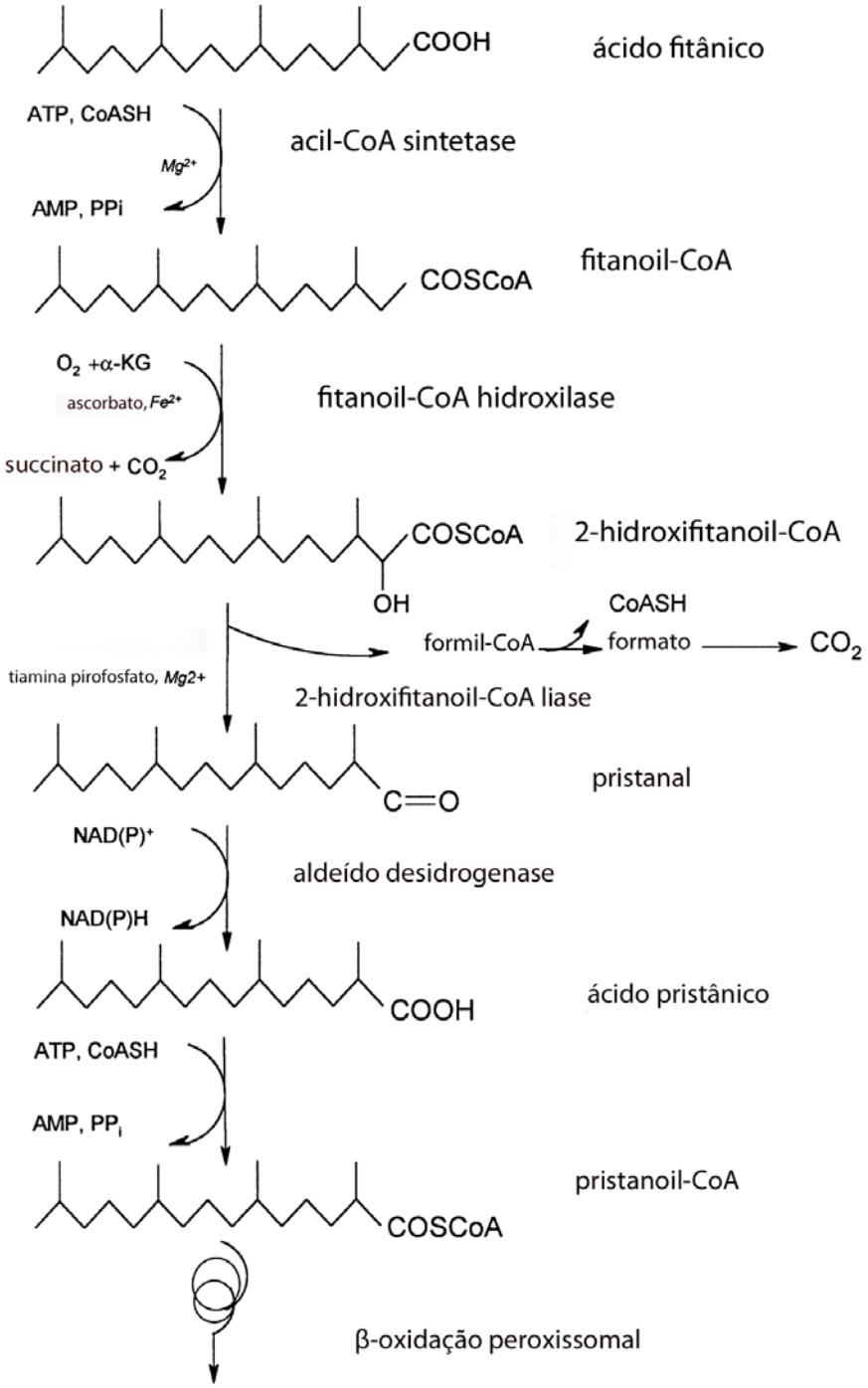

Figura 1. α-oxidação peroxissomal. Adaptado de (Verhoeven e Jakobs 2001).

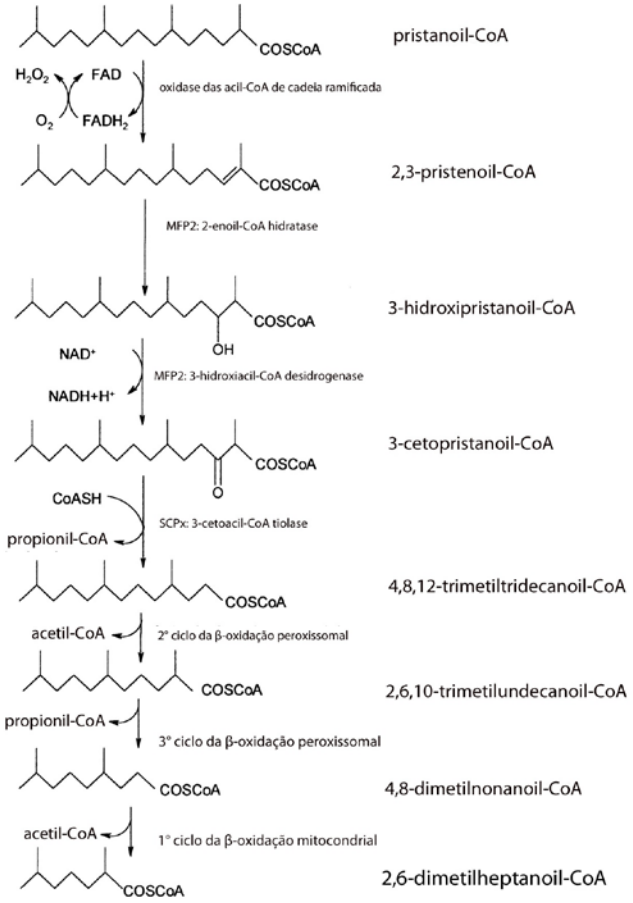

Figura 2. β-oxidação do ácido pristânico. Adaptado de (Verhoeven e Jakobs 2001)

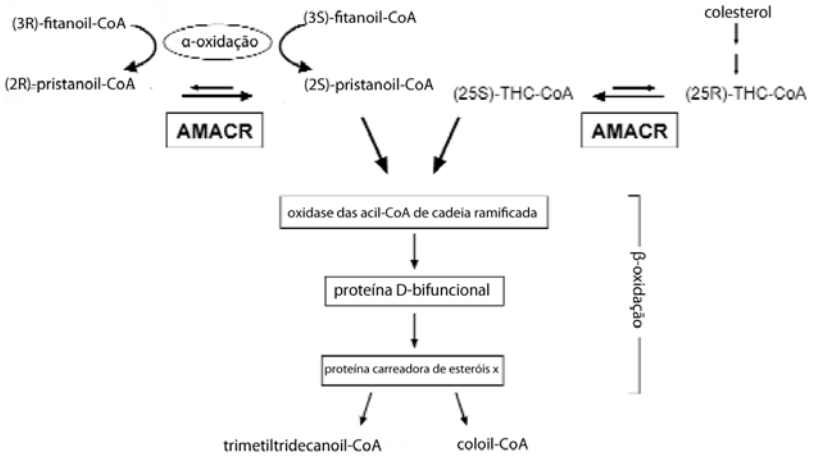

Figura 3. Função da enzima α-metil-acil-CoA racemase. Adaptado de (Ferdinandusse et al., 2000)

I.1.3. Peroxissomos

Os peroxissomos são organelas presentes em praticamente todas as células eucariotas. Na maioria das células humanas, sua presença pode variar de menos de cem a mais de mil peroxissomos por célula. Sua matriz granular contem mais de 50 enzimas que participam de vários processos metabólicos, incluindo a β-oxidação de certos ácidos graxos e também a biossíntese de fosfolipídeos, ácidos biliares e compostos isoprenóides (Scriver et al., 2001). Essa organela representa um dos principais sítios na célula onde radicais de oxigênio são gerados e eliminados (Yeldandi et al., 2000).

Há um amplo grupo de erros inatos do metabolismo (EIM) onde há alterações peroxissomais. A incidência estimada dessas desordens é de 1:20.000 a 1:100.000 nascidos vivos. Elas são divididas em dois grupos: a) defeitos com alteração em uma única proteína envolvida na α ou β-oxidação e b) doenças hereditárias causadas por defeitos na biogênese peroxissomal. Os ácidos graxos Fit e Prist se acumulam em diversas dessas doenças hereditárias de ambos os grupos (Mclean et al., 2002; Scriver et al., 2001).

I.1.4. Doenças com acúmulo dos ácidos fitânico e pristânico

I.1.4.1 Síndrome de Zellweger

A síndrome de Zellweger (SZ) (OMIM 214100) constitui-se em um grupo de desordens com diferentes defeitos genéticos que apresentam em comum a ausência de peroxissomos funcionais, levando a várias anormalidades bioquímicas, bem como sintomas clínicos variáveis. (Verhoeven e Jakobs 2001). A SZ é a doença peroxisomal que apresenta fenótipo clínico mais severo, sendo caracterizada por malformações que afetam o desenvolvimento do cérebro, fígado, rins e esqueleto. Os pacientes portadores dessa desordem apresentam anormalidades craniofaciais e oculares características, além de hipotonia, fraqueza e convulsões no período neonatal. Normalmente os

pacientes morrem no primeiro ano de vida. A SZ é originada a partir de mutações dos genes PEX, que codificam proteínas (peroxinas) necessárias para a formação de importantes proteínas na matriz e membrana peroxissomal (Müller et al., 2010). Por outro lado, alguns defeitos da biogênese peroxissomal podem manifestar sintomas menos severos, como retardo psicomotor, disfunção neurológica leve e expectativa de vida de mais de 10 anos. No plasma dos pacientes afetados pela SZ, Fit e Prist estão presentes em concentrações elevadas, podendo atingir níveis plasmáticos superiores a 500 e 90 μM, respectivamente (Ferdinandusse et al., 2002; Scriver et al., 2001; Verhoeven e Jakobs 2001).

I.1.4.2 Doença de Refsum

A doença de Refsum clássica ou adulta (OMIM 26650) é uma doença neurometabólica rara caracterizada por um defeito no gene estrutural que codifica a enzima fitanoil-CoA hidroxilase, levando ao acúmulo do Fit nos tecidos e líquidos biológicos dos pacientes. Essa enzima cataliza o primeiro passo da α-oxidação peroxissomal, convertendo fitanoil-CoA em 2-hidroxi-fitanoil CoA (Reiser et al., 2005; Wierzbicki e Lloyd 2007). Quando ocorre um bloqueio na atividade da enzima fitanoil-CoA hidroxilase, as concentrações plasmáticas de Fit nos pacientes podem atingir até 5000 μM (níveis em indivíduos normais: até 30 μM) (Wanders et al., 2003). Os pacientes afetados pela doença de Refsum clássica apresentam múltiplos sintomas clínicos que geralmente começam a aparecer durante a infância. Os principais sinais clínicos incluem retinite pigmentosa, polineuropatia periférica e ataxia cerebelar, todos neurológicos. Outros achados menos comuns incluem arritmias cardíacas, déficits auditivo e olfatório, ictiose, perda de visão, distúrbios psiquiátricos e proteinúria. Todos os pacientes afetados apresentam sinais de retinite pigmentosa (degeneração da retina), sendo considerado o mais importante sintoma inicial da doença, precedendo até mesmo os achados bioquímicos característicos. Essa degeneração retinal característica parece ser devida ao acúmulo excessivo de Fit no tecido ocular. Exames patológicos revelam quase uma perda completa dos fotorreceptores, afinamento da

camada nuclear interna e redução no número de células ganglionais da retina. A neuropatia periférica afeta a parte motora e sensorial, sendo caracterizada por fraqueza e atrofia muscular e distúrbios sensoriais. Diferentemente da retinite, pode não ser detectada no início da doença. Por outro lado, as disfunções cerebelares também são bem estabelecidas nesses pacientes, incluindo ataxia e tremores. Contudo, essas disfunções cerebelares se manifestam em um período mais tardio quando comparadas com a retinopatia e a neuropatia características (Wierzbicki 2007; Wierzbicki e Lloyd 2007).

I.1.4.3 Defeito da α-metil-acil CoA racemase

A deficiência da atividade da α-metil-acil CoA racemase (AMACR) (OMIM 604489) é uma desordem rara que resulta no acúmulo de R-isômeros do Prist e intermediários de sais biliares e se manifesta principalmente em adultos. Pacientes portadores dessa desordem apresentam neuropatia periférica que afeta a parte motora e sensorial, embora há o relato de pelo menos um caso com coagulopatia e colestase no período neonatal devido a anormalidade nos sais biliares (Setchell et al., 2003). Nos pacientes afetados observa-se o acúmulo de Fit e Prist (em torno de 25 e 300μM no plasma, respectivamente), além de intermediários de sais biliares (Ferdinandusse et al., 2002). A mutação predominante dessa enzima é a c.154T>C que foi encontrada na maioria dos casos descritos da deficiência de AMACR. Essa mutação tem caráter de herança autossômica recessiva e está relacionada tanto com a forma adulta quanto a forma neonatal dessa desordem (Smith et al., 2010).

