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ALTERAÇÕES METABÓLICAS E MITOCONDRIAIS NA ANALBUMINEMIA CONGÊNITA: ESTUDOS EM RATOS NAGASE ANALBUMINÊMICOS-DISLIPIDÊMICOS

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Epígrafes

"*One of the principal objects of theoretical research in any department of knowledge is to find the point of view from which the subject appears in its greatest simplicity*" J. Willard Gibbs (*1839-1903†).

Pensando no processo de pós-graduação e pesquisa no qual esta tese se inclui, esta epígrafe centenária, é extremamente contrastante à conduta contemporânea; e este contraste vale reflexão!

Parece-me, que a ciência aplicada assemelha-se mais com a ciência pura do que a ciência básica o faz. Na pesquisa básica não se permite mais a pureza e a simplicidade da hipótese sobre um objeto, há de se apresentar justificativas grandiosas, até mesmo fantasiosas. É facilmente identificável a devassidão em nome do financiamento, da aparente importância social (aplicação prática), do reconhecimento pessoal – isto tudo é muito menos evidente na pesquisa aplicada – daí a maior semelhança desta com a pesquisa pura. Temo que esta sistemática já tenha produzido impacto negativo na formação de pessoal, o principal produto da pós-graduação. A pesquisa pura de interesse acadêmico precisa sobreviver, é ela que poderá, eventualmente, ajudar a resgatar a filosofia e o artesanato que havia na formação de pessoal, fatores não privilegiados no ambiente atual.

Outro ponto importante é relacionado às aspirações brasileiras em ciência: o "Fordismo" que há nos processos acadêmicos nos E.U.A., agora é evidente na pós-graduação brasileira. Neste modelo, é preocupante a valorização da capacidade de trabalho (físico), talvez, em detrimento de outras habilidades.

Resumo

A analbuminemia congênita é uma doença autossômica recessiva caracterizada por níveis traços de albumina plasmática (< 1 mg/mL) e sintomas clínicos leves. Entre as comorbidades apresentadas pelos indivíduos e ratos analbuminêmicos (ratos Nagase – NAR), os distúrbios no metabolismo/transporte de lipídeos plasmáticos são as mais marcantes. A dislipidemia associada à analbuminemia é caracterizada por níveis aumentados de colesterol e triglicérides, e déficit de ácidos graxos livres (FFA). Nesta tese, são apresentados três estudos independentes sobre a analbuminemia, os quais objetivaram investigar: **1)** os mecanismos da hipertrigliceridemia e do déficit de FFA plasmático do NAR; **2)** o metabolismo de carboidratos no NAR; **3)** as funções mitocondriais no NAR. Também é apresentado um quarto estudo (aspectos metodológicos) sobre o uso do *probe* safranina para avaliar o potencial elétrico transmembrana mitocondrial. Os principais resultados destes estudos foram: **Estudo um**: as taxas de lipogênese (596 ± 40 *vs*. 929 ± 124 µmol $^{3}H_2O$/g/h) e de secreção de triglicérides para o plasma (4,25 ± 1,00 *vs.* 7,04 ± 1,68 mg/dL/min) foram mais lentas ($P \leq 0,05$) no NAR do que no rato controle Sprague-Dawley (SDR). As injeções de heparina ou de albumina no NAR promoveram um aumento de FFA plasmático em função do tempo. Noventa minutos após a injeção de albumina, os níveis de FFA plasmáticos nos NAR se elevaram de 0,36 ± 0,05 para 1,34 ± 0,16 mEq/L ($P \leq 0,05$), atingindo os níveis do SDR. Estes resultados indicam que a falta de albumina plasmática inibe a lipólise intravascular e causa o déficit de FFA plasmático na analbuminemia, e que a produção hepática de triglicérides não

contribui para a hipertrigliceridemia no NAR. **Estudo dois**: a concentração de glicose plasmática foi similar entre os NAR e os SDR alimentados ou em jejum, porém a insulinemia no estado alimentado foi maior nos NAR do que nos SDR ($P \leq 0,05$). O NAR apresentou maior tolerância à glicose quando comparado ao SDR ($P \leq 0,05$). Esta maior tolerância a glicose está associada à maior resposta insulinêmica à administração de glicose. Não houve diferença entre os grupos para a sensibilidade periférica a insulina. Apesar do conteúdo similar de glicogênio hepático no estado alimentado, o NAR apresentou menor conteúdo de glicogênio (40% do SDR) após 6 h de jejum. A injeção de piruvato (substrato neoglicogênico) promoveu um aumento mais rápido na glicemia do NAR em comparação ao SDR. Deste modo, os resultados indicam que o NAR apresenta metabolismo de glicose acelerado. **Estudo três**: a capacidade de retenção de Ca^{2+} pelas mitocôndrias isoladas do fígado do NAR aos três meses de idade foi ~50% daquela do SDR. Esta variável não se diferiu entre os grupos quando avaliada aos 21 dias de vida dos ratos. Foi observada uma depleção de ~20% no conteúdo de nitrosotiol e um aumento de ~30% na expressão de ciclofilina D nas mitocôndrias de fígado do NAR. Nenhuma das variáveis relacionadas ao estado redox mitocondrial diferiu entre NAR e SDR, tais como: o conteúdo de tióis reduzidos, de glutationa total, a taxa de liberação de H_2O_2, e o estado reduzido de NAD(P)H. Com isso, conclui-se que a maior expressão de ciclofilina D, um componente importante no processo de transição de permeabilidade mitocondrial, e o menor conteúdo de nitrosotiol nas mitocôndrias dos NAR podem explicar a sua menor capacidade de retenção de Ca^{2+}.

Abstract

Congenital analbuminemia is a rare autosomal recessive disorder characterized by a trace level of albumin in blood plasma and mild clinical symptoms. Analbuminemic patients and rats (Nagase analbuminemic rats – NAR) present associated abnormalities, among which the disturbances in plasma lipid metabolism and transport are hallmarks. The dyslipidemia associated with analbuminemia comprises a unique plasma lipid profile (*i.e.* high cholesterol and triglycerides, but a severe free-fatty acids deficiency). Three independent works on analbuminemia are presented in this PhD thesis, whose aims were: **1)** to investigate the mechanisms of NAR hypertriglyceridemia and plasma free-fatty acids deficiency; **2)** to study carbohydrate metabolism in NAR; **3)** to evaluate mitochondrial (dys)function in NAR. Also, a methodological study about the use of the dye safranine as a fluorescent probe for the assessment of mitochondrial transmembrane electrical potential is presented in this thesis. The main results from these studies were: **Study one**: lipogenesis (596 ± 40 vs. 929 ± 124 µmol ^3H_2O/g/h) and triglyceride secretion rates (4.25 ± 1.00 vs. 7.04 ± 1.68 mg/dL/min) were slower ($P \leq 0.05$) in fasted NAR than in control Sprague-Dawley rats (SDR). The injection of either heparin or albumin elicited an increase in NAR plasma FFA levels over time. FFA levels reached control levels 90 min after the albumin administration into NAR, increasing from 0.36 ± 0.05 to 1.34 ± 0.16 mEq/L ($P \leq 0.05$). These results indicate that the lack of plasma albumin inhibits intravascular lipolysis and causes the FFA deficit observed in NAR. Moreover, hepatic triglyceride output seems not to contribute to NAR hypertriglyceridemia. **Study**

two: plasma glucose levels were similar between fed and fasted NAR and SDR, but fed insulinemia was higher in NAR than in SDR ($P \leq 0.05$). NAR displayed increased glucose tolerance compared to SDR ($P \leq 0.05$). This enhanced glucose tolerance was associated with higher insulinemia after the glucose load, and with similar insulin sensitivity between the groups. Despite similar liver glycogen content in fully fed condition, NAR had lower glycogen content (40% of control) after 6-h fasting. The injection of pyruvate (gluconeogenic substrate) elicited a faster rise in glycemia of NAR than in SDR. Therefore**,** NAR display enhanced glucose metabolism. **Study three**: the Ca^{2+} retention capacity of the liver mitochondria isolated from 3-month-old NAR was about 50% that of the control. Interestingly, the assessment of this variable in 21-day-old NAR and SDR indicated that the mitochondrial Ca^{2+} retention capacity was preserved at this age. A 20% decrease in mitochondrial nitrosothiol content and a 30% increase in cyclophilin D expression were observed in NAR liver mitochondria. None of the variables related to mitochondrial redox state differed between the controls and NAR, *i.e.*, namely the contents of reduced mitochondrial membrane protein thiol groups and total glutathione, H_2O_2 release rate, and NAD(P)H reduced state. We conclude that the higher expression of cyclophilin D, a major component in the mitochondrial permeability transition process, and decreased nitrosothiol content in NAR mitochondria may underlie their lower Ca^{2+} retention capacity.

Lista de Abreviaturas

	Inglês	Português
SDR	*Sprague-Dawley rats*	Ratos Sprague-Dawley
NAR	*Nagase Analbuminemic rats*	Ratos Nagase analbuminêmicos
FFA	*Free-fatty acids*	Ácidos graxos livres
ANT	*Adenine nucleotide translocator*	Traslocador de nucleotídios de adenina
Tg	*Triglycerides*	Triglicerídios
Chol	*Cholesterol*	Colesterol
GSIS	*Glucose stimulated insulin secretion*	Secreção de insulina estimulada por glicose
KIC	*Keto-isocaproate*	Keto-isocaproato
CyD	*Cyclophilin D*	Ciclofilina D
NAD(P)H	*Nicotinamide adenine dinucleotide*	Nicotinamida adenina dinucleotídio
H_2O_2	*Hydrogen peroxide*	Peróxido de hidrogênio
EGTA	*Glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid*	Glicol-bis(2-aminoetileter)-*N*,*N*,*N'*,*N'*- acido tetraacético
CsA	*Cyclosporin A*	Ciclosporina A
Mito	*Mitochondria*	Mitocôndria

Sumário

1. Introdução

A albumina é a proteína mais abundante do plasma sanguíneo, compreendendo aproximadamente 60% do total de proteínas [1]. Ela é secretada pelo fígado para a circulação sanguínea na taxa de ~200 mg/kg/dia [2], e seu tempo de meia vida na circulação é de ~19 dias [1]. A albumina não se confina somente ao plasma. Há evidências que o total de albumina corporal é distribuído pelos compartimentos corporais e tecidos. Quantitativamente, o plasma sanguíneo detém a maior quantidade de albumina corporal, enquanto a pele e a musculatura esquelética contêm aproximadamente 80% do conteúdo de albumina extravascular no corpo. Nos outros tecidos, somente quantidades traços são observadas [1].

Inúmeras funções são atribuídas à albumina plasmática, entre as mais reconhecidas estão: a) maior determinante da pressão coloidosmótica do plasma, e deste modo da distribuição dos fluidos entre o compartimento circulatório e o espaço intersticial; b) seu papel como carreadora de várias substâncias corpóreas (ácidos graxos, bilirrubina, hormônios da tireóide, cálcio, cobre e outras) e xenobióticos (toxinas, drogas e seus metabólitos) - sua afinidade de ligação a vários desses ligantes é especialmente importante para o transporte de substâncias pouco solúveis em meio aquoso (ex. ácidos graxos) e para reduzir a fração livre e o *clearance* de outras substâncias, inclusive as tóxicas (ex.

bilirrubina, fármacos) [1][1]. Além destas funções, a albumina desempenha também outros papéis menos compreendidos e divulgados, como aqueles derivados de suas propriedades antioxidantes e citoprotetoras [3-5]. Experimentos *in vitro* indicam que a albumina inibe a oxidação de lipoproteína de baixa densidade (LDL) [3] e que também confere citoproteção em condições de agressão celular [4, 6]. Também há estudos *in vivo* que suportam a atribuição de uma função antioxidante à albumina, tais estudos mostram a oxidação aumentada das moléculas de albumina em doenças crônico-degenerativas [7, 8] e o aumento de produtos de peroxidação lipídica no plasma de ratos analbuminêmicos [9]. Um estudo recente realizado com hepatócitos isolados indica um novo papel para a albumina: transnitrosilação de proteínas celulares [4]. Neste estudo, foi observado que na presença de albumina há aumento da nitrosilação de proteínas intracelulares quando há s-nitrosotióis no ambiente extracelular.

Há condições fisiopatológicas onde a concentração de albumina plasmática está alterada em relação à faixa de referência que é de 30 a 54 mg/mL. Hiperalbuminemia geralmente reflete desidratação, enquanto a hipoalbuminemia pode ser uma alteração comum a várias doenças (ex: nefrose, hepatopatias) [10]. É importante ressaltar que a hipoalbuminemia é um fator de risco independente para doenças cardiovasculares e mortalidade de todas as causas [11].

Apesar da albumina exercer diferentes papéis fisiológicos no organismo, a vida humana e a de ratos é viável mesmo na ausência quase completa (< 1

[1] Uma revisão completa de todos os aspectos relacionados à albumina pode ser encontrada no livro "All about albumin" escrito por T. Peter. A CAPES disponibiliza acesso completo a este livro através da editora Elsevier.

mg/mL; faixa referência = 30-54 mg/mL) da albumina plasmática (*i.e.* analbuminemia) [12, 13]. Os primeiros casos de analbuminemia congênita ou familiar foram descobertos em 1954 por Benhold; esta doença é caracterizada pela ausência de albumina plasmática (< 1 mg/mL) associada a não perda de albumina pelo intestino ou pela filtração renal [12, 14]. A analbuminemia deriva de mutações no gene da albumina que levam a não síntese e secreção da mesma para o plasma [15]. Apesar de não haver dados absolutamente fidedignos, acredita-se que a freqüência de analbuminemia congênita seja de aproximadamente de um para um milhão de nascimentos [14, 15]. Nos aproximadamente 50 casos de analbuminemia congênita relatados na literatura, diferentes mutações do gene da albumina e comorbidades já foram descritas [14, 15]. As comorbidades mais comuns são dislipidemia, edema de membro inferior, fadiga, infecções recorrentes e lipodistrofia em mulheres [14, 16]. Em função da analbuminemia ser uma doença rara entre os humanos, o melhor entendimento das alterações secundárias se deu pelo estabelecimento de uma colônia de ratos mutantes analbuminêmicos [13, 16]. Estes ratos Nagase analbuminêmicos (NAR) são derivados da linhagem de ratos Sprague-Dawley (SDR) e carregam uma mutação no gene da albumina, imitando bem a etiologia e várias comorbidades da doença humana [16]. A subsessão 1.1 desta sessão de introdução (Pág. 18) revisa mais detalhadamente as mutações genéticas que causam a analbuminemia humana e do rato.

Em geral, a analbuminemia produz sintomas clínicos leves, porém várias alterações subclínicas podem ser observadas tanto no rato quanto no homem.