I.1.4.4 Defeito da poteína D-bifuncional

A deficiência da proteína D-bifuncional (OMIM 261515) pode ser classificada em 3 tipos: tipo I com deficiência das unidades hidratase e

desidrogenase, tipo II com deficiência isolada na hidratase e tipo III com deficiência isolada na desidrogenase. Os pacientes portadores dessa desordem normalmente morrem no primeiro ano de vida (Möller et al., 2001). Os achados clínicos são hipotonia, dismorfismo facial, atraso psicomotor, convulsões neonatais, defeitos de migração neonatal ou desmielinização. Os pacientes afetados acumulam ácidos graxos de cadeia muito longa, intermediários dos sais biliares, Prist (até 80 μM em plasma) e/ou Fit (mais de 190 μM em plasma) (Ferdinandusse et al., 2002; Khan et al., 2010; Verhoeven e Jakobs 2001).

I.1.5 Diagnóstico das doenças com acúmulo dos ácidos fitânico e pristânico

O diagnóstico das doenças metabólicas com acúmulo do Fit e Prist deve ser realizado com base na análise das concentrações plasmáticas desses ácidos graxos por cromatografia gasosa, além da medida da atividade da enzima defeituosa em fibroblastos para confirmar o defeito nos processos de α ou β-oxidação. Também pode-se realizar análise mutacional e medida de outros compostos que possam estar acumulados (Ferdinandusse et al., 2002; Johnson et al., 2003; Müller et al., 2010; Smith et al., 2010).

I.1.6 Tratamento

Os tratamentos disponíveis até o momento para as doenças peroxissomais são apenas de suporte, focados em tratar as convulsões, disfunções hepáticas e melhorar a qualidade de vida dos pacientes (Steinberg 2006). Alguns estudos mostraram que a administração oral de ácido biliar melhorou a função hepatobiliar em muitas crianças com SZ (Maeda et al., 2002; Setchell et al., 1992).

Considerando que o Fit é obtido exclusivamente da dieta, proveniente principalmente de carne vermelha e laticínios, o tratamento para a doença de Refsum e para a deficiência de AMACR é baseado na restrição dietética desse ácido graxo (Ferdinandusse et al., 2002; Smith et al., 2010). O primeiro paciente portador da doença de Refsum tratado com restrição dietética de Fit obteve a diminuição significativa nos níveis plasmáticos do metabólito, melhora significativa na polineuropatia, além da estabilização da retinite pigmentosa, indicando que o Fit é tóxico para células neurais (Wanders et al., 2001). Muitos outros pacientes que foram submetidos a esse tratamento obtiveram diminuição nas concentrações de Fit (Scriver et al., 2001). Contudo, no início do tratamento os níveis de Fit podem aumentar, sugerindo que reservas teciduais são mobilizadas quando a ingesta é diminuída. Dessa forma, além da restrição de Fit na dieta, é indicado um rigoroso controle na ingesta calórica para evitar a mobilização excessiva de gordura que possa causar um aumento significativo nos níveis de Fit. Entretanto, deve-se destacar que esse tratamento está longe de ser efetivo, provavelmente porque a fisiopatogenia da doença é pouco conhecida.

I.1.6 Fisiopatologia das doenças com acúmulo dos ácidos fitânico e pristânico

Os mecanismos de dano cerebral dos pacientes portadores de doenças com acúmulo dos ácidos Fit e/ou Prist não estão completamente esclarecidos, embora haja evidências apontando para uma ação tóxica dos mesmos.

Recentemente, um estudo estabeleceu um modelo genético knockout para a SZ, onde foi observado estresse oxidativo, morte celular neuronal e prejuízo no desenvolvimento cerebelar (Müller et al., 2010). Também foi desenvolvido recentemente um modelo knockout de ratos para a proteína D-bifuncional. Os animais apresentaram retardo severo no crescimento na primeira semana após o nascimento e 40% de mortalidade durante esse período (Baes et al., 2000). Por outro lado, Ferdinandusse e colaboradores (2003) demonstraram que houve dano oxidativo lipídico e ao DNA em plasma e

urina de pacientes portadores da deficiência da enzima D-bifuncional. Além disso, os pesquisadores mostraram também que houve um desequilíbrio entre a geração de peróxido de hidrogênio e a atividade da enzima catalase, responsável por sua degradação, em fibroblastos de pacientes. Também foi demonstrado que o Prist exerceu uma acentuada atividade citotóxica em células cerebrais, causada por alterações do Ca^{2+} intracellular, despolarização mitocondrial e morte celular. O Prist também induziu marcadamente a geração de espécies reativas de oxigênio (ERO), enquanto o Fit exerceu apenas efeitos moderados sobre esse parâmetro (Rönicke et al., 2009). Nosso grupo mostrou recentemente que o Fit induziu dano oxidativo e reduziu as defesas antioxidantes em ratos jovens (Leipnitz et al., 2010). Além disso, estudos recentes demonstraram que o Fit causa morte celular em astrócitos cultivados de hipocampo de ratos (Kahlert et al., 2005). Essa morte celular astrocitária é precedida por um aumento significativo na produção de ânion superóxido, despolarização do potencial da membrana mitocondrial e liberação do citocromo *c* (Kahlert et al., 2005). O efeito tóxico do Fit também tem sido objeto de intenso estudo em mitocôndrias isoladas de cérebro. Foi demonstrado que o Fit possui atividade protonófora, inibe a translocase ADP / ATP e o fluxo de elétrons em mitocôndrias e sinaptossomas de cérebro de ratos, levando a uma diminuição da produção de ATP mitocondrial (Komen et al., 2007; Schönfeld et al., 2004). Nesse contexto, também foi mostrado que este ácido facilita a abertura do poro de transição em mitocôndrias pré-tratadas com baixas quantidades de Ca^{2+} (Schönfeld et al., 2004).

Por outro lado, Ferdinandusse e colaboradores (2008a) demonstraram recentemente alterações patogênicas em um modelo animal da doença de Refsum obtido através do desenvolvimento de uma linhagem de camundongos knockout para a enzima fitanoil-CoA hidroxilase que foram submetidos a uma dieta suplementada com fitol, precursor do Fit. Este estudo verificou que os camundongos desenvolveram neuropatia periférica e ataxia cerebelar com perda de células de Purkinje. Também evidenciou que o acúmulo de Fit causou astrocitose, bem como desencadeou mecanismos de adaptação como o aumento nas concentrações de proteínas ligantes de Ca^{2+}. Esse último achado está de acordo com resultados prévios obtidos *in vitro* por evidenciando que

astrócitos hipocampais têm uma alta liberação de Ca^{2+} intracelular quando expostos ao Fit (Kahlert et al., 2005).

Outros estudos mostraram que o Fit age como ativador e receptores nucleares retinóide-X (RXR) e de receptores ativadores da proliferação de peroxissomos (PPAR). Esses receptores atuam como fatores de transcrição que regulam a expressão gênica de proteínas envolvidas no metabolismo de lipídios e glicose, bem como na proliferação dos peroxissomos. Além disso, os efeitos do PPAR também estão relacionados à regulação da apoptose (Roberts et al., 2002). O receptor PPAR possui três isoformas PPARα, PPARβ e PPARγ, sendo que o Fit é um ligante natural da PPARα. O PPARα está distribuído em tecidos com elevado metabolismo de ácidos graxos, tais como tecido adiposo marrom, fígado, rins e coração. O PPARα ativado se dimeriza com os RXR e este complexo se liga a fatores de resposta para proliferação de peroxissomos localizados nos promotores de vários genes (Berger e Wagner 2002).

Seedorf (1998) por sua vez demonstrou uma ação do Fit sobre receptores nucleares, modulando a expressão gênica. Utilizando camundongos knockout para a proteína carreadora de esteróis-2 (SCP-2) tratados com uma dieta rica em fitol, verificou que os camundongos mutantes desenvolveram letargia, ataxia, perda de peso corporal e neuropatia periférica. Outros estudos também evidenciaram uma ação do Fit sobre a expressão gênica demonstrando que uma dieta rica em fitol em camundongos knockout para a enzima da fitanoil-CoA hidroxilase leva ao acúmulo de Fit, ácido fitênico e Prist e que o Fit causa aumento na expressão de enzimas da β-oxidação mitocondrial e peroxissomal via ativação do PPARα, causando modificações no metabolismo de ácidos graxos (Gloerich et al., 2005).

Embora várias observações indicam que defeitos no metabolismo energético mitocondrial, bem como produção aumentada de ERO estão envolvidas na toxicidade do Fit e do Prist em pacientes portadores de doenças com acúmulo desses ácidos graxos, os mecanismos subjacentes que mediam esses efeitos deletérios são pouco conhecidos.

I.1.7 Metabolismo Energético Cerebral

O cérebro é um dos órgãos mais ativos metabolicamente, entretanto possui reservas energéticas extremamente pequenas em relação a sua demanda energética metabólica (Dickinson 1996).

A glicose é o principal metabólito energético no cérebro (Erecińska e Silver 1994). Em condições normais o metabolismo energético nos tecidos neurais é mantido, quase que exclusivamente, pelo metabolismo oxidativo da glicose (Sokoloff 1993). A oxidação da glicose no cérebro ocorre mais rapidamente do que em outros órgãos como fígado, coração ou rins. Em contraste com outros tecidos, o cérebro não necessita de insulina para captar e oxidar a glicose. Entretanto, no jejum, corpos cetônicos podem substituir mais de 50% das necessidades energéticas cerebrais (Dickinson 1996).

A oxidação da glicose através da via glicolítica forma piruvato, que é convertido a CO_2 e H_2O no ciclo do ácido cítrico e na cadeia transportadora de elétrons. O acoplamento entre a cadeia transportadora de elétrons e a fosforilação oxidativa gera grande parte do ATP necessário ao cérebro (Erecińska e Silver 1994).

É bem conhecido que no cérebro a fosforilação oxidativa fornece em torno de 95% de todo o ATP sintetizado. O mecanismo que auxilia a manutenção dos níveis cerebrais de ATP é o sistema da creatina quinase. A creatina quinase está presente tanto no citosol quanto ligada às membranas mitocondriais e catalisa a transferência reversível de um fosfato entre a fosfocreatina e o ATP. O alto fluxo da reação na direção da síntese de ATP, em situações de consumo de ATP, indica que a reação é crucial para a manutenção de concentrações constantes dos substratos energéticos no citosol. O sistema creatina/fosfocreatina/creatina quinase tem sido associado a algumas funções particularmente importantes para o cérebro: tamponamento energético (através da regeneração do ATP a da manutenção de níveis baixos de ADP) e transferência de ATP de sítios de produção para outros de consumo (Erecińska e Silver 1994).

I.1.8 Metabolismo Energético e Doenças Neurodegenerativas

Numerosas hipóteses têm sido propostas para explicar a fisiopatologia das doenças de Alzheimer, Huntington e Parkinson, sem, no entanto, obter até o momento uma explicação satisfatória para o dano cerebral dessas doenças. Entretanto acredita-se que possíveis mecanismos envolvam deficiência no metabolismo energético, estresse oxidativo e neurotoxicidade mediada por receptores glutamatérgicos do tipo NMDA, ou, possivelmente, um somatório desses fatores (Rose e Henneberry 1994). Uma das hipóteses é de que alterações na cadeia transportadora de elétrons seria o evento etiológico primário na maioria dessas doenças (Parker et al., 1990; Swerdlow et al., 1998).

O cérebro é altamente dependente de energia para seu funcionamento normal e a mitocôndria é a estrutura intracelular que mantém os suprimentos de energia para o cérebro. Uma alteração funcional nessa estrutura pode levar, portanto, a alterações patológicas nos neurônios e astrócitos (Beal 1995; Bowling e Beal 1995; Davis et al., 1995). Mutações no DNA mitocondrial e reações envolvendo geração de espécies reativas podem danificar a mitocôndria e diminuir a atividade dos complexos da cadeia respiratória. Um prejuízo no transporte de elétrons, além de causar um prejuízo na produção de ATP, leva a uma dispersão dos elétrons na forma de radicais livres potencialmente danosos a célula.

Numerosas evidências relacionam doenças neurodegenerativas a uma diminuição no metabolismo energético. Estudos demonstraram uma diminuição na atividade do complexo I da cadeia respiratória em cérebros *postmortem* de pacientes portadores de doença de Parkinson (Janetzky et al., 1994; Schapira et al., 1989). Também há relatos de defeitos nos complexos II e III da cadeia respiratória e na enzima α-cetoglutarato desidrogenase, importante enzima do ciclo do ácido cítrico, nessa doença (Mizuno et al., 1990).

Na doença de Alzheimer, a mais comum dentre as doenças neurodegenerativas, é encontrada principalmente uma redução na atividade do complexo IV da cadeia respiratória (Maurer et al., 2000). Estudos em cérebros postmortem demostraram uma diminuição na atividade do complexo enzimático

da piruvato desidrogenase e na atividade da enzima α-cetoglutarato desidrogenase na doença de Alzheimer (Gibson et al., 1988; Mastrogiacomo et al., 1993; Perry et al., 1980).

I.2. OBJETIVOS

I.2.1 Objetivo geral

Investigar a influência dos ácidos fitânico e pristânico sobre vários parâmetros do metabolismo energético cerebral *in vitro* em córtex cerebral de ratos jovens.

I.2.2 Objetivos específicos

- Avaliar o efeito do ácido fitânico nas concentrações de 1, 10, 100 e 200μM sobre a produção de CO_2 a partir de D-[U-^{14}C] glicose (via glicolítica) e ácido [1-^{14}C] acético (ciclo do ácido cítrico) em homogeneizado de córtex cerebral de ratos jovens.
- Avaliar o efeito do ácido pristânico nas concentrações de 10, 50, 100 e 200 μM sobre a produção de CO_2 a partir de ácido [1-^{14}C] acético (ciclo do ácido cítrico) em homogeneizado de córtex cerebral de ratos jovens.
- Avaliar o efeito dos ácidos fitânico e pristâncio sobre a atividade dos complexos enzimáticos I, I-III, II, II-III e IV da cadeia respiratória em homogeneizado de córtex cerebral de ratos jovens.
- Avaliar o efeito dos ácidos fitânico e pristânico sobre a atividade total da enzima creatina quinase (CK) em homogeneizado de córtex cerebral de ratos jovens.