Dentre estas alterações, a mais marcante, em ambas as espécies, é a dislipidemia [12, 14, 16-18]. A dislipidemia secundária à analbuminemia caracteriza-se pelos altos níveis plasmáticos de lipoproteínas, triglicérides, colesterol e pelo déficit de ácidos graxos livres (FFA) [14, 16, 19]. De fato, este déficit de FFA associado à hipertrigliceridemia é um perfil lipídico bastante singular.

A dislipidemia é um fator de risco clássico para doenças cardiovasculares e também pode causar lesões em outros órgãos como rins e fígado [20]. Há também uma importante associação entre hipertrigliceridemia e resistência periférica a insulina [21].

Os mecanismos da dislipidemia secundária à analbuminemia, especialmente os que delineiam o déficit de FFA plasmático, bem como suas prováveis repercussões metabólicas, ainda não são totalmente compreendidos. A subsessão 1.2 desta sessão de introdução (Pág. 20) contém uma revisão mais extensa da dislipidemia do NAR.

Recentemente, o grupo do professor Anibal Vercesi descreveu algumas alterações mitocondriais em dois modelos de dislipidemia primaria, o camundongo hipertrigliceridêmico (transgênico para ApoCIII) e o camundongo hipercolesterolêmico (*knockout* para o receptor de LDL) [22-26]. As alterações de função mitocondrial foram específicas para cada modelo. No camundongo hipertrigliceridêmico, a alteração mais marcante foi a maior taxa de respiração mitocondrial de repouso promovida pela ativação redox do canal mitocondrial de potássio sensível a ATP, a qual resultou no aumento da taxa metabólica de repouso do animal [22-24]. Diferentemente, o camundongo hipercolesterolêmico

apresentou maior taxa de geração mitocondrial de H_2O_2 e inabilidade da mitocôndria sustentar o NADP no estado reduzido; estas duas alterações sendo conseqüência da depleção de substrato mitocondrial associado à redução enzimática de NADP (*i.e*. citrato e isocitrato) [25, 26].

A disfunção mitocondrial comum entre estes dois modelos foi a maior susceptibilidade a transição de permeabilidade mitocondrial induzida por Ca^{2+} (MPT) [22, 25]. MPT é um processo induzido pelo acúmulo mitocondrial de Ca^{2+} que resulta na permeabilização não seletiva da membrana mitocondrial interna, permitindo a passagem de solutos com massa molecular de até 1,5 kDa [27-29]. Como consenquência desta permeabilização, ocorre inchamento osmótico da organela, dissipação do potencial elétrico transmembrana e falência energética, ruptura da membrana mitocondrial externa, e liberação de fatores apoptogênicos para o citoplasma da célula [29, 30]. Apesar de muitas propriedades do processo de MPT terem sido estabelecidas somente *in vitro*, hoje, há um enorme corpo de evidências de que este processo está envolvido em várias condições patológicas (ex. Dano causado por isquemia-reperfusão de tecidos) [29, 31-33]. A natureza molecular do poro formado na membrana mitocondrial interna pelo processo de MPT não é conhecida, mas quanto a sua regulação, sabe-se que algumas proteínas (ciclofilina D e o translocador de adenina nucleotídio - ANT), estresse oxidativo e nitrosilação de proteínas tem participação central no processo de MPT [27, 29, 34-37]. A ciclofilina D presente na matriz mitocondrial é uma proteína chave neste processo. Sua inibição farmacológica ou deleção genética resulta em menor susceptibilidade ao MPT induzida por Ca^{2+} e em citoproteção sob

condições catastróficas (ex. isquemia-reperfusão) [29, 35, 38]. O ANT, proteína que catalisa o antiporte ATP-ADP através da membrana mitocondrial interna, assume uma conformação na presença de ATP ou ADP que afeta profundamente a susceptibilidade da mitocôndria sofrer o processo de MPT (*i.e.* confere inibição do processo) [39]. Estudos bastante recentes tem mostrado a importância dos nitrosotióis mitocondriais no processo de MPT [34, 36]. O mais importante deles, publicado este ano, indicou que a modulação da ciclofilina D sobre o MPT se dá pela nitrosilação de um único resíduo de cisteína desta proteína [35].

Nesta tese, são apresentados, na forma de artigos independentes, três estudos conduzidos com o rato Nagase analbuminêmico (NAR) que objetivaram investigar: 1) os mecanismos da hipertrigliceridemia e do déficit de FFA plasmático; 2) a provável repercussão deste perfil lipídico raro (*i.e.* hipertrigliceridemia e déficit de FFA plasmático) sobre o metabolismo de carboidratos; 3) as (dis)funções mitocondriais frente à dislipidemia secundária do NAR.

1.1 Revisão sobre as mutações genéticas que causam analbuminemia

Há atualmente cerca de 80 mutações na albumina humana descritas na literatura. A maioria destas mutações é benigna e compreende alterações pontuais em resíduos de aminoácidos. Somente 15 destas mutações resultam em analbuminemia. As mutações que resultam em analbuminemia foram identificadas através do seqüenciamento do DNA de 40 indivíduos diagnosticados com a doença (diagnóstico realizado pela análise das proteínas plasmáticas, geralmente por eletroforese). As alterações moleculares do gene da albumina nos indivíduos analbuminêmicos são bastante heterogêneas, envolvendo defeitos na região codificante do gene da albumina e da junção exon-intron, entre elas: mutações *non sense*, *splice-site mutation*, *frameshift insertion*, *frameshift deletions* e *reading frameshift*. As mutações em heterozigose causam um fenótipo intermediário para a albuminemia (*i.e.* albumina plasmática de 28-35 mg/mL; refência = 30-54 mg/mL e valor médio = 42 mg/mL) [15, 40]. Quando em homozigose, a deficiência de albumina plasmática é tão severa que os métodos comumente empregados para dosagem de albumina plasmática não conseguem detectar os valores. O método colorimétrico utilizado em laboratório clínico para dosagem de albumina plasmática não apresenta validade na analbuminemia (alguns interferentes produzem valores falsamente elevados) [41]. Faz-se necessário o uso de métodos imunológicos de detecção da albumina plasmática ou de eletroforese de proteínas plasmática para a correta avaliação da analbuminemia [41].

As 15 diferentes mutações que resultam em analbuminemia ocorreram em 7 diferentes países, e desta forma, acredita-se que não estejam relacionadas e ocorreram espontaneamente [15]. É importante realçar que um grande contingente de indivíduos analbuminêmicos são filhos de casamentos consangüíneos, onde os pais eram, presumivelmente, heterozigotos para mutações relacionadas à analbuminemia [15].

Em relação à mutação dos ratos Nagase analbuminêmicos, primeiro identificou-se que não havia RNA mensageiro para a albumina no fígado destes animais[42], mas o mecanismo que determinava a ausência de RNA mensageiro não foi desvendado. Três anos mais tarde, o mesmo grupo de pesquisadores

identificou a mutação genética que causa a analbuminemia nestes animais: uma deleção de sete pares de base de DNA (5’ G-T-T-T-C-C-G 3’) de uma região intron do gene da albumina [43]. Esta deleção resulta na substituição da seqüência normal de nucleotídeo (5’ G-T-A-G-G-T-T-T-C-C 3’) pela seqüência 5’ G-T-A-G-C-G-A-G-C-T 3’, e no impedimento do *splicing* do RNA mensageiro da albumina.

1.2 Revisão sobre a dislipidemia do NAR

A colônia de NAR foi estabelecida através do cruzamento de ratos Sprague-Dawley que apresentavam espontaneamente hipercolesterolemia. Na primeira descrição desta colônia por Nagase e Shimamune [13], foi mostrado que o NAR apresentava colesterolemia 100% maior que a do rato controle e que havia uma alta correlação inversa (R = -0,92) entre os níveis de albumina plasmática e a colesterolemia [13]. Posteriormente vários estudos deste grupo e de outros pesquisadores caracterizaram bem o perfil lipídico do NAR [44, 45], porém os mecanismos de algumas destas alterações ainda não são ainda completamente entendidos. A distribuição de colesterol, triglicérides e de fosfolipídios através das lipoproteínas plasmática de diferentes densidades está representada na Figura 1 (modificada de Van tol et al [19]). Neste estudo [19], fica evidente que o NAR apresenta hipercolesterolemia, hipertrigliceridemia e hiperfosfolipidemia (Figura 1), e que estes lipídios se acumulam mais na partícula de LDL. Em adição, este estudo mostrou a inibição da síntese de colesterol pelo tratamento com pravastatina parece não ter eficácia em reduzir a hipercolesterolemia do NAR (Figura 1A), porém tal tratamento reduziu significativamente a concentração plasmática de triglicérides no NAR (Figura 1B).

Outra alteração marcante do perfil lipídico plasmático do NAR é o déficit de FFA. Os estudos indicam reduções de 65 a 80% na concentração de FFA plasmático [19, 44, 45] e também uma redistribuição dos FFA para as lipoproteínas [19], uma vez que não há o aceptor primário para o FFA (*i.e.* albumina). Esta deficiência de FFA plasmático parece ter repercussões fisiológicas importantes para o NAR, uma vez que estes animais apresentam algumas alterações relacionadas à menor disponibilidade de FFA [46], tais como o retardo de crescimento [13], a menor adiposidade [45], a intolerância ao exercício [47] e a intolerância a privação de carboidratos dietéticos quando expostos ao frio [48].

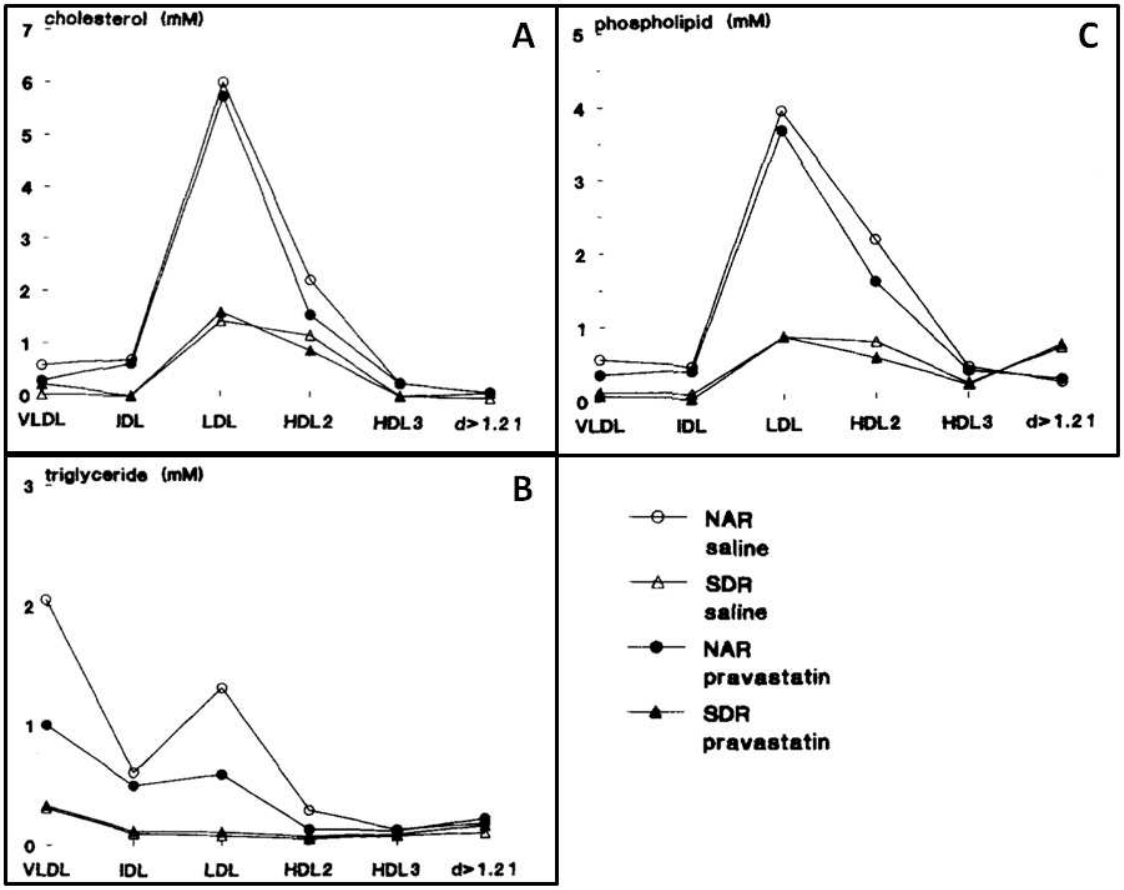

Figura 1. Distribuição do total de lipídios nas frações de lipoproteínas plasmáticas em ratas tratadas com salina ou pravastatina. As frações de lipoproteínas foram obtidas por gradiente de densidade e ultracentrifugação do plasma. SDR, ratos controles Sprague-Dawley. NAR, ratos Nagase analbuminêmicos. Figura modificada de Van tol et al[19].

O perfil lipídico do NAR é dependente do sexo. Especificamente, as fêmeas NAR apresentam uma hipertrigliceridemia mais severa do que o NAR macho quando comparados aos seus respectivos controles SDR [45, 49]. A figura 2, construída a partir de dados da literatura [17, 19, 45, 49, 50], mostra a diferença entre os sexos para colesterol, triglicérides e FFA plasmáticos. Trabalhos do grupo do Joles, um pesquisador muito ativo neste tema, demonstraram que o estrógeno é parcialmente responsável pela hipertrigliceridemia mais severa da fêmea NAR [49, 51]. Em um dos seus trabalhos, Joles e colaboradores observaram que a hipercolesterolemia da fêmea NAR também é mais severa em relação à do macho [49], porém este achado não se reproduziu em outro estudo [45] ou em nossas

mãos (*i.e*. o percentual de aumento na colesterolemia é similar entre macho e fêmea NAR quando comparado aos seus controles).

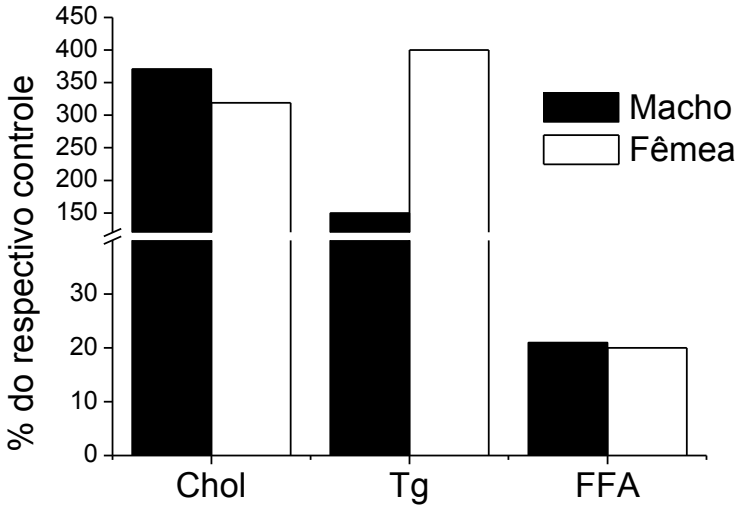

Figura 2. Percentual da alteração da concentração de lipídios plasmático do NAR em relação ao respectivo controle do mesmo sexo. Os dados são médias de valores disponíveis na literatura. Chol, colesterol. Tg, triglicérides. FFA, ácidos graxos livres.