- Avaliar o efeito dos ácidos fitânico e pristânico sobre a atividade da enzima Na^+,K^+-ATPase em membrana sináptica de córtex cerebral de ratos jovens.

PARTE II

Artigos Científicos

Capítulo I

In vitro evidence that phytanic acid compromises Na $^{+}$, K $^{+}$ -ATPase activity and the electron flow through the respiratory chain in brain cortex from young rats

Estela Natacha Brandt Busanello, Carolina Maso Viegas, Alana Pimentel Moura, Anelise Miotti Tonin, Mateus Grings, Carmen Regla Vargas, Moacir Wajner

Artigo científico publicado no periódico

Brain Research 1352 (2010) 231 – 238

BRAIN RESEARCH 1352 (2010) 231–238

available at www.sciencedirect.com

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www.elsevier.com/locate/brainres

BRAIN RESEARCH

Research Report

In vitro evidence that phytanic acid compromises Na^+, K^+-ATPase activity and the electron flow through the respiratory chain in brain cortex from young rats

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ARTICLE INFO

Article history:
Accepted 5 July 2010
Available online 29 July 2010

Keywords:
Phytanic acid
Peroxisomal disorders
Energy metabolism
Neurotransmission

ABSTRACT

Phytanic acid (Phyt) tissue concentrations are increased in Refsum disease and other peroxisomal disorders characterized by neurologic damage and brain abnormalities. The present work investigated the in vitro effects of Phyt, at concentrations found in these peroxisomal disorders, on important parameters of energy metabolism in brain cortex of young rats. The parameters analyzed were CO_2 production from labeled acetate and glucose, the activities of the citric acid cycle enzymes citrate synthase, aconitase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase, fumarase and malate dehydrogenase, as well as of the respiratory chain complexes I–IV, creatine kinase and Na^+,K^+-ATPase. Our results show that Phyt did not alter citric acid cycle enzyme activities, or CO_2 production from acetate, reflecting no impairment of the functionality of the citric acid cycle. In contrast, respiratory chain activities were reduced at complexes I, II, I–III, II–III and IV. Membrane synaptical Na^+,K^+-ATPase activity was also reduced by Phyt, with no alteration of creatine kinase activity. Considering the importance of the electron flow through the respiratory chain for brain energy metabolism (oxidative phosphorylation) and of Na^+,K^+-ATPase activity for maintaining membrane potential necessary for neurotransmission, the data indicate that Phyt impairs brain bioenergetics at the level of energy formation, as well as neurotransmission. It is presumed that Phyt-induced impairment of these important systems may be involved at least in part in the neurological damage found in patients affected by disorders in which brain Phyt concentrations are increased.

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Abbreviations: ANOVA, one-way analysis of variance; BSA, bovine serum albumin; CAC, citric acid cycle; F, value with the degrees of freedom; α-KGDH, α-ketoglutarate dehydrogenase; Phyt, phytanic acid

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doi:10.1016/j.brainres.2010.07.012

1. Introduction

Phytanic acid, a chlorophyll-derived, is not produced in the human gut, but microorganisms present in the gastrointestinal system of ruminants can break down chlorophyll to release phytol, which is then converted into phytanic acid (Allen et al., 2008). This long branched-chain fatty acid accumulates in massive amounts in various peroxisomal disorders, including Refsum disease (Brosius and Gärtner, 2002; Wanders et al., 1993; Zomer et al., 2000).

Refsum disease, an inherited disorder characterized by defective peroxisomal α-oxidation of branched-chain fatty acids, is caused by a defect on the structural gene encoding the phytanoyl-CoA-hydroxylase, which catalyzes the first step of the peroxisomal α-oxidation pathway, i.e., the conversion of phytanoyl-CoA to 2-hydroxy-phytanoyl-CoA (Reiser et al., 2005). As a consequence, phytanic acid (3,7,11,15-tetramethyl-hexadecanoic acid, Phyt), which is found predominantly in red meat and dairy products, accumulates in plasma and other tissues of the affected individuals, reaching concentrations above 1000 μM (normal individuals: 30 μM) (Ferdinandusse et al., 2002).

The peroxisomal disorders, including Refsum disease, are clinically characterized by neurological symptoms and progressive white matter abnormalities with cortical atrophy (Brosius and Gärtner, 2002; Gould et al., 2001; Wanders et al., 2001). Although the pathophysiology of the brain damage in these disorders is poorly known, the observations that reduction of dietary phytol intake leads to a decrease of Phyt levels and delays the progression of the symptoms in patients affected by Refsum disease indicate that this branched-chain fatty acid is neurotoxic (Eldjarn et al., 1966; Ferdinandusse et al., 2008; Gibberd et al., 1979; Hungerbuhler et al., 1985; Masters-Thomas et al., 1980). In this context, it has been demonstrated that Phyt exerts cytotoxic effects mediated by mitochondrial impairment in cultured hippocampus astrocytes (Reiser et al., 2005). It was also shown that Phyt acts as a mitochondrial uncoupler, inhibits the electron flow through the respiratory chain and the adenine nucleotide exchange in rat brain (Reiser et al., 2006; Schonfeld et al., 2004). Furthermore, Phyt acts as a protonophore, decreasing ATP synthesis, the mitochondrial membrane potential and NAD(P)H content in digitonin-permeabilized fibroblasts (Komen et al., 2007). It was also observed that the incorporation of Phyt into the inner mitochondrial membrane impairs the H^+ conductance and disturbs the protein-linked functions in energy coupling (Schönfeld et al., 2004). On the other hand, it has been suggested that Phyt induces oxidative stress in mitochondria (Schönfeld and Reiser, 2006) and nitric oxide-dependent apoptosis in vascular smooth muscle cells (Idel et al., 2002). Finally, some studies demonstrated that Phyt induces Ca^{2+} increase, mitochondrial depolarization, superoxide generation and cell death in hippocampal neurons, astrocytes and oligodendrocytes (Kahlert et al., 2005; Ronicke et al., 2009).

In the present study we investigated the in vitro influence of Phyt on citric acid cycle activity — CAC (CO_2 production from [1-^{14}C] acetate and CAC enzyme activities), aerobic glycolysis (CO_2 production from [U-^{14}C] glucose), respiratory chain function (activities of chain complexes I–IV), cellular energy transfer (creatine kinase) and neurotransmission (Na^+, K^+-ATPase) in brain cortex preparations from young rats in the hope to elucidate novel mechanisms of neurotoxicity of Phyt.

2. Results

2.1. *Phytanic acid does not alter citric acid cycle (CAC) function in rat cerebral cortex*

We first observed that Phyt, at concentrations as high as 200 μM, did not alter citrate synthase, aconitase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase, fumarase or malate dehydrogenase activities in rat cortical homogenates (Table 1), suggesting that it does not affect the CAC function. This hypothesis was reinforced by the observations that this branched-chain fatty acid also did not modify CO_2 production from the labeled substrates [U-^{14}C] acetate and [U-^{14}C] glucose (Fig. 1).

2.2. *Phytanic acid inhibits respiratory chain complexes I–IV activities in rat cerebral cortex*

The next step of our investigation was designed to evaluate the effect of Phyt on various respiratory chain complex activities in an attempt to elucidate whether the electron transfer flow could be compromised by this compound. We found that Phyt significantly inhibited complexes I (up to 70%) [$F_{(4,14)}=40.62$, $P<0.001$], II (up to 65%) [$F_{(5,18)}=19.88$ $P<0.001$], I–III (up to 40%) [$F_{(5,18)}=5.62$, $P<0.01$], II–III (up to 80%) [$F_{(5,30)}=27.50$, $P<0.001$] and IV activities (up to 34%) [$F(4,39)=5.38$, $P<0.01$] in a dose-dependent manner: complexes I [$\beta=-0.951$; $P<0.001$], II [$\beta=-0.884$; $P<0.001$], I–III [$\beta=-0.735$; $P<0.01$], II–III [$\beta=-0.833$; $P<0.001$] and IV [$\beta=-0.513$; $P<0.01$] (Fig. 2). These data strongly indicate that Phyt compromises respiratory chain function.

2.3. *Phytanic acid does not alter creatine kinase activity in rat cerebral cortex*

We then examined the effect of Phyt on creatine kinase (CK) activity, a crucial enzyme necessary for intracellular ATP transfer, in rat cerebral cortex. This parameter was not altered by Phyt at concentrations as high as 200 μM (Table 2). It seems therefore presumed that intracellular energy transfer is not mainly affected by Phyt.

2.4. *Phytanic acid inhibits Na^+,K^+-ATPase activity in synaptic plasma membranes from rat cerebral cortex*

Finally, we tested the influence of Phyt on Na^+,K^+-ATPase activity in synaptic plasma membranes prepared from cerebral cortex. Fig. 3 shows that purified synaptic membrane preparations' exposition to Phyt resulted in a significant inhibition of Na^+,K^+-ATPase activity (up to 59%) [$F_{(4,30)}=16.98$, $P<0.001$] in a dose-dependent manner [$\beta=-0.822$; $P<0.001$]. These results indicate that neurotransmission, which depends on normal Na^+,K^+-ATPase activity, is possibly impaired by Phyt.

Table 1 – Effect of phytanic acid (Phyt) on citric acid cycle enzyme activities in enriched mitochondrial fractions from rat cerebrum.

	Citrate sintase	Aconitase	Isocitrate dehydrogenase	α-Ketoglutarate dehydrogenase	Succinate dehydrogenase	Fumarase	Malate dehydrogenase
Control	747 ± 65.6	756 ± 160	131 ± 6.15	7.86 ± 2.18	11.2 ± 1.24	10.6 ± 0.55	37.9 ± 1.02
200 μM Phyt	740 ± 83.8	725 ± 132	152 ± 12.3	8.17 ± 2.45	12.0 ± 2.11	13.2 ± 1.97	37.4 ± 0.83

Values are mean ± standard deviation for four to five independent (animals) experiments per group. The activity of isocitrate dehydrogenase and malate dehydrogenase are expressed as nmol NADH min^{-1} mg $protein^{-1}$, whereas aconitase, α-ketoglutarate dehydrogenase, succinate dehydrogenase and fumarase activities are expressed as mM NADPH min^{-1} mg $protein^{-1}$, mM NADH min^{-1} mg $protein^{-1}$, nmol DCIP min^{-1} mg $protein^{-1}$ and nmol fumarate min^{-1} mg $protein^{-1}$, respectively. No significant differences were detected (Student *t* test for paired samples).

3. Discussion

Phyt, which is found in great amounts in Refsum disease and other peroxisomal disorders, was shown to inhibit the electron flow through the respiratory chain and induce generation of reactive species (Ferdinandusse et al., 2008; Wierzbicki, 2007; Wills et al., 2001). In the present study we investigated the effects of Phyt, whose tissue concentrations are increased in various peroxisomal diseases, on important parameters of energy homeostasis and neurotransmission in cerebral cortex preparations from young rats in order to better elucidate the mechanisms underlying the pathogenesis of the brain damage of patients affected by these disorders. We tested the effects of Phyt, at concentrations found in Refsum disease, on citric acid cycle (CAC) function, by measuring CAC enzyme activities and CO_2 production from acetate, on the electron flow through the respiratory chain by determining complex I–IV activities, on the intracellular ATP transfer by assessing creatine kinase activity and on the maintenance of the cell membrane potential necessary to a normal neurotransmission activity, by measuring membrane synaptic Na^+, K^+-ATPase activity.

We verified that Phyt did not change any of the CAC enzyme activities and CO_2 production from acetate, indicating that this branched-chain fatty acid does not affect CAC functioning in brain cortex. Similarly, the glycolytic pathway, assessed by CO_2 production from glucose, was also not impaired by Phyt. It should be however noted that a previous publication has shown

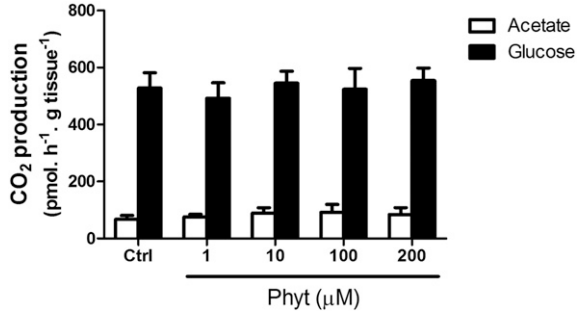

Fig. 1 – Effect of phytanic acid (Phyt) on $^{14}CO_2$ production from [U-^{14}C] glucose and [1-^{14}C] acetate in rat cerebral cortex. Values are mean ± standard deviation for five to nine independent experiments (animals) per group and expressed as pmol $^{14}CO_2$ h^{-1} g $tissue^{-1}$.

that aconitase activity is inhibited by Phyt probably through reactive species (Schönfeld and Reiser, 2006) since this enzyme is vulnerable to free radical attack (Chepelev et al., 2009; Kachadourian et al., 2001; Tretter and Adam-Vizi, 2000). The apparent discrepancy between this study and our present results may be possibly attributed to differences in methodology in view of the fact that the reaction medium that we used to measure aconitase activity contains the antioxidant cystein (Morrison, 1954) in the incubation reaction, which protects essential groups of the enzyme from oxidation, whereas the method used by Schönfeld and Reiser (2006) does not have cystein or any other antioxidant in the medium. Our present results therefore indicate that Phyt does not directly inhibit aconitase.