Quanto aos mecanismos que delineiam estas alterações de triglicérides, colesterol e FFA plasmáticos, somente a hipertrigliceridemia teve seus mecanismos determinantes melhor abordados. Nesta sessão, somente as questões pertinentes a hipercolesterolemia serão revisadas, uma vez que a introdução do artigo apresentado no capítulo I desta tese contempla os mecanismos relacionados à hipertrigliceridemia e à deficiência de FFA plasmáticos. Enquanto a hipertrigliceridemia do NAR parece ser devida a menor taxa de remoção de triglicérides do plasma (ver discussão no capítulo I), há menos estudos a respeito do mecanismo da hipercolesterolemia nestes animais.

Alguns estudos mensuraram a taxa de remoção de colesterol da circulação do NAR através da análise da cinética do desaparecimento de lipoproteínas radioativamente marcadas [51-53]. No entanto, há diferenças importantes entre estes estudos, tais como: densidade da lipoproteína analisada, fonte doadora da lipoproteína, e molécula radioativamente marcada (*i.e*. proteínas ou colesterol).

Dois deles avaliaram a remoção da partícula de HDL marcada radioativamente na proteína apoA1, e observaram que o NAR remove mais lentamente estas partículas do plasma [51-53]. O outro estudo, observou que o NAR também remove mais lentamente partículas de quilomicro marcadas radioativamente no colesterol [51]. Em um estudo conduzido com duas pacientes portadoras de analbuminemia congênita, também foi observado menor taxa de remoção de lipoproteínas que contém proteínas ApoB [54].

Dentre os estudos que abordaram a produção hepática de colesterol, a informação mais contundente é o achado de Joles et al mostrando que a síntese de colesterol hepática (incorporação de $^{3}H_2O$) é similar entre o NAR e o seu controle [50]. Quanto ao envolvimento da HMG-CoA redutase, enzima chave da via de biosíntese do colesterol, há um estudo que mostrou maior atividade desta enzima no fígado do NAR [49], enquanto dois outros mostraram não haver diferença entre NAR e seu controle [50, 55] e são, desta forma, concordantes com os resultados de Joles et al [50]. Outro fato que diminui a importância da produção hepática de colesterol na hipercolesterolemia do NAR é o achado que o tratamento do NAR com pravastatina (inibidor da HMG-CoA redutase) não reduz a hipercolesterolemia destes animais [19]. É importante destacar que o metabolismo de sais biliares e o fluxo biliar não diferem entre NAR e o seu controle [56]. Com base nestes estudos, parece que a hipercolesterolemia do NAR é primariamente determinada pela menor taxa de remoção de colesterol do plasma.

2. Objetivos:

A apresentação desta tese está organizada na forma de artigos; cada um dos três apresentados abaixo tem seu objetivo específico e compreende um capítulo desta tese. Há também um quarto artigo, metodológico, sobre o uso do *probe* safranina para se avaliar o potencial elétrico transmembrana mitocondrial, apresentado no Apêndice I.

Capítulo I	Lack of plasma albumin impairs intravascular lipolysis and explains the associated free fatty acids deficiency and hypertriglyceridemia.
	Objetivos: investigar os mecanismos da hipertrigliceridemia e do déficit de FFA plasmático.
Capítulo II	Chronic low plasma free-fatty acids in congenital albumin deficiency: enhanced insulin secretion and glucose tolerance despite of hypertriglyceridemia.
	Objetivos: investigar a provável repercussão do perfil lipídico, (déficit de FFA plasmático) sobre o metabolismo de glicose.
Capítulo III	The higher susceptibility of congenital analbuminemic rats to Ca^{2+}-induced mitochondrial permeability transition is associated with the increased expression of cyclophilin D and nitrosothiol depletion.
	Objetivos: estudar as funções mitocondriais neste modelo de dislipidemia secundária.
Apêndice I	Safranine as a fluorescent probe for the evaluation of mitochondrial membrane potential in isolated organelles and permeabilized cells.
	Objetivos: descrever o uso da Safranina como um indicador fluorescente de potencial elétrico transmembrana em suspensões de mitocôndria isolada e células permeabilizadas.

3.1 Capítulo I

Figueira TR, Vercesi AE, Oliveira HCF. Lack of plasma albumin impairs intravascular lipolysis and explains the associated free fatty acids deficiency and hypertriglyceridemia. **Lipids in Health and Disease**, 9: 146, 2010.
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Lack of plasma albumin impairs intravascular lipolysis and explains the associated free fatty acids deficiency and hypertriglyceridemia

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Abstract

Background: Abnormalities in lipid metabolism and transport are hallmarks in analbuminemic Nagase rats (NAR) and humans. Triglyceridemia is nearly 3- to 5-fold higher in female NAR than in control Sprague-Dawley rats (SDR). Also, NAR present with a severe plasma free fatty acid (FFA) deficit. There are conflicting results regarding the mechanisms underlying NAR hypertriglyceridemia.

Objective: We aimed at investigating whether liver lipogenesis and triglyceride secretion rates into the plasma contribute to the hypertriglyceridemia in NAR. We also studied whether heparin or albumin administration would release the hypothesized lipolysis inhibition in NAR.

Methods: The incorporation of tritiated water into lipids and the linear accumulation rate of plasma triglycerides after Triton WR1339 injection were the measures of liver lipogenesis and triglyceride secretion rates.

Results: Lipogenesis (596 ± 40 vs. 929 ± 124 μmol ^3H_2O/g/h) and triglyceride (4.25 ± 1.00 vs. 7.04 ± 1.68 mg/dL/min) secretion rates were slower ($P \leq 0.05$) in fasted NAR than in control SDR. The injection of either heparin or albumin elicited an increase in NAR plasma FFA levels over time. FFA levels reached control levels 90 min after the albumin administration, increasing from 0.36 ± 0.05 to 1.34 ± 0.16 mEq/L ($P \leq 0.05$). These results indicate that the lack of plasma albumin inhibits intravascular lipolysis and causes the FFA deficit observed in NAR.

Conclusion: NAR hepatic triglyceride synthesis and output do not contribute to NAR hypertriglyceridemia. We propose that the lack of albumin diminishes intravascular lipolysis which reduces the plasma triglyceride removal rate and explain both NAR hypertriglyceridemia and FFA deficiency.

Introduction

Albumin is the most abundant plasma protein in mammals and plays an important role as a carrier for a variety of molecules. Albumin possesses a high binding affinity for certain divalent cations, bilirubin, free fatty acids (FFA) and other molecules, including xenobiotics. Due to its high abundance and low molecular weight in relation to other major plasma proteins, albumin is responsible for about 80% of the total plasma oncotic pressure [1]. Therefore, albumin is a key regulator of fluid distribution between the plasma and interstitial compartments in physiological conditions; although the absence of plasma albumin can be well compensated by increased liver secretion of other proteins which help to maintain nearly normal plasma oncotic pressure [2]. Other less understood functions may also be ascribed to albumin, since its plasma depletion and redox modification have been demonstrated in several pathological conditions [3-5].

Both humans and rats (Nagase rats, NAR) suffering from congenital analbuminemia present with minor clinical symptoms despite the ascribed roles of albumin [6-8]. Abnormalities in fat storage and in lipid metabolism and/or transport are hallmarks of analbuminemic NAR and humans [9-15]. Plasma triglyceride (TG) levels are 3- to 5-fold higher in female NAR than in control Sprague-Dawley rats (SDR). A more severe dyslipidemia in female NAR seems to be partially driven by estrogen [13]. Compared to male, female NAR also develop more

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efficient adaptations to the lack of plasma albumin and do not present a deficit in total plasma proteins [2]. In analbuminemic plasma, very low density lipoprotein triglycerides (VLDL-TG) accounts for the largest fraction of total triglycerides [15]. Conversely, plasma FFA levels are severely decreased in NAR compared to control [14]. In addition, analbuminemic rats and human subjects present with hypercholesterolemia [6,9,11,12]. This lipemic phenotype predisposes the affected individuals to a higher risk of developing cardiovascular disease, while low levels of FFA may contribute to other metabolic-related alterations, such as the fatigue experienced by analbuminemic individuals [7,11,12,16].

Studies addressing the basis of hypertriglyceridemia in NAR have shown conflicting results. Catanozzi et al. [17] reported a faster triglyceride secretion rate from the liver and a slightly faster VLDL-protein plasma clearance rate. In contrast, others have demonstrated that NAR present with a slower plasma clearance rate of chylomicron-TG [16] or a similar clearance rate of VLDL- and chylomicron-TG [18]. In addition, lower post-heparin plasma lipoprotein lipase (LPL) activity has been described in NAR [13,19]. Regarding the hepatic production of triglycerides, Joles et al. [9] showed that the liver lipogenesis rate is not different between NAR and SDR. Therefore, no clear conclusions can be drawn concerning the main metabolic processes contributing to NAR hypertriglyceridemia. Differences in criteria for animal group pairing (by body mass or by age), sex, basal states (fasted vs. fed) and methodology may account for these data discrepancies.

Despite the known roles of albumin as a FFA acceptor and carrier [20], there are no data on the mechanism underlining the low levels of plasma FFA found in NAR. It is still unknown whether the FFA deficit is a consequence of lipolysis inhibition, either directly by lower LPL activity [13,19] or by a deficiency in plasma albumin, or by both. Abnormalities, such as lower body mass and adiposity [10] and the exercise and starvation intolerance observed in NAR [21,22], seem consistent with a condition of lower FFA availability and flux into tissues [16,23].

In this work, we found that liver lipogenesis and hepatic triglyceride output are decreased in fasted NAR compared to control. We also show that the low levels of plasma FFA are due to an inhibition of intravascular lipolysis caused by an acceptor (albumin) deficiency in NAR. All together, our data indicate that hepatic triglyceride production does not contribute to NAR fasting hypertriglyceridemia.

Materials and methods

Animal housing and plasma variable assessments

NAR founders were kindly donated by Dr. Eder Quintão from the Lipid Laboratory at the University of São Paulo Medical School and were bred and maintained in the animal facility of our department. The experiments were approved by the Committee for Ethics in Animal Experimentation at the University and are in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Academy of Sciences. The rats had free access to a standard laboratory rodent chow diet (Nuvital CR1) and water. Female rats, 12-14 weeks old, were housed at 22 ± 2°C on a 12 h light-dark cycle. Unless otherwise stated, all the measurements were carried out in female NAR after a 20-h fasting, and the blood samples were taken from the tip of the tail. Triglycerides (Roche Diagnostics), total cholesterol (Roche Diagnostics), total proteins (Bradford, Sigma) and free fatty acids (Wako Chemicals, Japan) were determined in the plasma using enzymatic-colorimetric methods according to the instructions of the manufacturers.

Total liver lipogenesis

The rate of total lipid synthesis was measured *in vivo*. The rats were injected intraperitoneally with 20 mCi of tritiated water (^3H_2O) dissolved in an isotonic saline solution as described previously [24]. One hour later, blood samples were obtained from the tip of the tail from anesthetized rats (ketamine 75 mg/kg and xylazine 10 mg/kg), and livers were excised, minced, and saponified; after which lipids were extracted with hexane [25]. Radioactivity in the lipid extract was measured in a Beta Counter (LS6000 Beckman Instruments, California, USA). The specific activity of ^3H_2O was measured in the plasma in triplicate. The rate of total lipid synthesis was calculated as μmol of ^3H_2O incorporated into the lipids per gram of tissue in one hour (μmol/g/h).

Triglyceride secretion rate

The hepatic triglyceride secretion rate to the plasma was measured after the administration of Tyloxapol WR1339 (Sigma), as described previously by Otway & Robinson [26]. Briefly, rats were bled to obtain baseline plasma samples and then injected intravenously (tail vein) with Tyloxapol WR1339 at a dose of 400 mg/kg (15% v/v solution in saline). Blood samples (50 μL) were collected at 15, 30, 60, and 90 min after the Tyloxapol WR1339 injection. Triglyceride levels were measured in the plasma samples. The slopes from the linear regression of the triglyceride concentrations vs. time curves were calculated (mg/dL/min). Experiments were performed between 10:00 and 13:00 h. Additional groups of overnight sucrose-fed (5% in drinking water) female and male NAR were also subjected to this experiment. These rats were fed with carbohydrate only to avoid interference of absorbed fat.

Albumin- or heparin-stimulated *in vivo* lipolysis

Sodium heparin (Cristália, Brazil) and bovine serum albumin (globulin and fatty acid free, Sigma) were diluted in

saline. The rats were injected with heparin (1,000 U/Kg, intraperitoneally) or with albumin (1.3 g/kg, intravenously). Blood samples were taken before (0 min) and after the injections (30 and 90 min). After blood plasma was separated by centrifugation (4°C, 3000 g for 10 min), plasma FFA levels were measured. FFA levels were also measured in an aliquot of the injected albumin solution but were not detected. In order to control for likely changes in plasma volume due to the intravascular albumin injection (0.27 mL per 100 g), additional NAR and SDR groups were injected with saline at the same volume. The amount of injected albumin (1.3 g/kg) was expected to elevate plasma albumin concentrations from nearly zero to above 17 mg/mL in NAR [17]. As conducted in females, the lipolysis induced by intravenous albumin injection was also studied in fasting male NAR, but plasma free glycerol levels, instead of plasma FFA, were measured enzymatically (Sigma).

Table 1 Body and liver masses and plasma lipid and protein concentrations in analbuminemic (NAR) and control (SDR) rats

	SDR	NAR
Body Mass (g)	285.8 ± 24.8 N = 9	216.4 ± 8.7* N = 9
Liver Mass (g/100 g)	3.4 ± 0.2 N = 9	5.5 ± 0.4* N = 9
Plasma Proteins (g/L)	59.3 ± 8.0 N = 4	55.7 ± 3.3 N = 4
Triglycerides (mg/dL)	62.2 ± 20.1 N = 10	316.8 ± 99.8* N = 10
Cholesterol (mg/dL)	72.6 ± 15.5 N = 10	189.2 ± 22.8* N = 10
FFA (mEq/L)	1.53 ± 0,22 N = 8	0.48 ± 0,06* N = 8

Mean ± SD. *$P \leq 0.05$ vs. SDR. Female Nagase rats (NAR) and Sprague-Dawley rats (SDR) were paired by age (12 to 14 weeks old). Liver mass expressed as gram per 100 g of total body mass. FFA: free fatty acids.