In contrast, Phyt markedly inhibited the activities of various respiratory chain complexes (I, II, I–III, II–III and IV), implying a drop of the electron transport chain flow and consequently of ATP produced in the cell. These results obtained with cerebral cortex of young rats are in agreement with previous reports showing that oxidative phosphorylation is compromised by Phyt in hippocampus and cerebral cortex (Kahlert et al., 2005; Schönfeld et al., 2004; Schönfeld and Reiser, 2006). It should be noted that Schönfeld and Reiser (2006) only analyzed complexes I and III of the respiratory chain and found that Phyt inhibited these activities, whereas we assessed activities of complexes and pathways I to IV and observed inhibitions of complex I and at other sites of the electron transfer flow.

Taken together, it is concluded that the first step of aerobic cellular respiration, in which electrons are released from energetic substrates (glycolytic pathway and CAC), is not compromised by Phyt. In contrast, the transference of these electrons through the redox pairs of the respiratory chain with simultaneous transference of energy to form ATP (oxidative phosphorylation) is severely impaired by this branched-chain fatty acid.

We also observed that creatine kinase activity, a crucial enzyme activity involved in intracellular ATP transfer, was not modified by this branched long chain fatty acid, indicating that energy transfer through the mitochondrial and cytosolic compartments is probably not affected by Phyt.

An interesting observation of the present study was the marked inhibition (60 %) of synaptic Na^+,K^+-ATPase activity provoked by Phyt, which may compromise neurotransmission since this enzyme is mainly responsible for the generation of the membrane potential necessary to maintain neuronal excitability (Erecinska et al., 2004; Erecińska and Silver, 1994; Wheeler et al., 1975). We cannot attribute the Phyt-induced

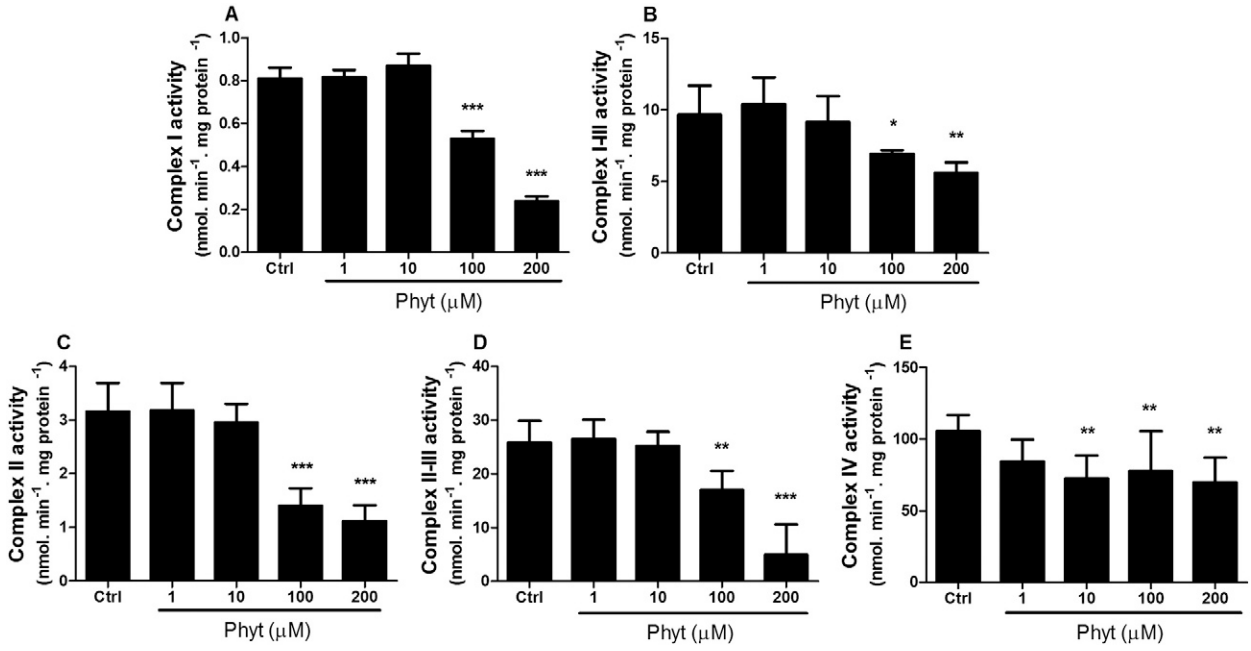

Fig. 2 – Effect of phytanic acid (Phyt) on the activities of the respiratory chain complexes I–IV in rat cerebral cortex. Values are mean ± standard deviation for four to nine independent experiments (animals) per group. The activity of complex I (*A*) is expressed as nmol ferricyanete reduced min^{-1} mg $protein^{-1}$, complex II (*B*) as nmol DCIP reduced min^{-1} mg $protein^{-1}$ and complex I–III (*C*) as nmol cytochrome *c* reduced min^{-1} mg $protein^{-1}$. The activities of complexes II–III (*D*) and IV (*E*) are expressed, respectively, as nmol cytochrome *c* reduced min^{-1} mg $protein^{-1}$ and nmol cytochrome *c* oxidized min^{-1} mg $protein^{-1}$. *P < 0.05, **P < 0.01 and ***P < 0.001 compared to control (Duncan multiple range test).

reduction of Na^+,K^+-ATPase activity to shortage of ATP since the in vitro assay that measures this activity is carried out with excess of ATP in the incubation medium.

We could tentatively attribute the inhibition of synaptic membrane Na^+,K^+-ATPase activity to alterations of membrane lipid arrangement and/or of interactions between proteins and lipids caused by this fatty acid since Phyt may be incorporated into cell membranes (Steinberg, 1995). Alternatively, since this enzyme is highly susceptible to free radical attack (Hitschke et al., 1994; Kurella et al., 1997; Muriel and Sandoval, 2000), we cannot rule out that Na^+,K^+-ATPase activity reduction could be indirectly caused through reactive species formation elicited by Phyt (Schönfeld and Reiser, 2006; Schönfeld and Wojtczak, 2007). At this point it should be emphasized that lower activity of Na^+,K^+-ATPase activity may compromise neuronal function and represent an important neurotoxic mechanism to neurons (Lees, 1991; Lees, 1993; Satoh and Nakazato, 1992; Sweadner, 1979).

Table 2 – Effect of phytanic acid (Phyt) on the activity of creatine kinase in rat cerebrum homogenates.

	Creatine kinase activity
Control	1.74 ± 0.71
200 μM Phyt	1.97 ± 0.61

Values are mean ± standard deviation for six independent (animals) experiments per group. The activity of creatine kinase is expressed as mmol of creatine min^{-1} mg $protein^{-1}$. No significant differences were detected (Student's *t*-test for paired samples).

We cannot precisely determine the pathophysiological relevance of our data. However, considering that the striking alterations of the electron transfer chain and Na^+,K^+-ATPase activity, which are necessary for normal brain functioning, were obtained at concentrations of Phyt similar or even lower than those found in plasma of patients affected by Refsum disease (Ferdinandusse et al., 2008), it is feasible that our present results may be of pathological significance.

In conclusion, this is the first report showing that Phyt markedly decreases in vitro the activity of synaptic Na^+,K^+-ATPase, a

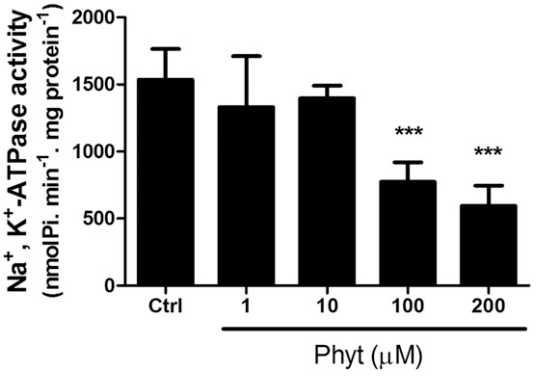

Fig. 3 – Effect of phytanic acid (Phyt) on Na^+,K^+-ATPase activity in purified synaptic plasma membranes prepared from rat cerebrum homogenates. Values are mean ± standard deviation of six independent experiments (animals) performed in triplicate and are expressed as nmol Pi min^{-1} mg $protein^{-1}$. ***P < 0.001 compared to control (Duncan multiple range test).

crucial enzyme necessary for normal neurotransmission. This work also found that Phyt blocks the electron transfer at various sites of the respiratory chain in brain cortex, in accordance with previous studies performed in hippocampus. It is expected that further studies carried out in vivo in mouse models for peroxisomal diseases in which Phyt accumulates would improve our knowledge on the pathogenesis of these disorders (Ferdinandusse et al., 2008).

4. Experimental procedures

4.1. Reagents

All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA, except for [U-^{14}C] glucose and [1-^{14}C] acetate, which were purchased from Amersham International plc, UK. Phytanic acid was first dissolved in methanol and then on the day of the experiments in the buffer used for each assay with pH adjusted to 7.4, so that the maximal concentration of methanol was 1%, which was previously shown not to alter any of the measured parameters.

4.2. Animals

Thirty-day-old Wistar rats obtained from the Central Animal House of the Department of Biochemistry, ICBS, UFRGS, were used in the assays. The animals had free access to water and to a standard commercial chow and were maintained on a 12:12 h light/dark cycle in an air-conditioned constant temperature (22 ± 1 °C) colony room. The "Principles of Laboratory Animal Care" (NIH publication no. 80-23, revised 1996) were followed in all experiments and the experimental protocol was approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. All efforts were made to minimize the number of animals used and their suffering.

4.3. Cerebral preparations

The animals were sacrificed by decapitation, the brain was rapidly removed and the cerebral cortex was isolated. For CO_2 production, the cerebral cortex was homogenized (1:10, w/v) in Krebs–Ringer bicarbonate buffer, pH 7.4. For the determination of the activities of the respiratory chain complexes I–III, II, II–III and IV, cerebral cortex was homogenized (1:20, w/v) in SETH buffer, pH 7.4 (250 mM sucrose, 2.0 mM EDTA, 10 mM Trizma base and 50 UI mL^{-1} heparin). The homogenates were centrifuged at 800×*g* for 10 min and the supernatants were kept at −70 °C until being used for enzyme activity determination. For the determination of the activities of citric acid cycle enzymes and complex I of the respiratory chain, mitochondrial enriched fractions from cerebrum were prepared according to Rosenthal et al. (1987), with slight modifications. For total creatine kinase activity determination, the cerebral cortex was homogenized (1:10 w/v) in isosmotic saline solution. The period between tissue preparation and measurement of the various parameters was always less than 5 days, except for complex I activity, citric acid cycle enzymatic and CO_2 production assays, which were performed on the same day of the preparations. We applied approximately 0.01 to 1 mg protein in the assays with homogenates, 0.1 mg protein for complex I activity that uses mitochondrial fractions and up to 15 mg protein in the techniques measuring the citric acid cycle enzyme activities.

4.4. Preparation of synaptic plasma membrane from rat cerebrum

Tissue was homogenized in 10 volumes of 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA. Synaptical plasma membranes were prepared afterwards according to the method of Jones and Matus (1974) using a discontinuous sucrose density gradient consisting of successive layers of 0.3, 0.8 and 1.0 mM. After centrifugation at 69,000×*g* for 2 h, the fraction at the 0.8–1.0 mM sucrose interface was taken as the membrane enzyme preparation.

The biochemical parameters were determined in the presence of various concentrations of Phyt (1, 10, 100 and 200 μM), whereas control groups did not contain this fatty acid in the incubation medium. We always carried out parallel experiments with various blanks (controls) in the presence or absence of Phyt and also with or without brain preparations in the reaction medium in order to detect any interference (artifact) of this fatty acid on the techniques utilized to measure the biochemical parameters.

4.5. Spectrophotometric analyses of the activities of citric acid cycle enzymes

The activities of the enzymes of the citric acid cycle (CAC) were determined using enriched mitochondrial fractions from cerebrum. Phyt was supplemented to the medium and brain supernatants were pre-incubated at 37 °C for 30 min.

Citrate synthase activity was measured according to Srere (1969), by determining DTNB reduction at λ = 412 nm. The activity of the enzyme aconitase was measuring according to Morrison (1954), following the reduction of $NADP^+$ at wavelengths of excitation and emission of 340 and 466 nm, respectively. Isocitrate dehydrogenase activity was accessed by the method of Plaut (1969), by following NAD^+ reduction at wavelengths of excitation and emission of 340 and 466 nm, respectively. The activity of α-ketoglutarate dehydrogenase complex was evaluated according to Viegas et al. (2009). The reduction of NAD^+ was recorded in a Hitachi F-4500 spectrofluorometer at wavelengths of excitation and emission of 340 and 466 nm, respectively. The activity of succinate dehydrogenase was determined as described by Fischer et al. (1985). Fumarase activity was measured according to O'Hare and Doonan (1985), measuring the increase of absorbance at λ = 250 nm. Malate dehydrogenase activity was measured according to Kitto (1969) by following the reduction of NADH at wavelengths of excitation and emission of 340 and 466 nm, respectively. The activities of the citric acid cycle enzymes were calculated as nmol min^{-1} mg protein^{-1}, mmol min^{-1} mg protein^{-1} or μmol min^{-1} mg protein^{-1}.

4.6. CO_2 production

Homogenates prepared in Krebs–Ringer bicarbonate buffer, pH 7.4, were added to small flasks (11 cm^3) in a volume of 0.45 mL. Flasks were pre-incubated at 35 °C for 30 min in the presence of Phyt (1–200 μM) in a metabolic shaker (90 oscillations min^{-1}) with 625 μM *n*-dodecyl-β-D-maltoside in order to permeabilize the

mitochondrial membranes. Controls did not contain the metabolite in the incubation medium. After pre-incubation, [U-^{14}C] glucose (0.055 μCi) plus 5.0 mM of unlabeled glucose or 0.055 μCi [1-^{14}C] acetate plus 1.0 mM of unlabeled acetate were added to the incubation medium. The flasks were gassed with an O_2/CO_2 (95:5) mixture and sealed with rubber stoppers Parafilm M. Glass center wells containing a folded 60 nm/4 nm piece of Whatman 3 filter paper were hung from the stoppers. After 60 min incubation at 35 °C in a metabolic shaker (90 oscillations min^{-1}), 0.2 mL of 50% trichloroacetic acid was supplemented to the medium and 0.1 mL of benzethonium hydroxide was added to the center of the wells with needles introduced through the rubber stopper. The flasks were left to stand for 30 min to complete CO_2 trapping and then opened. The filter paper were removed and added to vials containing scintillation fluid, and radioactivity was counted (Reis de Assis et al., 2004). Results were calculated as pmol CO_2 h^{-1} g tissue^{-1}.

4.7. *Spectrophotometric analysis of the respiratory chain complexes I–IV activities*

The activity of NADH-dehydrogenase (complex I) was determined in mitochondrial preparations from cerebrum according to Cassina and Radi (1996). The activities of succinate-2,6-dichloroindophenol (DCIP)-oxidoreductase (complex II) and succinate:cytochrome c oxidoreductase (complex II–III) were determined in homogenates from cerebral cortex according to Fischer et al. (1985). The activity of NADH:cytochrome c oxidoreductase (complex I–III) was assayed in cerebral cortex homogenates according to the method described by Schapira et al. (1990) and that of cytochrome c oxidase (complex IV) according to Rustin et al. (1994). The methods described to measure these activities were slightly modified, as described in details in a previous report (da Silva et al., 2002). Phyt (1–200 μM) was added to the reaction medium at the beginning of the assays, while no metabolite was added to controls. The activities of the respiratory chain complexes were calculated as nmol min^{-1} · mg protein^{-1} or mmol min^{-1} mg protein^{-1}.

4.8. *Spectrophotometric analysis of creatine kinase activity*

Creatine kinase (CK) activity was measured in total homogenates according to Hughes (1962) with slight modifications (Schuck et al., 2002). Briefly, the reaction mixture consisted of 50 mM Tris buffer, pH 7.5, containing 7.0 mM phosphocreatine, 7.5 mM $MgSO_4$, and 0.5–1.0 μg protein in a final volume of 0.1 mL. Phyt (1–200 μM) was supplemented to the medium and submitted to a pre-incubation at 37 °C for 30 min. The reaction was then started by addition of 4.0 mM ADP and stopped after 10 min by addition of 0.02 mL of 50 mM *p*-hydroxy-mercuribenzoic acid. The creatine formed was estimated according to the colorimetric method of Hughes (1962). The color was developed by the addition of 0.1 mL 20% α-naphtol and 0.1 mL 20% diacetyl in a final volume of 1.0 mL and read after 20 min at λ = 540 nm. Results were calculated as μmol of creatine min^{-1} mg protein^{-1}.

4.9. *Spectrophotometric analysis of Na^+,K^+-ATPase activity*

The reaction mixture for the Na^+,K^+-ATPase assay contained 5 mM $MgCl_2$, 80 mM NaCl, 20 mM KCl, 40 mM Tris–HCl buffer, pH 7.4, and purified synaptic membranes (approximately 3 μg of protein) in a final volume of 200 μL. The enzymatic assay occurred at 37 °C during 5 min and started by the addition of ATP (disodium salt, vanadium free) to a final concentration of 3 mM. The reaction was stopped by the addition of 200 μL of 10% trichloroacetic acid. Mg^{2+}-ATPase ouabain-insensitive was assayed under the same conditions with the addition of 1 mM ouabain. Na^+,K^+-ATPase activity was calculated by the difference between the two assays (Tsakiris and Deliconstantinos, 1984). Released inorganic phosphate (Pi) was measured by the method of Chan et al. (1986). Enzyme-specific activities were calculated as nmol Pi released^{-1} min^{-1} mg protein^{-1}.

4.10. *Protein determination*

Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard.

4.11. *Statistical analysis*

Unless otherwise stated, results are presented as mean ± standard deviation. Assays were performed in duplicate or triplicate and the mean or median was used for statistical analysis. Data was analyzed using one-way analysis of variance (ANOVA) followed by the post-hoc Duncan multiple range test when *P* was significant. Linear regression was also used to detect dose-dependent effects. The Student *t* test for paired samples was used for comparison of two means. Only significant F and *t* values are shown in the text. Differences between groups were rated significant at $P<0.05$. All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.

Acknowledgments

We are grateful to the financial support of CNPq, PROPESq/UFRGS, FAPERGS, PRONEX, FINEP Rede Instituto Brasileiro de Neurociência (IBN-Net) # 01.06.0842-00, and INCT-EN.

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Capítulo II

Neurochemical evidence that pristanic acid impairs energy production and inhibits synaptic Na^+,K^+-ATPase activity in brain of young rats

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Artigo científico submetido para publicação no periódico

Journal of Neural Transmission

Neurochemical evidence that pristanic acid impairs energy production and inhibits synaptic Na^+,K^+-ATPase activity in brain of young rats

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Number of pages: 20

Number of Figures: 3

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Abstract

Pristanic acid (Prist) accumulates in some peroxisomal abnormalities characterized by neurologic dysfunction and brain abnormalities. The present work investigated the *in vitro* effects of Prist, at concentrations found in these disorders, on important parameters of energy metabolism in brain cortex of young rats. The parameters analyzed were CO_2 production from labeled acetate, as well as the activities of the respiratory chain complexes I-IV, creatine kinase and synaptic Na^+,K^+-ATPase. Our results show that Prist significantly decreased CO_2 production from acetate, reflecting an impairment of the functionality of the citric acid cycle. Prist also inhibited the activities of the respiratory chain complexes I, II, II-III, but not of IV. Membrane synaptical Na^+,K^+-ATPase activity was also reduced by Prist, with no alteration of creatine kinase activity. Considering the importance of the citric acid cycle and the electron flow through the respiratory chain for brain energy production (oxidative phosphorylation) and of Na^+,K^+-ATPase activity for the maintenance of the membrane potential for neurotransmission, the data indicate that Prist compromises brain bioenergetics and neurotransmission. It is presumed that the impairment of these important systems necessary for normal brain development and function may be involved at least in part in the neurological damage found in patients affected by disorders in which brain Prist concentrations are increased.

Keywords: pristanic acid, energy metabolism, peroxisomal disease, rat brain.

1. Introduction

Pristanic acid (2,6,10,14-tetramethyl pentadecanoic acid, Prist) is the α-oxidation product of phytanic acid (3,7,11,15-tetramethylhexadecanoic acid, Phyt) and can be obtained from dietary sources (Wanders et al. 2001). Fatty acid metabolism is known to occur mainly via β-oxidation in mitochondria and peroxisomes. Phyt, a product from chlorophyll, is converted into Prist through the peroxisomal α-oxidation and then degraded via three cycles of β- oxidation in peroxisomes, followed by β-oxidation in mitochondria (Verhoeven et al. 1998). Phyt and Prist accumulate in a variety of inherited disorders caused by defects in the peroxisomal biogenesis or by single enzymatic peroxisomal defects in α or β-oxidation.

The most severe of the diseases of peroxisome biogenesis is Zellweger syndrome, a polymalformation condition, affecting the development of brain, liver, kidney and skeleton, in which both Phyt and Prist concentrations are elevated in plasma and tissues from the affected patients (Verhoeven and Jakobs 2001).

On the other hand, deficiency of α-methylacyl-CoA racemase (AMACR) due to impaired peroxisomal β-oxidation of Prist leads to large accumulation of this fatty acid and bile acid intermediates, and only mild elevations in Phyt. AMACR deficiency has been described in several patients, mainly adults with a late-onset sensorimotor neuropathy, epilepsy, encephalopathy, pyramidal tract signs and migraine (Ferdinandusse et al. 2000; Ferdinandusse et al. 2002; McLean et al. 2002; Smith et al. 2010; Thompson et al. 2008). AMACR

deficiency can also appear in the neonatal period with coagulopathy and mild cholestasis due to bile acid abnormalities (Setchell et al. 2003).

Deficiency of the D-bifunctional protein that catalizes the first cycle of Prist peroxisomal β-oxidaton is another enzyme defect leading to increased concentrations of Prist, bile acid intermediates and very long-chain fatty acids. Affected patients present dysmorfic features, severe central nervous system involvement, seizures and developmental disorders (Verhoeven and Jakobs 2001).

A few studies have demonstrated that Prist and Fit are deleterious to cells. Thus, it was shown that Prist exerts a strong cytotoxic activity on brain cells, deregulating intracellular Ca^{2+} concentrations and causing mitochondrial depolarization and cell death. It was also shown that Prist strongly induces generation of reactive oxygen species (ROS) in neural cell cultures (Rönicke et al. 2009). In addition, it was seen that Phyt and Prist activate the inducible nitric oxide synthase (iNOS) in endothelial cells, leading to increased production of the reactive nitrogen oxide (NO) and to apoptotic cell death via secretion of tumor necrosis factor α (TNFα) (Idel et al. 2002). We also recently found that Phyt induces lipid and protein oxidative damage and reduces antioxidant defenses in the brain (Leipnitz et al. 2010), besides compromising brain bioenergetics (Busanello et al. 2010).

Although Prist exerts strong cytotoxic effects on brain cells, the exact underlying mechanisms provoked by this fatty acid on brain bioenergetics is practically unknown. Therefore, in the present study we investigated the effects of Prist on the citric acid cycle (CAC) functionality, as well as on creatine kinase and Na^+,K^+-ATPase activities in cerebral cortex, which is mainly affected in

peroxisomal pathologies. We evaluated the *in vitro* influence of Prist on CO_2 production from [1-^{14}C] acetate (CAC), as well as on the respiratory chain complexes I-IV (respiratory chain), creatine kinase (cellular energy transfer) and Na^+,K^+-ATPase (neurotransmission) activities in brain cortex preparations from young rats in the hope to clarify the neurotoxic effects of Prist.

2. Experimental procedures

2.1 Reagents

All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA, except for [1-^{14}C] acetate, which was purchased from Amersham International plc, UK.

2.2 Animals

Thirty-day-old Wistar rats obtained from the Central Animal House of the Department of Biochemistry, ICBS, UFRGS, were used in the assays. The animals had free access to water and to a standard commercial chow and were maintained on a 12:12 h light/dark cycle in an air-conditioned constant temperature (22 ± 1°C) colony room. The “Principles of Laboratory Animal Care” (NIH publication no. 80-23, revised 1996) were followed in all experiments and the experimental protocol was approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. All efforts were made to minimize the number of animals used and their suffering.

2.3 Cerebral preparations

The animals were sacrificed by decapitation, the brain was rapidly removed and the cerebral cortex was isolated. For CO_2 production, the cerebral cortex was homogenized (1:10, w/v) in Krebs-Ringer bicarbonate buffer, pH 7.4. For the determination of the activities of the respiratory chain complexes II, II-III and IV, cerebral cortex was homogenized (1:20, w/v) in SETH buffer, pH 7.4 (250 mM sucrose, 2.0 mM EDTA, 10 mM Trizma base and 50 UI. mL^{-1} heparin). The homogenates were centrifuged at 800 x *g* for 10 min and the supernatants were kept at -70°C until being used for enzyme activity determinations. For the determination of complex I of the respiratory chain, mitochondrial enriched fractions from cerebrum were prepared according to Rosenthal et al. (1987), with slight modifications. For total creatine kinase activity determination, the cerebral cortex was homogenized (1:10 w/v) in isosmotic saline solution. The period between tissue preparation and measurement of the various parameters was always less than 5 days, except for complex I activity and CO_2 production assays, which were performed on the same day of the preparations.

2.4 Preparation of synaptic plasma membrane from rat cerebrum

Tissue was homogenized in 10 volumes of 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA. Synaptical plasma membranes were prepared afterwards according to the method of Jones and Matus (1974) using a discontinuous sucrose density gradient consisting of successive layers of 0.3, 0.8 and 1.0 mM. After centrifugation at 69,000 x *g* for 2 h, the fraction at the 0.8-1.0 mM sucrose interface was taken as the membrane enzyme preparation.

The biochemical parameters were determined in the presence of various concentrations of Prist (10, 50, 100 and 200 µM). Prist was dissolved on the day of the experiments in the buffer used for each technique with pH adjusted to 7.4. We always carried out parallel experiments with various blanks (controls) in the presence or absence of Prist and also with or without brain preparations in the reaction medium in order to detect any interference (artifact) of this fatty acid on the techniques utilized to measure the biochemical parameters.