In vitro serum lipolysis

Blood samples from resting rats were taken without anti-clogging agents and immediately centrifuged (4°C, 3000 g for 10 min) to separate the serum. Aliquots of the serum (120 μL) were incubated with exogenous albumin (final concentration of 30 mg/mL) or its vehicle (saline) at 37°C for 10 min with continuous shaking. Aliquots (20 μL) were withdrawn and quickly frozen in liquid nitrogen for later FFA analysis. Lipolysis was calculated as an increase in FFA concentration between the time points (zero to 10 min).

Statistical analysis

Data are represented as mean ± SD. Differences between means were assessed by a Student's t-test or one- or two-way analysis of variance (ANOVA) with repeated measures. A one-way ANOVA was run within the NAR albumin group to test for the effects of the albumin injection (Figure 2A) because we compared the pre-and post-injection values within this group only. The significance level was set at $P \leq 0.05$.

Results

The body and liver masses and the concentrations of the plasma lipids and proteins from NAR and SDR are shown in Table 1. As compared to SDR of the same age, 12-14 week old female NAR exhibited lower body and higher liver masses, a 5-fold increase in TG levels, a 2.6-fold increase in cholesterol levels, and one-third of the FFA concentrations ($P \leq 0.05$). Enlarged NAR livers probably reflect hypertrophy to compensate for the albumin deficiency, leading to additional synthesis and secretion of other plasma proteins.

The liver lipogenesis rate (596 ± 40 vs. 929 ± 124 μmol ^3H_2O/g/h) and the hepatic TG secretion rate into the plasma (4.25 ± 1.00 vs. 7.04 ± 1.68 mg/dL/min) were significantly ($P \leq 0.05$) reduced in the 20-h fasted NAR compared to the SDR controls (Figure 1). In overnight sucrose-fed female (n = 4 each group) and male (n = 6 each group) rats, the hepatic TG secretion rates into the plasma were not significantly different between NAR and SDR: 5.27 ± 0.73 vs 6.08 ± 0.56 mg/dL/min for females, and 2.95 ± 0.77 vs 3.67 ± 0.82 mg/dL/min for males. Therefore, irrespective of feeding state and sex, liver triglyceride output does not explain the higher TG plasma levels in NAR.

Data from early *in vitro* studies with purified LPL [20,27] showed that albumin allows lipolysis to proceed by acting as a FFA acceptor. Here, we show that an intravascular injection of albumin into NAR elicited an almost linear increase in the plasma FFA levels over time (Figure 2A). NAR plasma FFA levels increased 4-fold from a baseline of 0.36 ± 0.05 to nearly the control levels of 1.34 ± 0.16 mEq/L 90 min after the injection ($P \leq 0.05$) (Figure 2A). SDR and NAR groups were also injected with saline to control for possible changes in plasma volume, which actually did not cause significant fluctuations in plasma FFA levels (Figure 2A). A heparin injection, which is known to increase the plasma activity of LPL [28], promptly increased ($P \leq 0.05$) the plasma FFA levels in NAR but had no effect on SDR (Figure 2B). The highest increase in plasma FFA levels in NAR was observed 30 min after the heparin injection (from a baseline of 0.52 ± 0.12 to 1.40 ± 0.41 mEq/L, $P \leq 0.05$) and decreased thereafter (Figure 2B). Despite the lack of albumin acting as a FFA acceptor in NAR, the heparin injection was effective in acutely increasing plasma FFA levels, probably because lipoproteins can carry an additional load of FFA [29]. The lack of an observable effect of heparin on SDR's plasma FFA levels is probably due

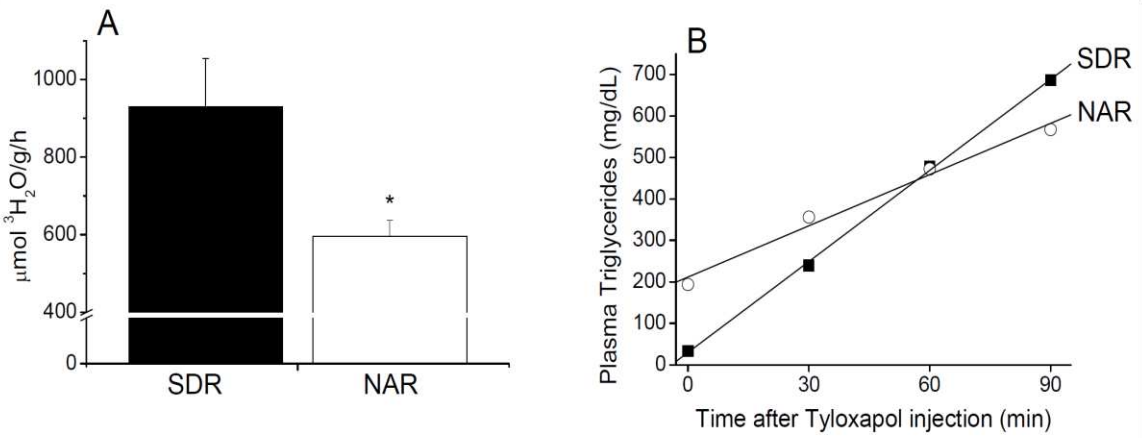

Figure 1 Total liver lipogenesis rate and triglyceride secretion rate into the plasma. A. Mean ± SD of tritiated water incorporation into total lipids after one hour of ^3H_2O injection. N = 4 in each group. B. Representative curves of plasma triglyceride response to Tyloxapol WR1339 injection. After the Tyloxapol WR1339 inhibition of triglyceride lipolysis, the rate of plasma triglyceride accumulation was taken as the measure of triglyceride secretion into the plasma. The mean ± SD of the triglyceride secretion rates were 7.04 ± 1.68 and 4.25 ± 1.00 mg/dL/min ($P \leq 0.05$) for SDR and NAR, respectively. N = 5 in each group. *$P \leq 0.05$.

to low concentrations of substrate (TG-rich lipoproteins) after the long fasting period.

The effect of exogenous albumin (final concentration of 30 mg/mL) on *in vitro* lipolysis was also studied. The addition of albumin to NAR serum promoted a nearly 3-fold stimulation in the amount of released FFA (Figure 3). The extent of lipolysis, taken as FFA accumulation above the initial values in NAR serum, increased ($P \leq 0.05$) from 120.3 ± 21.1 (saline control) to 310.2 ± 51.5 uEq/L in the presence of exogenous albumin.

Since female NAR present with more prominent abnormalities in lipid metabolism compared to male NAR [12,30], we also investigated whether intravenous albumin injection would stimulate plasma lipolysis in fasted male NAR. Similarly to female NAR, plasma lipolysis was stimulated in male NAR as indicated by a 2-fold increase ($P \leq 0.05$) in plasma free glycerol levels 90 min after the intravenous injection of albumin (from a pre-injection level of 125.0 ± 17.7 to 260.4 ± 31.3 umol/L at the 90th min after the injection).

Discussion

Our results indicate that hypertriglyceridemia in analbuminemic rats does not occur as a result of increased hepatic triglyceride synthesis and output (Figure 1). Consequently, a slower removal rate of the plasma

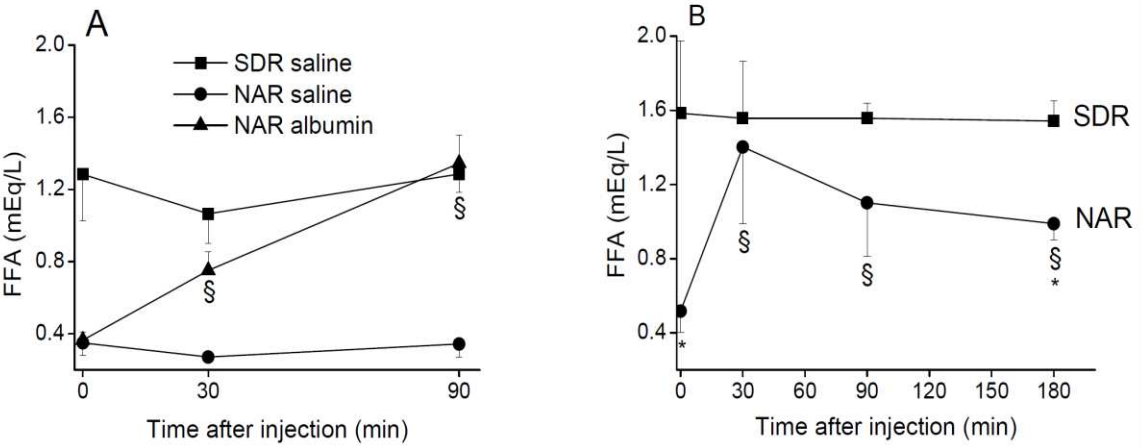

Figure 2 Plasma free fatty acid (FFA) response to albumin and heparin injections. Mean ± SD of the plasma FFA concentrations. A. Rats were injected (intravenous) with either albumin or saline. B. Rats were injected (intraperitoneal) with heparin. N = 4 in each group. The effects of the albumin injection were assessed within the NAR albumin group with a one-way repeated ANOVA. A two-way repeated ANOVA was used for the heparin study. §$P \leq 0.05$ vs. pre-injection values within the NAR group. * $P \leq 0.05$ vs. the SDR group at the same time point.

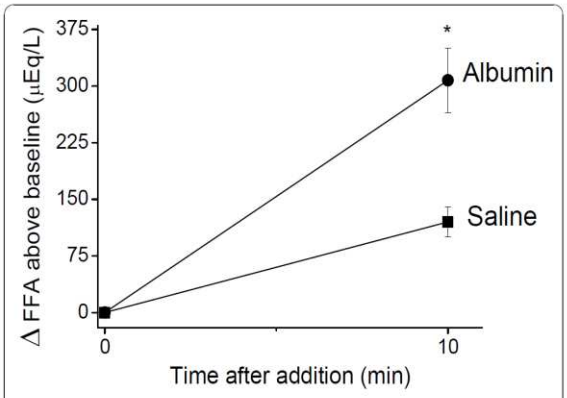

Figure 3 Exogenous albumin stimulates *in vitro* free fatty acid (FFA) release into fresh NAR serum. NAR serum was incubated at 37°C in the presence of exogenous albumin or vehicle (saline). Immediately after these additions and after 10 min of incubation, aliquots were taken for FFA analysis. Data are mean ± SD of the change in FFA concentration above the baseline value. N = 3 and the samples were incubated in duplicate. * $P \leq 0.05$ vs. saline at the same time point.

triglycerides seems to delineate the origin of NAR hypertriglyceridemia. Triglyceride removal from the circulation by targeted tissues occurs after their breakdown into glycerol and FFA [31]. Most of the FFA (~80%) that are derived from the LPL-mediated lipolysis of plasma triglyceride-rich lipoproteins are mixed within the pool of albumin-bound FFA before their transport into tissues [23,32]. Our data show that plasma lipolysis is stimulated by the exogenous administration of albumin in NAR, both *in vivo* (Figure 2A) and *in vitro* (Figure 3). These data support that the lack of albumin limits LPL activity and thus, slows down the removal rate of plasma triglyceride-rich lipoproteins.

Regarding the contribution of hepatic triglyceride production our findings differ from previous studies that reported this process is enhanced in fasted female NAR [13] and in glucose-fed male NAR [17]. However, in sucrose-fed NAR groups, our results are in accordance with data from Joles et al. [9] that showed similar liver lipogenesis rates between fed NAR and controls. Some discrepancies may be due to important differences in study design. First, we paired the rat groups by age (3-month old) and not by body mass as did Catanozzi et al. [17]. It is noteworthy that NAR are lighter than SDR at the same age. Second, the time of the day at which the experiments were conducted must be considered, since circadian rhythms are well known modifiers of sterol and lipid biosynthesis. While we performed the metabolic studies in the morning from 10:00 to 13:00 h, Catanozi et al. [17] evaluated TG secretion rates from 17:00 to 19:00 h. Third, fasting period and/or type of diet prior to the experiments could also interfere in the results. Thus, based on our findings one can conclude that, either during fasting or fed states, hepatic triglyceride production does not contribute to NAR hypertriglyceridemia. However, since we measured liver lipogenesis only, we may not exclude that extra-hepatic lipogenesis may contribute to NAR hyperlipidemia as previously shown by Joles et al. [9].

A plasma FFA deficit (Table 1) has long been described in NAR [14] and is also observed in analbuminemic humans [7]. However, its physiological basis has never been experimentally addressed. Our attempts to stimulate plasma lipolysis, either by releasing the hypothesized lipolysis inhibition (albumin injection) or by increasing plasma LPL activity (heparin injection), caused a significant increase in plasma FFA levels in NAR, although only albumin induced a virtually complete correction of the NAR plasma FFA deficit (Figure 2A). The fast rise in plasma FFA levels induced by the injection of heparin in NAR (Figure 2B) reveals that the restraint on intravascular lipolysis is not due to defective LPL activity. Therefore, the metabolic machinery involved in plasma triglyceride breakdown and FFA release is not severely compromised in NAR and the plasma FFA deficit reflects an impaired lipolysis due mainly to a lack of plasma albumin as an acceptor. However, since lower LPL activity was reported in NAR parametrial adipose and heart tissues [10,19], it is possible that the lower tissue-specific LPL may constitute another restraint for FFA flux into the NAR tissues [23].

Previous *in vitro* enzymatic studies using purified LPL clearly demonstrated that albumin acts as a FFA acceptor and allows lipolysis to proceed [20,27]. Yet, this fundamental role of albumin in the control of lipolysis was not known to operate *in vivo*. The present results (Figure 2A) expand these previous *in vitro* findings of albumin playing a permissive role in intravascular lipolysis. To avoid any misinterpretation between albumin having a FFA sparing effect or stimulating lipolysis, we used an *in vitro* setup to assess the effects of exogenous albumin on the NAR serum lipolysis rate (Figure 3). This result clearly indicates that albumin is affecting lipolysis, i.e. stimulating the appearance of FFA in NAR plasma.

In conclusion, although NAR present with marked liver hypertrophy, the hepatic triglyceride synthesis and output rates do not contribute to the analbuminemic hypertriglyceridemia. Instead, an impaired triglyceride removal from the circulation may cause this alteration. It is proposed that a lack of plasma albumin to act as a FFA acceptor inhibits the intravascular lipolysis rate, which in turn hampers plasma triglyceride breakdown and its plasma removal.

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Authors' contributions
TRF designed some experiments, conducted the experiments and the assays, analyzed the data, and wrote the manuscript. **AEV** designed the study, critically discussed the results, proposed new experiments, and critically reviewed the final version of the manuscript. **HCFO** designed the experiments, discussed the experimental protocols, discussed the data, and wrote the manuscript. All authors have read and approved the final version of this manuscript.