2.5 CO_2 production

Homogenates prepared in Krebs-Ringer bicarbonate buffer, pH 7.4, were added to small flasks (11 cm^3) in a volume of 0.45 mL. Flasks were pre-incubated at 35°C for 30 min in a metabolic shaker (90 oscillations. min^{-1}) with 625 µM *n*-dodecyl-β-D-maltoside in order to permeabilize the mitochondrial membranes. After pre-incubation, 0.055 µCi [1-^{14}C] acetate plus 1.0 mM of unlabeled acetate were added to the incubation medium. In some experiments, the incubation medium also contained 1.0 mM coenzyme A. The flasks were gassed with a O_2/CO_2 (95:5) mixture and sealed with rubber stoppers Parafilm M. Glass center wells containing a folded 60 nm/4 nm piece of Whatman 3 filter paper were hung from the stoppers. After 60 min incubation at 35°C in a metabolic shaker (90 oscillations. min^{-1}), 0.2 mL of 50% trichloroacetic acid was supplemented to the medium and 0.1 mL of benzethonium hydroxide was added to the center of the wells with needles introduced through the rubber stopper. The flasks were left to stand for 30 min to complete CO_2 trapping and then opened. The filter papers were removed and added to vials containing

scintillation fluid, and radioactivity was counted (Reis de Assis et al. 2004). Results were calculated as ρmol CO_2 . h^{-1}. g $tissue^{-1}$.

2.6 Spectrophotometric analysis of the respiratory chain complexes I-IV activities

The activity of NADH-dehydrogenase (complex I) was determined in mitochondrial preparations from cerebrum according to Cassina and Radi (1996). The activities of succinate-2,6-dichloroindophenol (DCIP)-oxidoreductase (complex II) and succinate:cytochrome *c* oxidoreductase (complex II-III) were determined in homogenates from cerebral cortex according to Fischer et al (1985). The activity of cytochrome *c* oxidase (complex IV) was assayed in cerebral cortex homogenates according to the method described by Rustin et al (1994). The methods described to measure these activities were slightly modified, as described in details in a previous report (da Silva et al. 2002). The activities of the respiratory chain complexes were calculated as nmol . min^{-1}. mg $protein^{-1}$ or mmol . min^{-1}. mg $protein^{-1}$.

2.7 Spectrophotometric analysis of creatine kinase activity

Creatine kinase (CK) activity was measured in total homogenates according to Huges (1962) with slight modifications (Tonin et al. 2009). Briefly, the reaction mixture consisted of 50 mM Tris buffer, pH 7.5, containing 7.0 mM phosphocreatine, 7.5 mM $MgSO_4$, and 0.5-1.0 μg protein in a final volume of 0.1 mL. Prist was supplemented to the medium and submitted to pre-incubation for 30 min at 37ºC. The reaction was then started by addition of 4.0 mM ADP and stopped after 10 min by addition of 0.02 mL of 50 mM *p*-hydroxy-

mercuribenzoic acid. The creatine formed was estimated according to the colorimetric method of Huges (1962). The color was developed by the addition of 0.1 mL 20% α-naphtol and 0.1 mL 20% diacetyl in a final volume of 1.0 mL and read after 20 min at λ= 540 nm. Results were calculated as μmol of creatine. min^{-1}. mg $protein^{-1}$.

2.8 Spectrophotometric analysis of Na^+, K^+-ATPase activity

The reaction mixture for the Na^+, K^+-ATPase assay contained 5 mM $MgCl_2$, 80 mM NaCl, 20 mM KCl, 40 mM Tris–HCl buffer, pH 7.4, and purified synaptic membranes (approximately 3 μg of protein) in a final volume of 200 μL. The enzymatic assay occurred at 37 ºC during 5 min and started by the addition of ATP (disodium salt, vanadium free) to a final concentration of 3 mM. The reaction was stopped by the addition of 200 μL of 10% trichloroacetic acid. In some experiments, the incubation medium also contained 200 μM GSH. Mg^{2+}-ATPase ouabain-insensitive was assayed under the same conditions with the addition of 1 mM ouabain. Na^+, K^+-ATPase activity was calculated by the difference between the two assays (Tsakiris and Deliconstantinos 1984). Released inorganic phosphate (Pi) was measured by the method of Chan et al (1986). Enzyme-specific activities were calculated as nmol Pi released $^{-1}$. min $^{-1}$ mg $protein^{-1}$.

2.9 Protein determination

Protein was measured by the method of Lowry et al (1951) using bovine serum albumin as standard.

2.10 Statistical analysis

Unless otherwise stated, results are presented as mean ± standard deviation. Assays were performed in duplicate or triplicate and the mean or median was used for statistical analysis. Data were analyzed using one-way analysis of variance (ANOVA) followed by the post-hoc Duncan multiple range test when *F* was significant. Linear regression was also used to detect dose-dependent effects. The Student *t* test for paired samples was used for comparison of two means. Only significant F and *t* values are shown in the text. Differences between groups were rated significant at $P < 0.05$. All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.

3.Results

3.1 Pristanic acid inhibits citric acid cycle (CAC) function in rat cerebral cortex

The effect of Prist on CO_2 production from labeled acetate was first investigated in rat cortical homogenates. Figure 1A shows that CO_2 production from [1-^{14}C] acetate was significantly inhibited by Prist (up to 25%) [$F_{(4,23)} = 3.76$, $P<0.001$]. In addition, Prist-induced inhibition of CO_2 formation did not change when 1.0 mM coenzyme A (CoA) was added to the medium [$F_{(3,18)} = 3.59$; $P<0.05$] (Figure 1B), ruling out a shortage of (CoA) due to a possible competition between Prist and acetate for the generation of acetyl-CoA. These data indicate a reduction of citric cycle acid (CAC) function caused by Prist possibly due to an inhibitory effect of this acid on one or more enzymatic steps of the CAC or secondary to a blockage of the respiratory chain enzyme complexes.

3.2 Pristanic acid inhibits respiratory chain complex activities in rat cerebral cortex

The next step of our investigation was designed to evaluate the effect of Prist on various respiratory chain complex activities in an attempt to elucidate whether the electron transfer flow could be compromised by this compound. We found that Prist significantly inhibited complexes I (up to 65%) [$F_{(4,15)}$ = 201.5, *P*<0.001], II (up to 40%) [$F_{(4,25)}$ = 10.56 *P*<0.001], II-III (up to 95%) [$F_{(4,25)}$ = 159.6 *P*<0.001] in a dose dependent manner (complexes I [β=-0.964; *P*<0.001], II [β=-0.626; *P*<0.001], and II-III [β=-0.835; *P*<0.001]). In contrast, the activity of complex IV was not altered by Prist (Figure 2). Taken together, it is concluded that this fatty acid markedly compromises the respiratory chain function.

3*.3 Pristanic acid does not alter creatine kinase activity in rat cerebral cortex*

We then examined the effect of Prist on creatine kinase (CK) activity in homogenates from rat cerebral cortex. This parameter was not altered by 200 □M Prist (mmol of creatine. min-^{1}. mg protein^{-1}; Control: 1.77 $\pm$ 0.32; Prist: 2.23 $\pm$ 0.08, n=6). Considering that CK activity is crucial for intracellular ATP transfer and buffering, it is presumed that intracellular energy transfer is not mainly affected by Prist.

3.4 Pristanic acid inhibits synaptic Na^+,K^+-ATPase activity from rat cerebral cortex

Finally, we tested the influence of Prist on Na^+,K^+-ATPase activity in synaptic plasma membranes prepared from cerebral cortex. Figure 3A shows that exposition of purified synaptic membrane preparations to Prist resulted in a

significant inhibition of this activity (up to 78%) [$F_{(4,24)}$ = 23.47, P <0.001] in a dose dependent manner [β=-0.816; P<0.001]. We also observed that the addition of 200 μM GSH to the medium did not change Prist-induced inhibition of Na^+, K^+-ATPase activity [$F_{(4,19)}$ = 7.318; *P*<0.001] (Figure 3B), suggesting that sulfhydryl or other groups of the enzyme did not suffer an oxidative attack. These results indicate that neurotransmission, which depends on normal Na^+,K^+-ATPase activity, is possibly impaired by Prist.

4. Discussion

Prist accumulates in various peroxisomal disorders in which neurological symptoms and brain abnormalities are predominant (McLean et al. 2002; Smith et al. 2010; Verhoeven and Jakobs 2001). The pathogenesis of these disorders is poorly established, although it could be presumed that this fatty acid is neurotoxic. In this context, it has been recently shown that Prist is cytotoxic to neural cells probably via reactive species generation (Rönicke et al. 2009). However, considering that the exact underlying neurotoxic mechanisms provoked by Prist are far from understood, in the present study we investigated the *in vitro* effects of this accumulating metabolite on important parameters of energy production and transfer, as well as on Na^+, K^+-ATPase activity in cerebral cortex of young rats in the hope to verify whether Prist could compromise brain energy homeostasis.

We initially verified that acetate oxidation was significantly decreased (up to 25%) by Prist and that this reduction was not due to a shortage of coenzyme A, implying a true blockage of the citric acid cycle (CAC). It is conceivable that this effect could be due to an inhibition of one or more enzymatic activities of

the CAC or, alternatively, secondary to a blockage of the electron transfer flow through the respiratory chain. Thus, we next tested the effects of Prist on the respiratory chain function by measuring the activities of complexes I-IV. We found that Prist significantly inhibited the activities of complexes I (up to 65%), II (up to 40%) and II-III (up to 95%), without affecting complex IV activity. These data indicate a strong reduction of the electron transport chain flow, which is necessary for almost all energy (ATP) produced in the cell. At this point, it may be presumed that the inhibition of electron transport through the respiratory chain may secondarily lead to inhibition of the CAC, as observed in the present study by the reduction of CO_2 formation from acetate. Taken together, the present data indicate that energy production is compromised by Prist in cerebral cortex of young rats.

In contrast, the activity of creatine kinase, a crucial enzyme involved in intracellular ATP transfer and buffering, was not modified by this branched chain fatty acid, indicating that energy transfer is probably not affected by Prist and highlighting the selective role of Prist towards energy production.

Prist also strongly inhibited (up to 78%) plasma synaptic membrane Na^+, K^+- ATPase activity, which is necessary to maintain neuronal excitability and cellular volume control through the generation and maintenance of the membrane potential by the active transport of sodium and potassium ions in the CNS. This enzyme activity is present at high concentrations in the brain, consuming about 40–50% of the ATP generated in this tissue, highlighting its importance for normal brain functioning. Alterations of this enzyme activity are mainly due to free radical attack (Kurella et al. 1997; Lees 1993) or to changes in membrane fluidity (Wheeler et al. 1975). Under our experimental conditions, it

is unlikely that free radicals could provoke oxidative damage on vulnerable groups of the enzyme leading to a reduction of its activity since GSH, a well known protector of thiol groups and scavenger of reactive species, was not able to change this effect. However, we cannot rule out that incorporation of Prist into the membrane could alter its fluidity finally causing Na^+, K^+- ATPase inactivation, as seen for other hydrophobic fatty acids (Abeywardena et al. 1983; Carfagna and Muhoberac 1993; Kimelberg and Papahadjopoulos 1974; Lee 1976). In this context, the strong inhibitory activity of some compounds on this activity was thought to be due to their property of penetrating into the hydrophobic interior of the membrane disturbing the interactions of ionic and hydrophobic groups on the enzyme necessary for its normal function (Carfagna and Muhoberac 1993). The binding of such substances to biomembranes can alter fluidity, ion permeability and the phospholipid interactions (Abeywardena et al. 1983; Kimelberg and Papahadjopoulos 1974; Lee 1976). Therefore, it is tempting to speculate that increased Prist brain concentrations may disrupt the activity of Na^+, K^+- ATPase activity by interfering with the membrane environment where the enzyme protein is embedded.

Relatively to the possible consequences of the marked inhibition of Na^+, K^+- ATPase activity provoked by Prist to neural cellular function, there is increasing evidence suggesting that Na^+, K^+- ATPase activity is critical for normal brain function and reduction of this activity is related to selective neuronal damage in rat and human brain (Cousin et al. 1995; Lees 1993). Furthermore, inhibition of Na^+,K^+-ATPase has also been associated with excitotoxicity and epilepsy (Choi and Rothman 1990; Cousin et al. 1995; Grisar 1984; Lees and Leong 1995).

It is difficult to determine at the present the pathophysiological relevance of our *in vitro* data. However, it should be stressed that the significant alterations of the biochemical parameters observed in our present study occurred within Prist concentrations similar or even lower than those found in plasma of patients affected by some peroxisomal diseases (Ferdinandusse et al. 2008).

In conclusion, this is the first report showing that Prist markedly disturbs brain bioenergetics and decreases synaptic Na^+,K^+-ATPase activity. It is therefore presumed that impairment of energy production and neurotransmission may contribute synergistically with other factors to the neurological damage found in patients affected by peroxisomal disorders in which Prist accumulates.

Acknowlegments

We are grateful to the financial support of CNPq, PROPESq/UFRGS, FAPERGS, PRONEX, FINEP Rede Instituto Brasileiro de Neurociência (IBN-Net) # 01.06.0842-00, Instituto Nacional de Ciência e Tecnologia em Excitotoxicidade e Neuroproteção (INCT-EN).

The authors declare that they have no conflict of interest.

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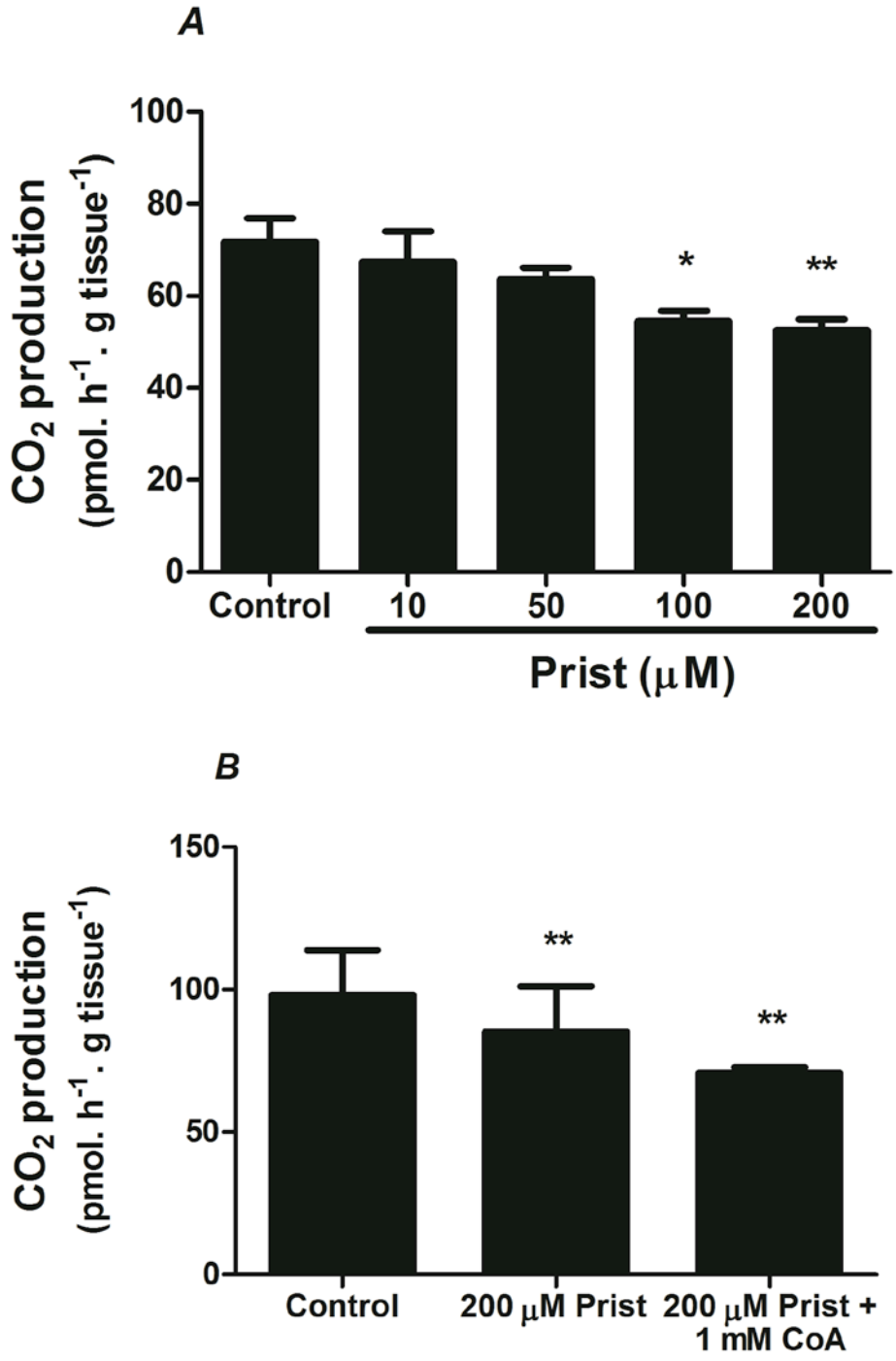
Legends to figures

Figure 1. Effect of pristanic acid (Prist) on CO_2 production from [1-^{14}C] acetate in rat cerebral cortex (*A*). In some experiments, CO_2 production was measured in the presence of 1 mM coenzyme A (CoA) (*B*). Values are mean ± standard deviation for five to nine independent experiments (animals) per group and expressed as pmol CO_2. h^{-1}. g $tissue^{-1}$. **P*<0.05 and ** *P*<0.01 compared to control (Duncan multiple range test).

Figure 2. Effect of pristanic acid (Prist) on the activities of the respiratory chain complexes I-IV in rat cerebral cortex. Values are mean ± standard deviation for four to nine independent experiments (animals) per group. The activity of complex I (*A*) is expressed as nmol ferricyanete reduced. min^{-1}. mg $protein^{-1}$ and of complex II (*B*) as nmol DCIP reduced. min^{-1}. mg $protein^{-1}$. The activities of complexes II–III *(C)* and IV *(D)* are expressed, respectively, as nmol cytochrome *c* reduced. min^{-1}. mg $protein^{-1}$ and nmol cytochrome *c* oxidized. min^{-1}. mg $protein^{-1}$. **P*<0.05 and *** *P*<0.001 compared to control (Duncan multiple range test).

Figure 3. Effect of pristanic acid (Prist) on Na^+, K^+-ATPase activity in purified synaptic plasma membranes prepared from rat cerebrum homogenates (*A*). In some experiments, Na^+, K^+-ATPase activity was measured in the presence of 200 μM GSH (*B*). Values are mean ± standard deviation of six independent experiments (animals) performed in triplicate and are expressed as nmol Pi . min^{-1}. mg $protein^{-1}$. *** *P*<0.001 compared to control (Duncan multiple range test).

Figure 1


A
100
80
60
40
20
0
CO2 production
(pmol. h-1. g tissue-1)
*
**
Control
10
50
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200
Prist (μM)
B
150
100
50
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CO2 production
(pmol. h-1. g tissue-1)
**
**
Control
200 μM Prist
200 μM Prist +
1 mM CoA

Figure 2

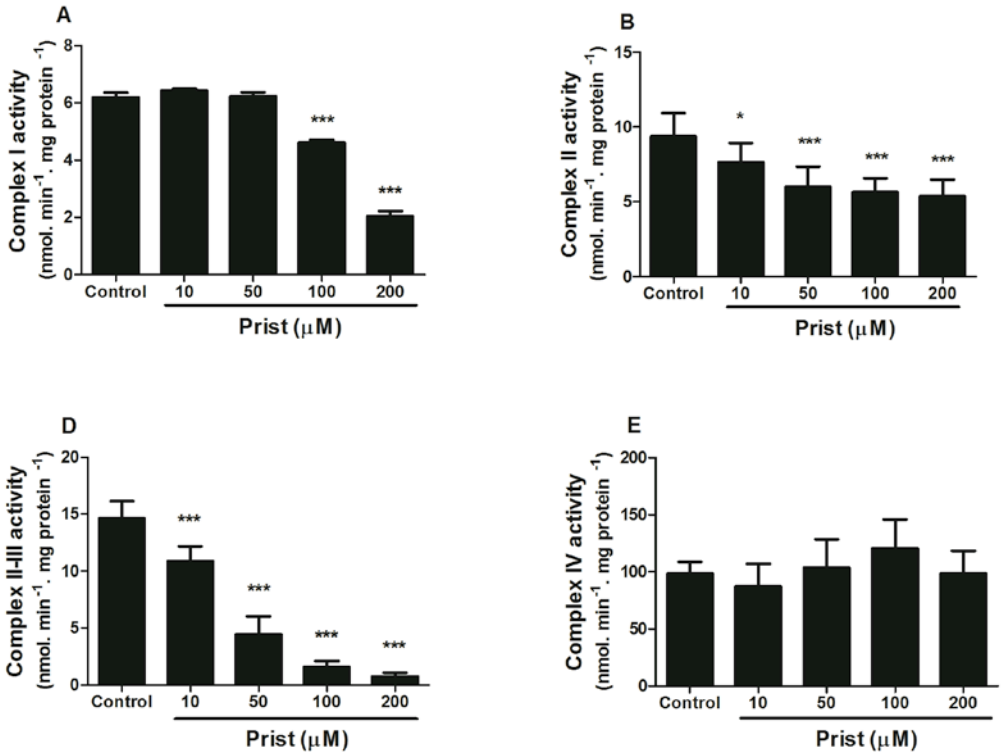
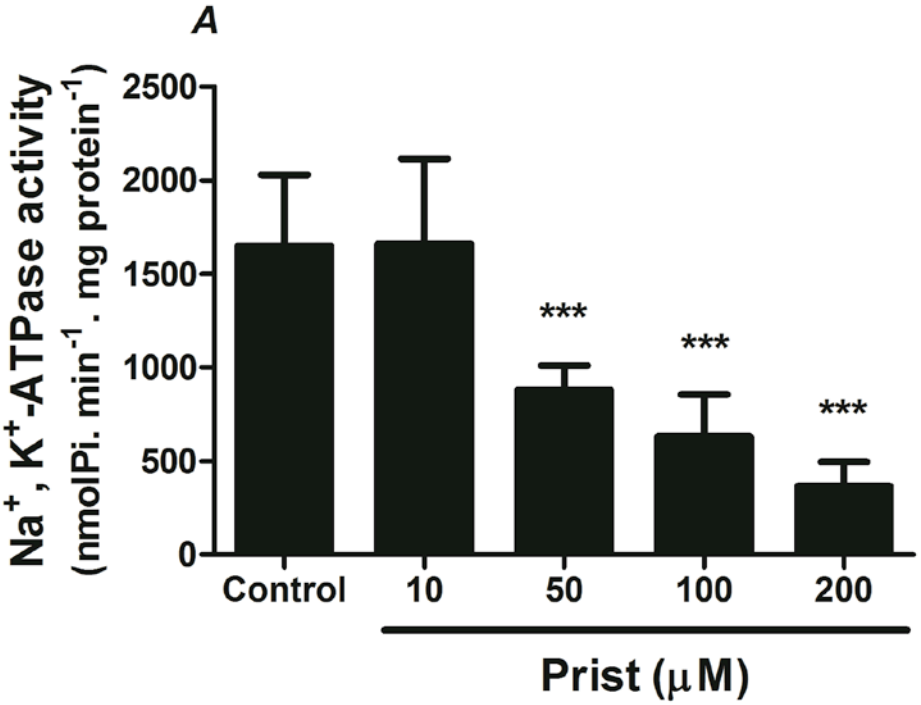
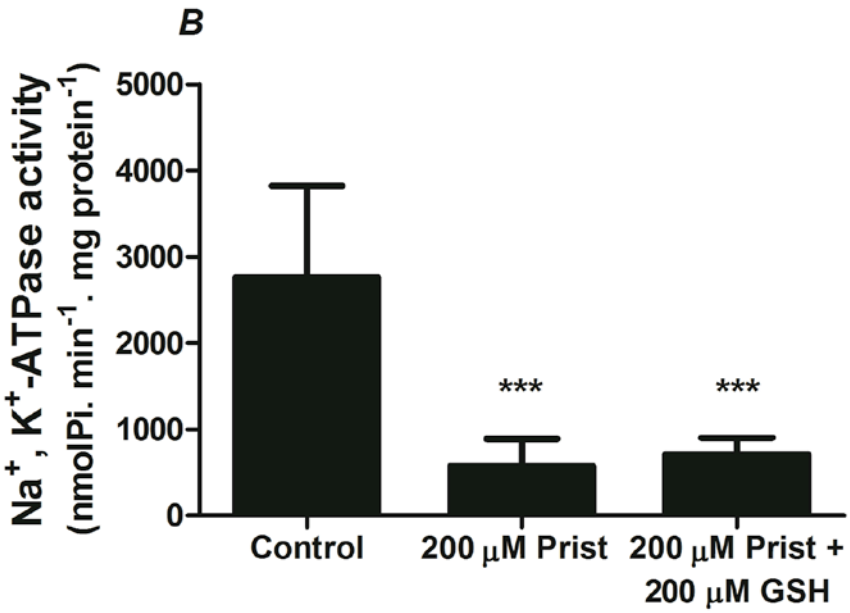

A
Complex I activity
(nmol. min-1. mg protein -1)
Control
10
50
100
200
Prist (μM)
B
Complex II activity
(nmol. min-1. mg protein -1)
Control
10
50
100
200
Prist (μM)
D
Complex II-III activity
(nmol. min-1. mg protein -1)
Control
10
50
100
200
Prist (μM)
E
Complex IV activity
(nmol. min-1. mg protein -1)
Control
10
50
100
200
Prist (μM)

Figure 3


A
Na+, K+-ATPase activity
(nmolPi. min-1. mg protein-1)
2500
2000
1500
1000
500
0
***
***
***
Control
10
50
100
200
Prist (μM)


B
Na+, K+-ATPase activity
(nmolPi. min-1. mg protein-1)
5000
4000
3000
2000
1000
0
***
***
Control
200 μM Prist
200 μM Prist +
200 μM GSH

PARTE III

Discussão e Conclusões

III.1. DISCUSSÃO

As desordens peroxissomais são doenças do metabolismo causadas por defeitos na biogênese do peroxissomo ou pela ausência de uma atividade enzimática peroxisomal específica, resultando no acúmulo do ácido fitânico (Fit) ou/e do ácido pristânico (Prist) em concentrações distintas. No seu conjunto, essas desordens apresentam uma incidência estimada de 1:20.000 a 1:100.000 nascidos vivos (Mclean et al., 2002; Scriver et al., 2001). As manifestações clínicas dos afetados por essas doenças são variáveis, embora sintomas neurológicos e anormalidades cerebrais sejam muito freqüentes (Verhoeven e Jakobs 2001). Pouco se sabe sobre os mecanismos fisiopatológicos do dano cerebral que acomete os pacientes portadores dessas desordens, embora o acúmulo do Fit e do Prist nessas doenças sugiram efeitos deletérios desses compostos sobre o sistema nervoso central (Kahlert et al., 2005; Leipnitz et al., 2010; Rönicke et al., 2009; Schönfeld et al., 2004). Neste sentido, foi verificado que o Fit causa morte celular em astrócitos de hipocampo de ratos precedida por um aumento significativo na produção de ânion superóxido, despolarização do potencial da membrana mitocondrial e liberação do citocromo *c* (Kahlert et al., 2005). Além disso, foi observado que este ácido graxo de cadeia ramificada induz dano oxidativo a lipídios e proteínas, além de reduzir as defesas antioxidantes no cérebro (Leipnitz et al., 2010). Por outro lado, demonstrou-se que o Prist possui ação citotóxica para diversas linhagens de células neurais, determinada através de um aumento pronunciado na concentração intracelular de Ca^{2+}, bem como despolarização mitocondrial e morte celular. Foi também verificado que o Prist induz marcadamente a geração de espécies reativas de oxigênio (ERO) predominantemente em

neurônios e o que of Fit possui uma ação semelhante, mas de menor grau (Rönicke et al., 2009). Baseando-se em seus resultados, os autores preconizam que esses ácidos graxos poderiam provocar disfunção mitocondrial e morte de células neurais com envolvimento de estresse oxidativo.