Competing interests
The authors declare that they have no competing interests.

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3.2 Capítulo II

Figueira TR, Ribeiro RA, Ignacio-Souza LM, Vercesi AE, Carneiro EM, Oliveira HCF. Chronic low plasma free-fatty acids in congenital albumin deficiency: enhanced insulin secretion and glucose tolerance despite of hypertriglyceridemia. **Submetido**.

Chronic low plasma free-fatty acids in congenital albumin deficiency: enhanced insulin secretion and glucose tolerance despite of hypertriglyceridemia

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Background: Congenital analbuminemic individuals and rats (NAR) present several metabolic abnormalities, such as hypertriglyceridemia and plasma free fatty acids (FFA) deficiency. Plasma FFA are known modulators of insulin secretion and action on target tissues, thus affecting glucose metabolism. **Objective**: we aimed at studying glucose homeostasis and insulin secretion in female NAR. **Methods**: plasma biochemical variables and insulin were measured in fed and fasted NAR and controls (SDR, Sprague-Dawley rats). Pancreatic islet insulin secretion and glucose homeostasis tests were performed in fasted rats. **Results**: plasma glucose levels were similar between NAR and SDR, irrespective of feeding status. However, fed insulinemia was higher (~37%, $P \leq 0.05$) in NAR than in SDR. NAR displayed increased glucose tolerance compared to SDR ($P \leq 0.05$). This enhanced glucose tolerance was associated with higher insulinemia after the glucose load, and with similar insulin sensitivity between the groups. Compared to SDR, isolated islets from NAR secreted more insulin in response to glucose, leucine, alpha-ketoisocaproate and KCl. Despite similar liver glycogen content in fully fed condition, NAR had lower glycogen content (~40%, $P \leq 0.05$) than SDR after 6-h fasting. The injection of a gluconeogenic substrate, pyruvate, elicited a faster rise in NAR glycemia than that of SDR. **Conclusions**: NAR display enhanced glucose tolerance, insulin secretion, and glyconeogenic flux. The higher glucose tolerance is attributed to enhanced islet responsiveness to secretagogues, while peripheral insulin sensitivity seems not to be involved in such alteration. Supposedly, the enhanced glucose metabolism is a chronic compensatory adaptation to decreased FFA availability in NAR.

Keywords: analbuminemia, analbuminemic, glycogen depletion, carbohydrate metabolism, fuel metabolism, gluconeogenesis

Abbreviations: SDR, Sprague-Dawley rats; NAR, Nagase analbuminemic rats; FFA, free fatty acids; GSIS, glucose stimulated insulin secretion; GDH, glutamate dehydrogenase; KIC, α-ketoisocaproate; GTT, glucose tolerance test; ITT, insulin tolerance test

1. INTRODUCTION

Congenital analbuminemia is a rare autosomal recessive disorder characterized by very low levels of plasma albumin (< 1 mg/mL) in the absence of renal and intestinal protein loss (1). This abnormality results from a negligible hepatic albumin production due to mutations in the albumin gene (2). The first case of human analbuminemia was reported in 1954 (3), thereafter several other cases of human analbuminemia have been uncovered (4). By selective breading of spontaneously hyperlipidemic Sprague-Dawley rats (SDR), Nagase and Shumiya (5) established a colony of rats that were finally found to be in fact analbuminemic: the Nagase analbumimemic rats (NAR). These rats resemble well many features of the human familial analbuminemia (6) first reported in the 50s.

The most remarkable metabolic alterations in congenital analbuminemic humans and rats are the high levels of plasma lipids and lipoproteins (7-10), but a myriad of co-morbidities have been described in the reported cases of human analbuminemia and in NAR (3, 10-12). Many endocrine-metabolic alterations have been described in NAR, such as hypothyroidism, hyperparathyroidism, lower adiposity and body mass, and plasma free fatty acid deficiency (7, 13-16). However, the clinical-physiological significance of these abnormalities may have been overlooked because NAR live well under standard laboratory housing conditions.

Kawaguchi et al (17) showed that NAR are severely intolerant (*i.e.* die earlier) to food deprivation at 5°C ambient temperature. Interestingly, these authors found that the survival time was greatly extended when NAR were fed with carbohydrate, while fat feeding did not extend survival. It has long been known that NAR present a plasma FFA deficiency (16). Recent data from our group indicate that NAR plasma FFA deficiency arises from diminished intravascular lipolysis due to the lack of albumin to act as a FFA acceptor (18). Importantly, nearly 80% of lipolysis-derived FFA are mixed within the pool of albumin-bound FFA before their uptake into peripheral tissues (19, 20). Therefore, the absence of plasma albumin and the FFA deficiency in NAR is expected to reduce FFA flux into tissues. The lower NAR adiposity (7) and the carbohydrate-dependent intolerance to starvation (17) are all in accordance with a lower FFA availability and flux into tissues (20). Taken together, these studies suggest that NAR energy metabolism relies more on carbohydrate than on fat. An impaired capacity to shift from carbohydrate to fat oxidation could also be expected under conditions where the utilization of endogenous carbohydrate stores is normally spared.

Pancreatic insulin secretion and insulin action on peripheral tissues are affected by FFA levels in the circulation (21-23). Chronic high levels of plasma FFA may lead to glucose intolerance due to the impairment of glucose-stimulated insulin secretion (GSIS) and of insulin sensitivity in target tissues (21, 24). Conversely, acute rising of plasma FFA levels enhances GSIS (25, 26), whereas an impaired GSIS is observed when plasma FFA levels are acutely lowered (25, 27). However, the effects of chronic low levels of plasma FFA on insulin secretion or action have never been investigated. Hypothetically, the chronic low plasma FFA availability might facilitate glucose metabolism in NAR tissues (21, 23). Nonetheless, the influence of chronic low plasma FFA levels on GSIS seems less predictable.

From this framework, our general hypothesis was that NAR present enhanced carbohydrate metabolism. Indeed, in the present study, we demonstrate that NAR rely more on liver glycogen stores upon fasting and present a higher glucose tolerance. The higher glucose tolerance was associated with enhanced GSIS, while there was no difference for peripheral insulin sensitivity between NAR and their controls (SDR). In addition, we showed that the acute correction of NAR plasma FFA deficiency does not affect the glucose tolerance in NAR.

2. MATERIAL and METHODS

2.1 Materials

^{125}I human insulin was purchased from Genesis (São Paulo, SP, Brazil). Unless

otherwise stated, all other reagents were the highest grade from Sigma (St Louis, MO, USA).

2.2 Animal housing and plasma variable assessments:

Nagase analbuminemic rats (NAR) founders were kindly donated by Dr. Eder Quintão from the Lipid Laboratory at the University of São Paulo Medical School and were bred and maintained in the animal facility of our department. Control rats (SDR, Sprague-Dawley rats) were from the breeding colony at our university. The experiments were approved by the Committee for Ethics in Animal Experimentation of the university. Female rats, 12-14 weeks old, were housed at 22 ± 2 °C on a 12 h light-dark cycle. Body masses (mean ± SD) were 286 ± 25 g and 216 ± 9 g, respectively, SDR and NAR. The rats had free access to standard laboratory rodent chow diet (Nuvital CR1, Parana, Brazil) and water. The measurements of variables were carried out in the fed state and/or after 20-h fasting. Blood samples were taken from the tip of tail for the determination of triglycerides (Roche diagnostics, Mannheim, Germany), total cholesterol (Roche diagnostics, Mannheim, Germany) and free fatty acids (Wako chemicals, Osaka, Japan) in plasma using colorimetric enzymatic methods according to the instructions of the manufacturer. Plasma insulin levels were measured by radioimmunoassay as described elsewhere (28) and blood glucose with a portable analyzer (Accu-Chek Advantage, Roche Diagnostic, Switzerland).

2.3 Glucose tolerance test (GTT):

Fasted rats were intraperitoneally injected with glucose (2 g/kg body weight). Blood samples were taken from the tip of the tail before (0 min) and after (30, 60 and 120 min) the glucose injection for the determination of blood glucose. The glucose response to GTT was calculated as the area under the glucose curve of each rat (Microcal Origin 8.0, Northampton, MA, USA). Another set of animals were used to measure plasma insulin and glucose levels at time points (0, 15 and 60 min) during the GTT.

2.4 Insulin tolerance test

Fasted rats were intraperitoneally injected (1.5 U/kg body weight) with human recombinant insulin (Biohulin®, Biobrás, São Paulo, Brazil). Blood samples were taken from the tip of the tail before (0 min) and after (4, 8, 12, 16 and 20 min) the insulin injection for blood glucose analysis.

2.5 Islet isolation, static insulin secretion and islet insulin content

Islets were isolated by collagenase digestion of the pancreas. For static incubations, four islets from each group were first incubated for 30 min at 37 °C in Krebs–bicarbonate (KBR) buffer with the following composition (in mM): 115 NaCl, 5 KCl, 2.56 $CaCl_2$, 1 $MgCl_2$, 10 $NaHCO_3$, 15 HEPES, supplemented with 5.6 mM glucose, 3 g/L BSA, and equilibrated with a mixture of 95% O_2/5% CO_2 to give a pH of 7.4. This medium was then replaced with fresh buffer and the islets were incubated for 1 h with crescent glucose concentrations (2.8, 5.6, 8.3, 11.1, 16.7 or 33.3 mM glucose) or with 2.8 mM glucose without or with: 10 mM leucine, 15 mM alpha-ketoisocaproic acid (KIC) and 30 mM KCl. At the end of the incubation period, the insulin content of the medium was measured by radioimmunoassay (RIA). For islet insulin content, groups of 4 islets were collected and transferred to tubes of 1.5 mL. One mL of deionized water was added to these samples, followed by sonication of the pancreatic cells (3 times, 10 sec pulses), and the islet insulin content was measured by RIA.

2.6 Effects of acute correction of NAR plasma FFA deficiency on glucose tolerance:

We recently showed, in another study conducted in parallel (18), that intravascular albumin injection into NAR elicits an elevation in NAR plasma FFA levels over time. A complete abrogation of NAR plasma FFA deficiency was observed at the 90th min after the albumin injection. To investigate whether the acute correction of plasma FFA deficiency rescues the higher glucose tolerance in NAR, these rats were injected with albumin (1.3 g/kg, intravenously) and the intraperitoneal GTT was performed 90 min later, as described above. SDR and NAR

control groups were injected an isovolume of saline (2.7 mL *per* kg).

Table I
Plasma biochemical variables in analbuminemic (NAR) and control (SDR) rats

		SDR	NAR
Glucose (mg/dL)	Fasting	70.4 ± 3.5	69.7 ± 2.3
	Fed	97.7 ± 2.5	98.4 ± 3.8
Insulin (ng/mL)	Fasting	0.24 ± 0.06	0.29 ± 0.06
	Fed	0.95 ± 0.11	1.3 ± 0.12*
TG (mg/dL)	Fasting	78.5 ± 11.7	406.9 ± 34.3*
	Fed	59.9 ± 4.8	269.7 ± 46*
Chol (mg/dL)	Fasting	67.5 ± 5.8	215.7 ± 22.0*
	Fed	58.3 ± 6.8	191.4 ± 11.8*
FFA (mM)	Fasting	0.94 ± 0.1	0.35 ± 0.03*
	Fed	0.6 ± 0.04	0.11 ± 0.01*

Mean ± SEM. TG, triglycerides; Chol, cholesterol; FFA, free fatty acids. *$P \leq 0.05$ *vs*. SDR. Female NAR and SDR were paired by age (12 to 14 weeks old)

2.7 The response of liver glycogen content to short-term fasting:

The livers were harvested after the decapitation of fed and fasted rats. The fed rats had full access to food and were sacrificed at 2:00 h. Other groups of fasted rats were sacrificed after 6 and 14h following food withdraw. Glycogen was measured by the phenolsulfuric method (29) in liver samples (pieces weighting 15-20 mg of the two major lobules) after KOH digestion and ethanol precipitation of glycogen. The content of liver glycogen was calculated against a standard curve built with D-glucose.

2.8 Glycemic response to pyruvate injection:

The increase in blood glucose after pyruvate injection is dependent on neoglucogenesis flux (30). Fourteen hours fasted rats were injected intraperitoneally with pyruvate (2g *per* kg of body mass). A 0.333 g/mL stock solution of sodium pyruvate salt (Merck, Germany) was prepared in saline just before use. Blood glucose was measured before and after (15, 30, 60, 90, 120 and 180 min) the injection. The time to reach peak glycemic value after pyruvate injection was taken as an index of glucose augmentation rate in the blood.

2.9 Statistical Analysis:

Data are presented as means ± SEM. Differences among means were assessed by Student t-test, on-way or two-way ANOVA followed by the Newman-Keuls post-hoc test. Mann-Whitney test was used for non-parametric data. Correlation was assessed by Person coefficient. The significance level was set at $P \leq 0.05$ in all tests.

3. RESULTS

Plasma levels of lipids, glucose and insulin from fasted and fed rats are reported in **Table 1**. NAR show higher plasma levels of triglycerides and cholesterol irrespective of feeding state ($p < 0.05$), while FFA levels were lower in both fed and fasted NAR than SDR ($P \leq 0.05$). Plasma glucose did not differ between NAR and SDR. Insulinemia was higher in fed NAR than in fed SDR ($P \leq 0.05$), but there was no difference between groups in the fasted state (**Table 1**).

An intraperitoneal GTT revealed a higher ($P \leq 0.05$) glucose tolerance in NAR than in SDR (**Fig.1A and 1B**). The insulinemic response during GTT was also assessed at the 0, 15 and 60 min time points. NAR presented higher ($P \leq 0.05$) insulinemia at the 15^{th} and 60^{th} min after the glucose load (**Fig. 1C**). The peripheral insulin sensitivity, as indicated by glycemic decay after the intraperitoneal injection of insulin (ITT), did not differ between NAR and SDR (**Fig. 2**). These data suggest that the higher glucose tolerance in NAR seems to be caused by enhanced GSIS. Therefore, we measured insulin secretion rates from pancreatic islets isolated from both groups of rats. **Figure 3A** shows the pattern of static insulin secretion in response to increasing concentrations of glucose (2.8 – 33.3 mM). NAR presented an increased insulin secretion in response to all concentrations of glucose tested ($P \leq 0.05$).

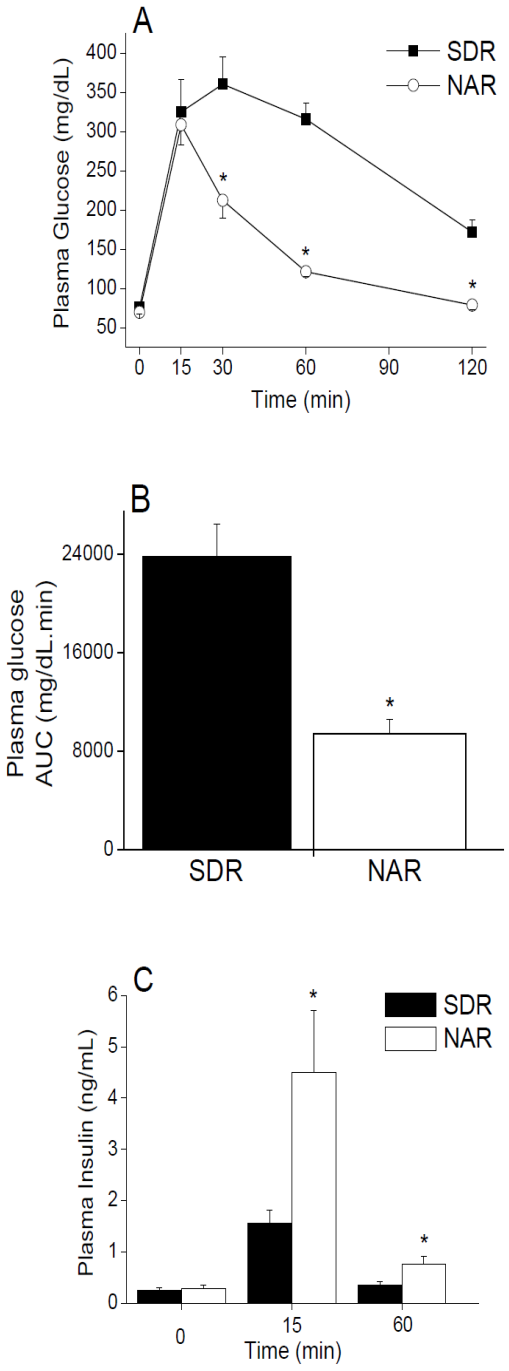

Figure 1. **Enhanced glucose tolerance and insulin response to a glucose load in NAR**. Mean ± SEM. **A**, blood glucose responses to intraperitoneal glucose tolerance test (GTT). **B**, area under the curves (AUC) of blood glucose during GTT. **C**, plasma insulin levels during the GTT. * P ≤ 0.05 *vs.* SDR.

These *in vitro* data are in agreement with the enhanced insulin response to the glucose load observed *in vivo* (**Fig. 1C**). The total content of insulin *per* islet was not different between NAR and SDR, respectively, 47.5 ± 4.7 and 49.4 ± 6.0 ng/islet.

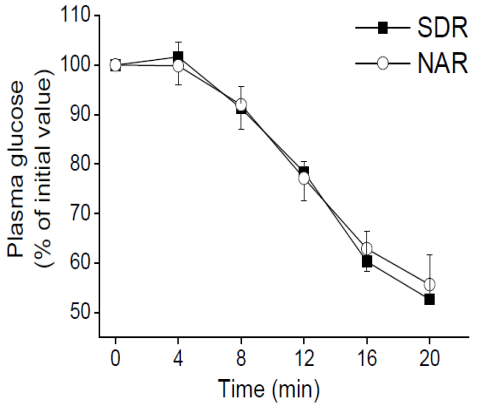

Figure 2. Blood glucose response to the insulin tolerance test (ITT). Nearly identical decay in glycemia after intraperitoneal injection of insulin between SDR and NAR.

We also evaluated the effect of L-leucine on insulin secretion (**Fig. 3B**). L-leucine is known to stimulate insulin secretion by two mechanisms; firstly, by L-leucine catabolism, and secondly by allosteric activation of glutamate dehydrogenase (GDH) that promotes an increase in the formation of α-ketoglutarate from glutamate (i.e. anaplerosis) (31, 32). We found higher insulin secretion from NAR islets ($p < 0.05$) upon L-leucine stimulation (**Fig. 3B**). However, KIC, a product of L-leucine catabolism that does not possess the GDH stimulatory action, elicited similar stimulation of insulin secretion in NAR and SDR islets (**Fig. 3B**). Thus, the higher NAR islet responsiveness to L-leucine arises from its stimulation of GDH activity. An enhanced insulin secretion was also observed in NAR islet ($p < 0.05$) in response to direct membrane depolarization by KCl (**Fig. 3B**), a stimuli that bypass islet nutrient metabolism.

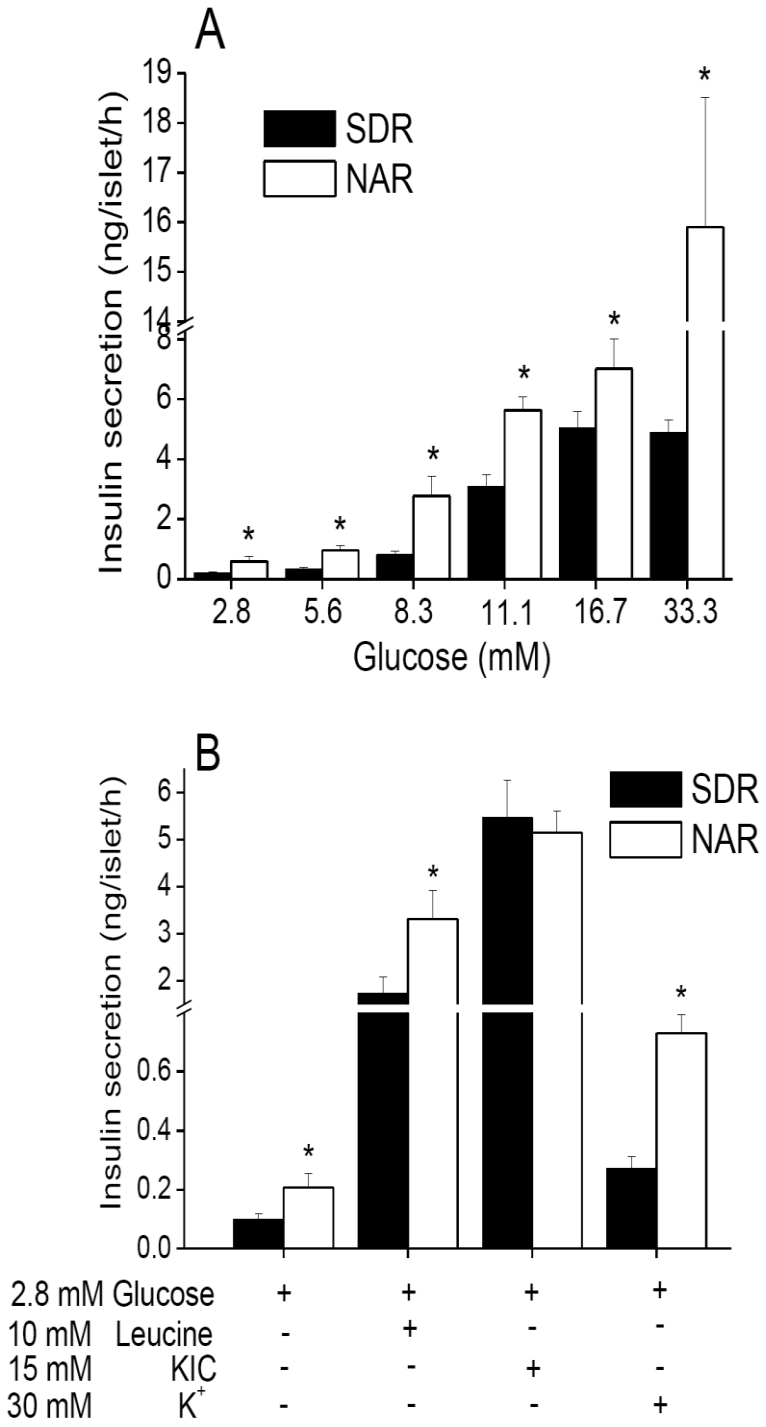

Figure 3. Enhanced insulin secretion from isolated pancreatic islets. **A**, insulin secretion rates in response to increasing glucose concentrations.**B**, insulin secretion rates in response to leucine, α-ketoisocaproate (KIC) and potassium chloride (K^+). *P ≤ 0.05 *vs.* SDR.

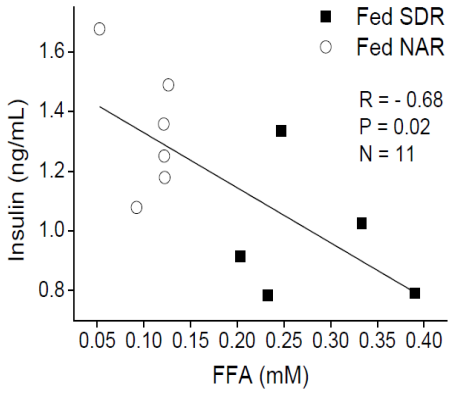

Figure 4. Correlation between the levels of plasma FFA and insulin. Data from fed SDR and NAR groups were pooled together and analyzed with the Pearson correlation test.

These data on islet insulin secretion seem to explain the higher *in vivo* insulinemia in fed NAR than in SDR (**Table 1**). It is noteworthy that the levels of plasma insulin were inversely correlated ($r = -0.68$, $P \leq 0.05$) with the plasma levels of FFA when data from fed NAR and SDR were analyzed together (**Fig. 4**). In attempt to directly approach the influence of FFA on the glucose homeostasis, a GTT was performed after the correction of NAR plasma FFA deficiency by injecting the rats with albumin (18). However, this acute abrogation of NAR plasma FFA deficiency did not affect the enhanced glucose tolerance in NAR (**Fig. 5**).

Since NAR are more tolerant to glucose (**Fig. 1**) and secretes more insulin (**Fig. 3**), it is expected that the metabolism of these animal relies more on carbohydrate than on fat stores. Thus, liver glycogen depletion in response to short-term fasting was evaluated (**Fig. 6**). Liver glycogen content does not differ between NAR and SDR in the fully fed state. Six hours of fasting induced a nearly 40% depletion in NAR liver glycogen stores ($p \leq 0.05$), while the SDR liver glycogen content was still unchanged at this time-point. Prolongation of fasting to 14 hours led to a marked depletion and a similar content of liver glycogen between NAR and SDR. These data on glycogen depletion raised the hypothesis that gluconeogenesis may

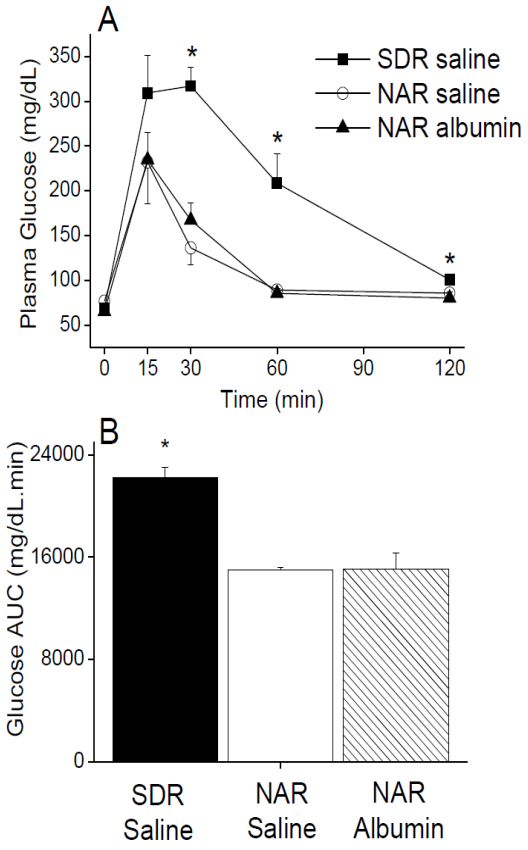

Figure 5. Acute correction of FFA deficiency in NAR by intravascular albumin injection does not restore the enhanced glucose tolerance. A, blood glucose response to intraperitoneal glucose tolerance test (GTT) in NAR injected with albumin (NAR albumin) and experimental controls (SDR and NAR injected with saline). **B**, area under the curves of blood glucose during the GTT. *$P \leq 0.05$ *vs.* other groups.

also be enhanced in order to cope with a greater carbohydrate utilization in NAR. Indeed, the injection of the gluconeogenic precursor pyruvate into fasted rats elicited a faster increase in blood glucose over time in NAR than in SDR (**Fig. 7A**). The peak glycemia was attained earlier in NAR (30 min) than in SDR (~120 min) (**Fig. 7B**), thus indicating a faster gluconeogenic rate in the former. The faster decay of glycemia after peak value observed in NAR may be a consequence of enhanced islet insulin responsiveness to high blood glucose (**Fig. 1C**).

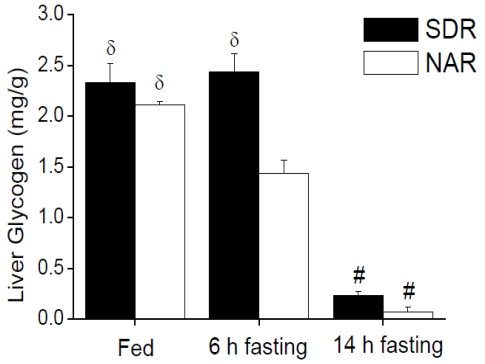

Figure 6. Faster liver glycogen depletion in response to short-term fasting in NAR. Liver glycogen content was measured in different set of rats at fully fed state and after 6 and 14h fasting. Bars not sharing the same symbol are different from each other ($P \leq 0.05$).

4. DISCUSSION

In this work we showed that analbuminemic rats (NAR) display improved glucose tolerance as a consequence of enhanced GSIS (**Fig. 1C and 3A**). The enhanced GSIS from isolated NAR pancreatic islet was elicited by both metabolic and depolarizing stimulus (**Fig. 3C**). Increased *in vivo* fed insulinemia inversely correlated with FFA plasma levels (**Fig. 4**). Peripheral insulin sensitivity seems not to be altered in this condition of high plasma triglycerides and low FFA availability (**Fig. 2**).

It is known that the glucose intolerance associated with metabolic diseases can be improved by interventions such as weight loss, exercise or pharmacotherapy (33), but very few conditions/interventions are associated with improved glucose tolerance above the normal levels. Exercise trained individuals are good examples of enhanced glucose tolerance above normal (34). Generally, these interventions that improve glucose tolerance in health or in diseases do so by mainly increasing insulin sensitivity in target tissues, with no or mild involvement of pancreatic insulin secretion (33). Another point is that only rare metabolic states present enhanced islet insulin secretion above the peripheral tissue needs, such as the insulinoma (35), the hypothalamic obesity syndrome (36), and the congenital hyperinsulinemia (37). These are all unhealthy abnormal states associated with enhanced insulin secretion in response to glucose or other stimulus (*e.g.* glucagon, pyruvate) and often results in hypoglycemic crisis (35, 37). The enhanced insulin secretion in NAR may comprise the first evidence of a positive compensatory adaptation of pancreatic islet to cope with a limitation of oxidative fuels. Lower plasma FFA availability in NAR could shift substrates partitioning towards carbohydrate (21).