A mitocôndria, além de ser importante na geração de espécies reativas, desempenha papel central na geração de energia para a homeostase dos processos celulares, através da manutenção dos níveis de ATP, além de participar ativamente da manutenção dos níveis intracelulares de cálcio (Nicholls e Akerman 1982) e estar envolvida em diversos processos que levam à morte celular (Liu et al., 1996).

O objetivo da presente investigação foi avaliar o efeito do Fit e do Prist sobre importantes parâmetros de metabolismo energético em ratos jovens. Avaliamos o efeito desses compostos, nas concentrações em que se encontram em doenças peroxisomais, sobre o ciclo do ácido cítrico (CAC, produção de CO_2 a partir de acetato), sobre o transporte de elétrons pela cadeia respiratória (atividades dos complexos I-IV), sobre a transferência intracelular de energia (atividade da creatina quinase) e sobre a atividade da Na^+,K^+-ATPase em cérebro de ratos jovens. Avaliamos também o efeito do Fit sobre a via glicolítica (produção de CO_2 a partir de glicose), bem como sobre as atividades enzimáticas das várias reações do CAC.

Observamos no capítulo I que o Fit não alterou a produção de CO_2 a partir de [U-^{14}C]glicose e [1-^{14}C]acetato, indicando que esse metabólito não prejudica a via glicolítica ou o CAC. Verificamos também que a atividade das enzimas do CAC não foi alterada pela presença de Fit, reforçando os achados obtidos com a produção de CO_2 a partir de acetato. No entanto, devemos

salientar que em um estudo prévio foi demonstrado que o Fit reduz a atividade da enzima aconitase, efeito não observado no presente estudo (Schönfeld e Reiser 2006). Contudo, deve-se observar que nossa metodologia na medida da atividade da aconitase inclui a adição do antioxidante cisteína ao meio de incubação, o que protege grupos essenciais da enzima de ataque oxidativo para o seu funcionamento normal. Já o método usado por aqueles investigadores não utiliza cisteína, outro antioxidante ou agente redutor, sugerindo que o efeito do Fit observado por esses investigadores foi provavelmente mediado por espécies reativas. Neste particular, outros estudos revelaram que a aconitase é altamente suscetível à oxidação, sendo a mesma considerada um parâmetro de estresse oxidativo (Chepelev et al., 2009; Tretter e Adam-Vizi 2000).

Por outro lado, demonstramos que o Fit foi capaz de diminuir marcadamente as atividades dos complexos I, I-III, complexo II e II-III nas concentrações de 100 e 200 μM. Já a atividade do complexo IV foi inibida pelo Fit nas concentrações de 10, 100 e 200 μM. Esses resultados atestam um efeito inibidor do Fit sobre o fluxo de elétrons da cadeia respiratória, podendo potencialmente diminuir a produção de ATP. Esses resultados estão de acordo com estudos anteriores mostrando que a fosforilação oxidativa é comprometida pelo Fit em hipocampo e córtex de ratos adultos (Schönfeld et al., 2004; Schönfeld e Reiser 2006).

Também verificamos que a atividade da enzima creatina quinase (CK) não foi alterada pelo Fit em concentrações tão altas quanto 200 μM, indicando que esse ácido graxo não interfere com uma atividade essencial para a transferência intracelular de energia.

Por outro lado, o Fit inibiu acentuadamente a atividade da enzima Na^+,K^+-ATPase nas concentrações de 100 e 200μM. Esses resultados sugerem que a neurotransmissão possa estar prejudicada pelo Fit, já que esta enzima é responsável pela manutenção do potencial de membrana celular necessário para a excitabilidade neuronal (Erecinska et al., 2004; Erecińska e Silver 1994).

No capítulo II, nosso primeiro passo foi investigar o efeito do Prist sobre a produção de CO_2 a partir de [1-^{14}C]acetato. Esse parâmetro foi inibido nas concentrações de 100 e 200μM do metabólito, indicando um efeito inibitório desse ácido graxo sobre o CAC. Na tentativa de elucidar se o efeito do Prist poderia ter sido provocado pela falta de CoA devido a uma possível competição entre Prist e acetato pela ligação com essa coenzima, formando acetil-CoA, acrescentamos excesso de CoA no meio de incubação e observamos que a inibição provocada pelo Prist não foi alterada, sugerindo que esse efeito inibitório poderia estar dirigido a um ou mais passos enzimáticos do CAC, ou a um bloqueio na transferência de elétrons pela cadeia respiratória.

Assim, observamos que as atividades dos complexos I, II e II-III da cadeia respiratória foram inibidos de maneira acentuada pelo Prist, o que não ocorreu com a atividade do complexo IV. Esses dados mostram que esse ácido graxo compromete a função da cadeia respiratória, indicando um comprometimento da fosforilação da oxidativa. Poder-se-ia sugerir que a inibição da cadeia respiratória poderia levar secundariamente a um bloqueio do CAC, verificado pela diminuição da produção de CO_2 por esse ácido.

Por outro lado, similarmente ao Fit, o Prist não alterou a atividade da enzima CK na concentração de 200μM, sugerindo que a transferência de

energia intracelular não parece ser alterada pelo Prist. A falta de efeito do Prist sobre as atividades do complexo IV e creatina quinase indicam um efeito seletivo desse metabólito sobre as outras atividades alteradas pelo mesmo.

Observamos também uma inibição marcada na atividade da enzima Na^+,K^+-ATPase causada pelo Prist nas concentrações de 50, 100 e 200μM, indicando que a neurotransmissão possa estar afetada pelo Prist. Também verificamos que o efeito inibitório do Prist sobre essa atividade não foi alterado pelo antioxidante GSH, tornando pouco provável um dano oxidativo por espécies ativas causado pelo ácido a grupos suscetíveis da enzima. Não se pode afastar a possibilidade de que o Prist se insira na membrana plasmática alterando sua fluidez e indiretamente reduzindo a atividade da Na^+,K^+-ATPase, como ocorre com outros compostos hidrofóbicos (Abeywardena et al., 1983; Schönfeld e Struy 1999; Steinberg 1995).

A partir dos resultados obtidos, podemos sugerir que os efeitos causados pelo Fit observados sobre as atividades dos complexos da cadeia respiratória e sobre a atividade da enzima Na^+,K^+-ATPase possam ser devidos a dano oxidativo à enzima ou a lipídios de membrana plasmática causado pela geração de espécies ativas que foram demonstradas ser induzidas por esses ácido (Leipnitz et al., 2010; Rönicke et al., 2009; Schönfeld e Reiser 2006; Schönfeld e Wojtczak 2007). Alterações na disposição dos lipídeos e proteínas de membrana também podem ser potencialmente causadas pelo Fit por meio de sua incorporação a membranas celulares devido a suas propriedades anfipáticas (Steinberg 1995), levando à inibição da Na^+,K^+-ATPase. Já no que se refere ao Prist, não se pode afastar a possibilidade de que em parte as

alterações das atividades enzimáticas possam ser devidas a dano oxidativo, embora a inibição da Na^+,K^+-ATPase causada pelo ácido seja possivelmente devida a alteração de fluidez de membrana onde a enzima está ancorada.

Deve-se aqui enfatizar que de uma forma geral os efeitos obtidos pelo Prist foram de maior intensidade relativamente aos causados pelo Fit, considerando-se as doses semelhantes empregadas nos vários ensaios. No entanto, devemos ainda levar em conta que o Fit acumula-se em acentuadas concentrações na doença de Refsum (5000 μM), enquanto na doença de Zellweger (Fit= 500 μM; Prist= 90 μM) e na deficiência da proteína D-bifuncional (Fit= 190 μM; Prist= 80 μM) os dois ácidos se acumulam com um predomínio do Fit. Além disso, o Prist possui concentrações mais elevadas (300 μM) relativamente as do Fit (25 μM) na deficiência da AMACR. Tomados essas considerações em seu conjunto, podemos postular que o Fit poderia exercer efeitos neurotóxicos principalmente na doença de Refsum, enquanto os dois ácidos poderiam agir sinergicamente na doença de Zellweger e nas deficiências da proteína D-bifuncional e AMACR. Quanto à última doença peroxissomal, é também possível que o principal agente neurotóxico seja o Prist.

Os resultados da presente investigação indicam que os principais metabólitos acumulados em desordens peroxissomais exercem efeitos deletérios importantes sobre o sistema nervoso central que podem estar envolvidos na disfunção neurológica e nas anormalidades cerebrais característicos nos pacientes afetados por dessas doenças

Dessa forma, é possível que a disfunção mitocondrial, aliada a outros mecanismos como o estresse oxidativo, atuem sinergicamente, levando ao dano neurológico nessas doenças. Acreditamos, portanto, que os achados deste trabalho possam auxiliar na elucidação na patogenia dos pacientes com estas desordens, servindo como base para o desenvolvimento de novas estratégias terapêuticas no tratamento e na melhora da qualidade de vida dos portadores das doenças peroxissomais.

III.2. CONCLUSÕES

III.2.1 Efeitos do ácido fitânico sobre o metabolismo energético

- O ácido fitânico (Fit) não alterou a produção de CO_2 a partir de [U-^{14}C]glicose e [1-^{14}C]acetato, sugerindo que não houve alteração da via glicolítica e no ciclo do ácido cítrico (CAC).
- O Fit não alterou a atividade das enzimas do CAC, corroborando o achado anterior de que não houve alteração da atividade do CAC
- O Fit inibiu as atividades dos complexos I, I-III, II, II-III e IV, o que indica que esse ácido graxo compromete a função da cadeia respiratória, prejudicando o fluxo de elétrons e potencialmente causando diminuição na síntese de ATP.
- O Fit não alterou a atividade da enzima creatina quinase (CK), sugerindo que esse metabóito não compromete a transferência intracelular de energia.
- O Fit diminuiu a atividade da enzima Na^+,K^+-ATPase, indicando que esse ácido graxo potencialmente prejudica a neurotransmissão.

III.2.2 Efeitos do ácido pristânico sobre o metabolismo energético

- O ácido pristânico (Prist) inibiu a produção de CO_2 a partir de [U- [1-^{14}C]acetato, sugerindo que houve prejuízo da atividade do ciclo do ácido cítrico (CAC). A diminuição na produção de CO_2 não foi devida a falta de CoA no meio, descartando uma possível competição entre o Prist e o acetato pela CoA.

- O Prist inibiu as atividades dos complexos I, II e II-III sem alterar a atividade do complexo IV, o que indica que esse ácido graxo pode estar comprometendo o fluxo de elétrons pela cadeia respiratória, levando a uma possível diminuição de ATP.
- O Prist não alterou a atividade da enzima CK, sugerindo que esse ácido graxo não compromete a transferência intracelular de energia.
- O Prist diminuiu a atividade da enzima Na^+,K^+-ATPase, indicando que esse ácido graxo pode prejudicar o potencial de membrana necessário para a manutenção da excitabilidade neuronal e a neurotransmissão. Essa inibição não foi prevenida pela adição de GSH ao meio, indicando que um ataque oxidativo causado pelo ácido sobre grupos vulneráveis da enzima, interferindo no estado redox dos grupamentos SH da mesma, é pouco provável.

III.3. PERSPECTIVAS

- Avaliar o efeito *in vitro* dos ácidos fitânico (Fit) e pristânico (Prist) sobre parâmetros do metabolismo energético em cerebelo e coração de ratos;
- Avaliar o efeito *in vitro* do ácido pristânico (Prist) sobre a atividade das enzimas do ciclo do ácido cítrico
- Avaliar o efeito *ex vivo* dos ácidos fitânico (Fit) e pristânico (Prist) sobre parâmetros do metabolismo energético e estresse oxidativo em cérebro de ratos;
- Avaliar o efeito dos ácidos fitânico (Fit) e pristânico (Prist) sobre parâmetros de metabolismo energético e estresse oxidativo em cultura de astrócitos e neurônios de cérebro de ratos;
- Avaliar o efeito do ácido pristânico (Prist) sobre parâmetros respiratórios obtidos pelo consumo de oxigênio em oximetria em cérebro de ratos.

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