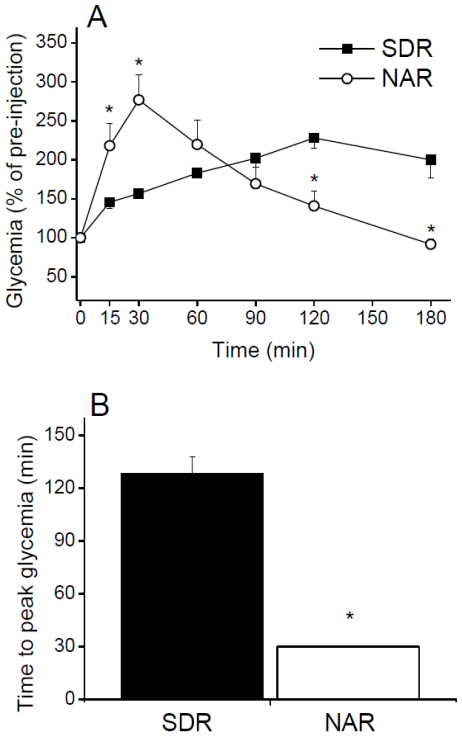

Figure 7. Blood glucose response to intraperitoneal injection of the gluconeogenic precursor pyruvate (2 g/kg). **A**, mean ±_SEM of glycemia expressed as percent of pre-injection value. **B**, mean ± SEM of the time to achieve peak glycemia after pyruvate injection. *$P \leq 0.05$ *vs.* SDR at the same time point (panel A). Mann-Whitney test was used for data in panel B.

Indeed, our results show greater NAR liver glycogen depletion (**Fig. 6**) and enhanced gluconeogenic flux (**Fig. 7**) in response to fasting, both supporting the hypothesis that NAR energy metabolism rely more on carbohydrate than on

FFA. In addition, a previous study showed that carbohydrate but not fat feeding can extend the survival of NAR maintained in cold environment (17). Therefore, the enhanced GSIS may be functionally important for NAR fuel metabolism.

Chronic high levels of plasma FFA may cause insulin resistance and impair GSIS (21, 24). Acute lowering of plasma FFA is experimentally feasible and impairs insulin secretion (22, 25, 27), but models to study the effects of chronic low FFA are lacking. Plasma FFA deficiency in analbuminemia has long been known (6, 16). However, our attempt to directly address this question only indicates that the acute correction of NAR plasma FFA deficiency does not affect glucose tolerance (**Fig. 5**). On the other hand, the adaptations for enhanced insulin secretion in NAR seem not to be acutely reversible since the isolated islets also present enhanced secretory capacity in the *in vitro* milieu (**Fig. 3**). Thus, the direct investigation of the role of plasma FFA deficiency on enhanced glucose tolerance may require a chronic correction of NAR plasma FFA deficit. Nonetheless, an inverse statistical correlation (**Fig. 4**) between plasma FFA levels and insulinemia strengthens the hypothesis that a decreased plasma FFA levels may play a role in the enhanced insulin secretion in NAR.

It is also important to consider that NAR also display lower adiposity among their metabolic abnormalities, so it can not be ruled out that endocrine signals from adipocytes, such as leptin and adiponectin, may contribute to the enhanced insulin secretion.

Hypertriglyceridemia is generally associated with insulin resistance and type II diabetes (38). High plasma levels of triglycerides increases FFA availability to tissues as a result of their peripheral hydrolysis by lipoprotein lipase. Overexpression of LPL leads to FFA oversupply to tissues and cause cellular lipotoxicity and insulin resistance (39). Shedding light on this issue, the unique NAR lipidemic phenotype, *i.e.* simultaneous hypertriglyceridemia and FFA deficiency, indicate that hypertriglyceridemia *per se* does not negatively affect glucose tolerance nor insulin sensitivity. So, the relationship between hypertriglyceridemia and glucose intolerance may arise from the fact that hypertriglyceridemia is generally accompanied by high levels of plasma FFA.

We conclude that analbuminemic rats present enhanced carbohydrate metabolism, namely higher glycogen disposal, neoglucogenic flux and glucose tolerance. The latter is a consequence of enhanced insulin secretion in response to glucose and does not involve abnormal peripheral insulin sensitivity. The mechanisms through which analbuminemia results in enhanced insulin secretion are not known and deserve further research.

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3.3 Capítulo III

Figueira TR, Castilho RF, Saito A, Oliveira HCO, Vercesi AE. The higher susceptibility of congenital analbuminemic rats to Ca^{2+}-induced mitochondrial permeability transition is associated with the increased expression of cyclophilin D and nitrosothiol depletion. **Molecular Genetics and Metabolism**, epub ahead.

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The higher susceptibility of congenital analbuminemic rats to Ca^{2+}-induced mitochondrial permeability transition is associated with the increased expression of cyclophilin D and nitrosothiol depletion

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ABSTRACT

Congenital analbuminemia is a rare autosomal recessive disorder characterized by a trace level of albumin in blood plasma and mild clinical symptoms. Analbuminemic patients generally present associated abnormalities, among which dyslipidemia is a hallmark. In this study, we show that mitochondria isolated from different tissues (liver, heart and brain) from 3-month-old analbuminemic rats (NAR) present a higher susceptibility to Ca^{2+}-induced mitochondrial permeability transition (MPT), as assessed by either Ca^{2+}-induced mitochondrial swelling, dissipation of membrane potential or mitochondrial Ca^{2+} release. The Ca^{2+} retention capacity of the liver mitochondria isolated from 3-month-old NAR was about 50% that of the control. Interestingly, the assessment of this variable in 21-day-old NAR indicated that the mitochondrial Ca^{2+} retention capacity was preserved at this age, as compared to age-matched controls, which indicates that a reduced capacity for mitochondrial Ca^{2+} retention is not a constitutive feature. The search for putative mediators of MPT sensitization in NAR revealed a 20% decrease in mitochondrial nitrosothiol content and a 30% increase in cyclophilin D expression. However, the evaluation of other variables related to mitochondrial redox status showed similar results between the controls and NAR, *i.e.*, namely the contents of reduced mitochondrial membrane protein thiol groups and total glutathione, H_2O_2 release rate, and NAD(P)H reduced state. We conclude that the higher expression of cyclophilin D, a major component of the MPT pore, and decreased nitrosothiol content in NAR mitochondria may underlie MPT sensitization in these animals.

1. Introduction

Due to albumin's abundance in relation to total plasma proteins, albumin exerts a major contribution to total plasma coloidosmotic pressure and fluid distribution across body compartments under normal physiology [1]. Many other important roles of plasma albumin are usually less highlighted. At nearly 42 mg/mL, plasma albumin is quantitatively the main plasma free radical scavenger [2]. Nearly 80% of total plasma reduced thiol groups and nitrosothiols also reside in albumin molecules [3]. Owing to its binding properties, albumin can act as a sink, which confers protection against the toxicity of certain substances, including xenobiotics and also some endogenous metabolites such as bilirubin [1, 4]. Prominently, a low level of plasma albumin, which is a common feature of certain diseases, is an independent risk factor for cardiovascular disease and all-cause mortality [5, 6].

Astonishingly, despite the mentioned roles of plasma albumin, humans and rats are viable with only trace levels of plasma albumin (<1 mg/mL) [7, 8]. Human congenital or familiar analbuminemia is a rare autosomal recessive disorder where the primary defects are mutations in the albumin gene [10]. These mutations result in a lack of liver albumin synthesis and, consequently, very low levels of plasma albumin. Although congenital analbuminemia is rare among humans, the understanding of some of its features was aided by the establishment of a colony of mutant Nagase analbuminemic rats (NAR), which closely resemble the human disease [11]. Similar to human patients, albumin is not produced in the liver of NAR, which ultimately results in a severe plasma albumin deficiency (values as low as 0.042 mg/mL) [9, 12]. In both species, the total plasma protein level is slightly below the reference range as the lack of albumin is quite well-compensated by the secretion of other proteins, mainly globulins [7, 8, 11].

A hallmark of analbuminemia is an abnormal plasma lipid and lipoprotein profile. NAR and individuals with analbuminemia present high levels of triglycerides, cholesterol and lipoproteins [13-15]. A unique feature of analbuminemic dyslipidemia is a plasma free fatty acid (FFA) deficiency [13, 14, 16]. Hyperlipidemia is a major risk factor for many diseases that generally involve lipotoxicity and cellular

Abbreviations: SDR, Sprague-Dawley rats; NAR, Nagase analbuminemic rats; FFA, free-fatty acid; MPT, mitochondrial permeability transition.

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stress [17]. Our group has recently reported on abnormal mitochondrial functions in primary hypertriglyceridemic (ApoCIII transgenic mice) and hypercholesterolemic (LDL receptor knockout mice) mice [18–22]. A common alteration between these two hyperlipidemic mouse models previously studied by our group is a higher susceptibility to Ca^{2+}-induced inner mitochondrial membrane permeability transition (MPT) [18, 21]. MPT is a process that can lead to cell death as it is followed by mitochondrial energy failure and the release of pro-apoptotic factors from this organelle [23]. MPT is involved in many pathological processes, which encompass cellular Ca^{2+} overload and oxidative stress (*e.g.*, ischemia–reperfusion) [23]. In Ca^{2+}-loaded mitochondria, the occurrence of MPT is favored by oxidative stress [24-30], and more recently nitric oxide and nitrosothiols have emerged as important players in MPT regulation as well [31–33].

In this paper, we aimed at studying mitochondrial (dys)function in dyslipidemic analbuminemic rats.

2. Material and methods

2.1. Chemicals and reagents

Unless otherwise stated, all utilized chemicals were of the highest grade and purchased from Sigma (St. Louis, MO, USA). Chemical abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid). EDTA, 2,2′,2″,2‴-(ethane-1,2-diyldinitrilo)tetraacetic acid. EGTA, glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid. FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone. HEPES, 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid. SDS, sodium dodecyl sulfate. t-BOOH, *tert*-butyl hydroperoxide.

2.2. Animal housing and plasma variable assessments

NAR founders were kindly donated by Dr. Eder Quintão (University of São Paulo Medical School) and were bred and maintained in the animal facility of our department. Control rats (SDR, Sprague-Dawley rats) were from the breeding colony at our university. The experiments were approved by the Committee for Ethics in Animal Experimentation at the university and are in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Academy of Sciences. Rats were housed at 22 ± 2 °C on a 12-h light-dark cycle with free access to a standard laboratory rodent chow diet (Nuvital CR1, Nuvital, Curitiba, PR, Brazil) and water. Groups of 3-month-old male rats were used for the measurements described below.

Plasma variable measurements were carried out after 20 h of fasting, and blood samples were taken from the tip of the tail. Triglycerides (Roche Diagnostics, Mannheim, Germany), total cholesterol (Roche Diagnostics, Mannheim, Germany), total proteins (Bradford reagent, St. Louis, MO, USA) and free fatty acids (Wako Chemicals, Osaka, Japan) were determined in the plasma using enzymatic-colorimetric methods according to the manufacturer's instructions. For the remaining experiments with isolated mitochondria, organs were harvested from fed rats after their sacrifice by decapitation in the morning (9–10 AM). Livers were also harvested from 21-day-old male NAR and SDR rats for mitochondrial isolation after sacrifice by cervical dislocation.

2.3. Mitochondrial isolation, incubation conditions and respirometry

Mitochondria from liver, heart, and brain were isolated by conventional differential centrifugation, as detailed and described elsewhere [34–36]. The protein concentration of final mitochondrial suspensions was determined using a modified Biuret assay. Unless otherwise stated, all the experiments with isolated mitochondria were performed at 28 °C in a standard reaction medium (125 mM sucrose, 65 mM KCl, 2 mM K_2HPO_4, 1 mM $MgCl_2$, 10 mM HEPES buffer (pH 7.2)) containing a cocktail of NADH-linked substrate (3.4 mM malate, 1.86 mM α-ketoglutarate, 2.1 mM pyruvate, 2.1 mM glutamate). Oxygen consumption was measured using a Clark-type electrode (Yellow Springs Instruments, Yellow Springs, OH, USA) in a 1.3-mL glass chamber equipped with a magnetic stirrer and filled with standard reaction medium supplemented with EGTA (200 μM). Stimulated respiration by oxidative phosphorylation (State 3 respiration) was elicited by the addition of ADP to a final concentration of 300 μM. Phosphorylation efficiency (ADP/O) was calculated as the molar ratio between the amount of added ADP and the oxygen consumed during State 3. For all experiments with functioning mitochondria, the equipments employed had magnetic stirrers and a temperature-controlled water bath. The final concentration of mitochondrial proteins was 0.5 mg/mL in the respiratory and MPT assays.

2.4. Assessment of Ca^{2+}-induced Mitochondrial Permeability Transition (MPT)

In suspensions of isolated mitochondria, cyclosporin A-sensitive Ca^{2+}-induced MPT results in osmotic swelling, disruption of transmembrane electrical potential, and Ca^{2+} release to the medium; all of which can be spectrophotofluorometrically followed over time.

Mitochondrial swelling was accessed by incubating organelles in 2 mL of standard reaction medium, and the decrease in the absorbance of the mitochondrial suspension (measured at 540 nm in a Hitachi U-3000 spectrophotometer, Tokyo, Japan) was taken as mitochondrial swelling. Specifically for brain mitochondria, the light scattering (excitation and emission wavelength set at 540 nm) of the suspension was measured on a Hitachi 4010 spectrofluorometer (Hitachi, Tokyo, Japan) instead of taking absorbance measurements.

Disruption of the transmembrane electrical potential ($\Delta\psi$) was evaluated by incubating mitochondria in 2 mL of standard reaction medium supplemented with safranine (5 μM), and $\Delta\psi$ was assessed by following safranine O fluorescence changes on a spectrofluorometer (Shimadzu RF-5301 PC, Kyoto, Japan) operated at excitation and emission wavelengths of 495 and 586 nm, respectively, and slit widths of 3 nm [37].

Ca^{2+} retention capacity was determined in mitochondria incubated in 2 mL of standard reaction medium supplemented with 0.2 μM Calcium Green-5N (Molecular Probes, Invitrogen, Carlsbad, CA). Levels of external free Ca^{2+} were measured by recording the fluorescence of Calcium Green-5N on a spectrofluorometer (Shimadzu RF-5301 PC, Kyoto, Japan) operated at excitation and emission wavelengths of 506 and 532 nm, respectively, and slit widths of 5 nm. Three min after the addition of mitochondria (0.5 mg/mL) to the cuvette, boluses of 5 nmol of $CaCl_2$ were sequentially added every third min until the mitochondria began to release Ca^{2+} into the medium. The amount of $CaCl_2$ added prior to mitochondrial Ca^{2+} release was taken as the mitochondrial Ca^{2+} retention capacity, a quantitative approach to compare MPT between groups [38].

2.5. Mitochondrial releasable endogenous Ca^{2+}

Isolated mitochondria were suspended to a final concentration of 1.0 mg/mL in 2 mL of standard reaction buffer supplemented with 2 μM ruthenium red (an inhibitor of the calcium uniporter and mitochondrial Ca^{2+} uptake) and 40 μM arsenazo III (an indicator of external free Ca^{2+}). The differential absorbance of arsenazo III (685–665 nm) was measured on a dual wavelength spectrophotometer (DW 2000 Aminco, SLM Instruments, Urbana, IL, USA). After recording the initial differential absorbance of energized mitochondria, mitochondria were deenergized by FCCP to promote the release of endogenous Ca^{2+} into the medium. The amount of released Ca^{2+} was estimated by EGTA titration considering a 1:1 stoichiometry under this condition [39, 40].

2.6. Citrate synthase (CS) activity

The conversion of oxaloacetate and acetyl-CoA to citrate and SH-CoA catalyzed by citrate synthase was monitored by measuring the colorimetric product thionitrobenzoic acid [41]. Liver homogenates (16–20 μg/mL) were incubated at 30 °C in a buffer containing 50 mM Tris-HCl (pH 8.0), 0.1% Triton X-100, 250 μM oxaloacetate, 50 μM acetyl-CoA, and 100 μM 5,5′-dithiobis(2-nitrobenzoic acid). The increase in absorbance at 412 nm was monitored for 6 min using a microplate reader (PowerWave XS 2, BioTek Instruments, Winooski, VT, USA).

2.7. Mitochondrial hydrogen peroxide release (H_2O_2)

Mitochondrial suspensions (0.5 mg/mL) were incubated in standard reaction medium plus Amplex red (10 μM; Molecular Probes, Invitrogen, Carlsbad, CA) and horseradish peroxidase 1 U/mL. The fluorescence was monitored over time on a spectrofluorometer (Shimadzu RF-5301 PC, Kyoto, Japan) operated at excitation and emission wavelengths of 563 and 587 nm, respectively, and slit widths of 5 nm. The slope of the linear increase in fluorescence over time represents the rate of H_2O_2 released from isolated mitochondria. A standard curve was built with known concentrations of hydrogen peroxide.

2.8. Redox state of mitochondrial pyridine nucleotides

The autofluorescence of reduced pyridine nucleotides (NAD(P)H) in mitochondrial suspensions (1 mg/mL) was followed on a spectrofluorometer (Hitachi F-4500, Tokyo, Japan) operated at excitation and emission wavelengths of 366 and 450 nm, respectively, and slit widths of 5 nm. For this assay, rotenone (2 μM) was added to the incubation medium, and 5 mM of succinate was used as an energizing substrate instead of complex I-linked substrates [22]. As experimental controls, the prooxidant *tert*-butyl hydroperoxide (20 μM) was added to promote the oxidation of NADPH, and the substrate isocitrate (1 mM) was added at the end of each trace to feed isocitrate dehydrogenase and re-reduce $NAD(P)^+$ if oxidation had occurred. Known amounts of NADPH were added as a reference [22].

2.9. Glutathione levels

Glutathione (GSH) and oxidized glutathione (GSSG) were measured in isolated mitochondria by following the enzymatic recycling method described by Teare et al. [42]. Briefly, 250 μg of mitochondrial proteins were suspended to a final volume of 25 μL in water, and then a solution (1:1 ratio) of sulfosalicilic acid (11%) and Triton X-100 (0.11%) was added to these samples. After a brief incubation for 5 min at 4 °C under continuous shaking, the samples were centrifuged at 10,000 *g* for 10 min (4 °C), and the supernatant was saved for subsequent analyses of glutathione levels. To measure only oxidized glutathione, 10 μL of this supernatant were added to 110 μL of a reduced glutathione masking buffer (phosphate buffer (100 mM), EDTA (1 mM), 2-vinylpyridine (1.1%), pH 7.4) and incubated for 1 h at room temperature. The samples prepared for GSH and GSSG were then subjected to enzymatic recycling analyses in a recycling buffer system containing NADPH (300 μM), DTNB (225 μM), glutathione reductase (1.6 U/mL) and EDTA (1 mM) in 100 mM phosphate buffer (pH 7.4). The linear increase in absorbance at 412 nm over time was monitored using a microplate reader (PowerWave XS 2, BioTek Instruments, Winooski, VT, USA). A standard curve was built with known amounts of GSH and GSSG.

2.10. Reduced thiols of mitochondrial membrane proteins

The content of reduced thiols of mitochondrial membrane proteins was measured using the DTNB method, as detailed and described elsewhere [43, 44].

Table 1
General characteristics of SDR and NAR

	SDR	NAR
Body mass (g)	437 ± 47	357 ± 50*
Liver mass (g/100 g)	3.6 ± 0.4	4.5 ± 0.6*
Plasma protein (g/L)	60.7 ± 3.9	45.3 ± 6.5*
Triglycerides (mg/dL)	70.8 ± 30.2	112.1 ± 33.3*
Cholesterol (mg/dL)	76.7 ± 24.0	161.1 ± 30.9*
FFA (mEq/L)	1.09 ± 0.31	0.30 ± 0.05*

Mean ± SD. Liver mass was normalized by total body mass (g/100 g). Triglycerides and cholesterol levels were measured in blood plasma. FFA: plasma free fatty acid (mEq/L). N = 10 to 20.
* $P<0.05$.

2.11. S-nitrosothiols (S-NO)

The content of mitochondrial S-NO was determined using a modified Saville assay, as detailed by Park and Kostka [45] and Leite et al. [32].

2.12. Cyclophilin D expression

The abundance of cyclophilin D in mitochondrial suspensions was analyzed by western blotting. Ten micrograms of sample proteins were electrophoretically resolved in 12% acrylamide SDS-PAGE gel (150 V for 70 min). The proteins were then cold electrotransferred to nitrocellulose membranes (100 V for 90 min). Thereafter, we strictly followed the western blot protocol provided for the primary cyclophilin-D monoclonal antibody (MitoScience, Eugene, OR, USA). Briefly, the membrane was incubated with the primary antibody (1:1000 dilution in Tris-base saline plus 1% defatted milk) for 2 h at room temperature. The membrane was developed by a colorimetric method (amplified alkaline phosphatase immunoblot kit, Bio-Rad, Hercules, CA, USA) after incubation with the secondary biotinylated anti-mouse antibody (1:1000 dilution; ab6788, Abcam, Cambridge, MA, USA). The relative optical densities of bands were analyzed using the gel tool of the software Image J (N.I.H., USA). As a loading control, the samples were electrophoresed, and the gel was silver stained (Pierce Silver Stain Kit, Thermo Scientific, Rockford, IL, USA) for total proteins.

2.13. Statistical analyses

Data are expressed as mean ± SD. The normality of data was assessed using Shapiro–Wilk's test, and the differences between the groups were analyzed using Student's *t*-test. The significance level was set at $P<0.05$. All tests were performed using the software SigmaStat 3.1 (Systat, San Jose, CA, USA).

3. Results

Some characteristics of NAR and SDR are shown in Table 1. NAR presented a lower body mass, total plasma proteins and plasma FFA levels while liver masses, plasma cholesterol and triglycerides were

Table 2
Liver mitochondrial respiration and phosphorylation efficiency.

	SDR	NAR
V4 (nmol O/mg/min)	9.8 ± 3.6	8.9 ± 3.2
V3 (nmol O/mg/min)	62.1 ± 26.3	67.4 ± 27.3
RCR	6.9 ± 2.3	7.7 ± 2.1
ADP/O	2.52 ± 0.38	2.56 ± 0.38

Mean ± SD. The maximal respiration rate (V3) was elicited by ADP 300 μM. The resting respiration rate (V4) was considered the state after all of the added ADP had been phosphorylated. RCR, ratio between V3 and V4. Phosphorylation efficiency (ADP/O) is the ratio between the amount of added ADP and the amount of oxygen consumed to phosphorylate all the added ADP. N = 14.

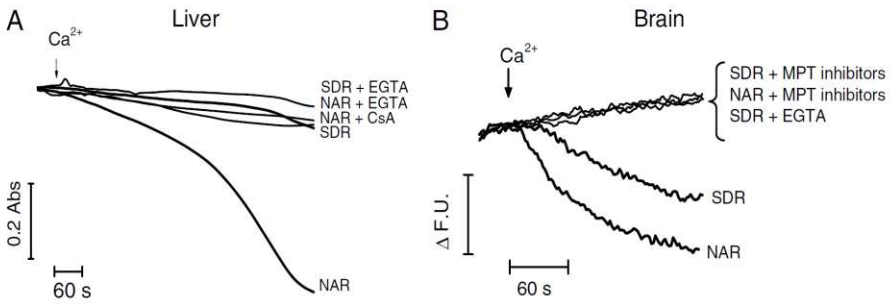

Fig. 1. Representative traces of Ca^{2+}-induced swelling in isolated mitochondria from liver and brain. SDR, Sprague-Dawley control rats. NAR, Nagase analbuminemic rats. A, calcium-induced swelling in liver mitochondria, N = 13. B, calcium-induced swelling in brain mitochondria, N = 5. Where indicated, EGTA (200 μM) or cyclosporin A (1 μM) was present as an experimental control. In A and B, the lower the ordinate value is, the higher the mitochondrial volume is.

Ca^{2+} concentration was 20 μM in both assays

increased. These characteristics are typical for the NAR phenotype and have also been reported by others [11, 46]. Table 2 depicts resting (V4) and ADP-stimulated (V3) liver mitochondrial respiration, the respiratory control ratio (RCR), and the phosphorylation efficiency (ADP/O ratio). None of these mitochondrial respiratory variables significantly differed between the NAR and SDR. Citrate synthase activity was assayed in liver homogenates as a marker of mitochondrial density in the tissue. Nearly identical values for citrate synthase activity were observed between the SDR and NAR, respectively, 131.2 ± 8.84 and 123.0 ± 9.0 mU/mg.

Figs. 1 and 2 show representative experiments conducted to assess MPT in mitochondria isolated from liver, brain and heart. In Fig. 1A and B, the swelling assay for MPT revealed that NAR liver and brain mitochondria undergo more extensive swelling than organelles from SDR when incubated in the presence of micromolar Ca^{2+} concentrations. Under similar incubation conditions, the safranine fluorescence assay for mitochondrial transmembrane electrical potential depicts an earlier disruption of transmembrane electrical potential for NAR heart mitochondria compared to SDR heart mitochondria (Fig. 2). The MPT inhibitor cyclosporin A (CsA) and the Ca^{2+} chelator EGTA were used as experimental controls, and these conditions are shown in the figures. Full inhibition of MPT in brain mitochondria requires the use of cyclosporin A plus ADP [38]. Under our experimental conditions, MPT results in minor changes in absorbance or light scattering of heart mitochondrial suspensions, so the safranine fluorescence assay for mitochondrial transmembrane electrical potential was conducted instead. Thus, these assays indicate that NAR mitochondria from different tissues were more susceptible to Ca^{2+}-induced MPT.

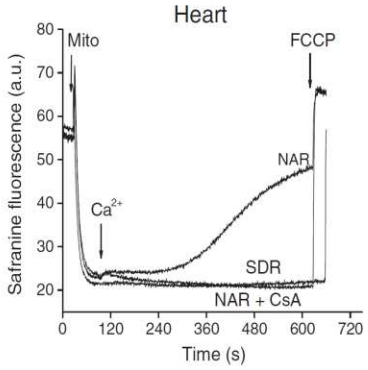

Fig. 2. Representative traces of Ca^{2+}-induced dissipation of heart mitochondrial transmembrane potential ($\Delta\Psi$), N = 4. Where indicated, EGTA (200 μM) or cyclosporin A (1 μM) was present as an experimental control. In this assay, the lower the safranine fluorescence signal was, the higher the mitochondrial $\Delta\Psi$ was. The uncoupler FCCP was added at the end to dissipate the mitochondrial $\Delta\Psi$.

Ca^{2+} concentration was 60 μM.

After the initial screening for MPT susceptibility across tissues, more in depth studies on the mechanisms of MPT sensitization in NAR were conducted with isolated liver mitochondria. The mitochondrial Ca^{2+} retention capacities are shown in Fig. 3. A representative raw experiment is depicted in Fig. 3A. A nearly 40% lower Ca^{2+} retention capacity was observed for NAR liver mitochondria compared to SDR (Fig. 3B). Under basal incubation conditions, the amount of releasable endogenous Ca^{2+} upon mitochondrial deenergization by FCCP did not significantly differ between the NAR and SDR liver mitochondria (2.64 ± 0.67 and 3.24 ± 0.69 nmol/mg, respectively), which indicated that the difference in the Ca^{2+} retention capacity between the groups upon exogenous Ca^{2+} challenging is not associated with different levels of preloaded Ca^{2+} into the organelles. To address the question of whether a plasma albumin-bound substance could leak into NAR cells and promote mitochondrial toxicity, experiments were also conducted in the presence of exogenous albumin. Under these experimental conditions, the NAR mitochondria still displayed a lower Ca^{2+} retention capacity (Fig. 3C). To investigate the hypothesis that the lower Ca^{2+} retention capacity was not a constitutive alteration present in NAR, we assessed this variable in 21-day-old rats (Fig. 3D), and no significant difference was observed at this age when compared to age-matched SDR.

Because young NAR (21-day-old) did not present MPT sensitization and oxidative stress is strictly involved in the MPT process [25-30], we evaluated whether MPT sensitization in NAR could result from a more oxidizing mitochondrial environment. Fig. 4A shows representative traces of mitochondrial NAD(P)H autofluorescence over time. Neither mitochondria from NAR nor SDR displayed spontaneous NAD(P)H oxidation over time, and a similar recovery of NAD(P)H fluorescence was also observed when mitochondria were challenged with *tert*-butyl hydroperoxide, which is metabolized by the glutathione peroxidase/reductase system at the expense of NADPH [47]. The rate of H_2O_2 release from mitochondria did not differ between organelles isolated from NAR and SDR (Fig. 4B). Also, the content of mitochondrial membrane-protein reduced thiols and total mitochondrial glutathione were not different between the NAR and SDR, Figs. 4C and D, respectively. Fig. 4E depicts the significantly lower content of nitrosothiols in NAR mitochondria compared to the control (~20%, $P<0.05$).

Last, we assessed mitochondrial cyclophilin D (CyD) protein expression, a major protein involved in the MPT process [48, 49]. Western blot analyses, which are shown in Fig. 5, revealed that CyD expression was approximately 30% greater ($P<0.05$) in NAR mitochondria compared to SDR.

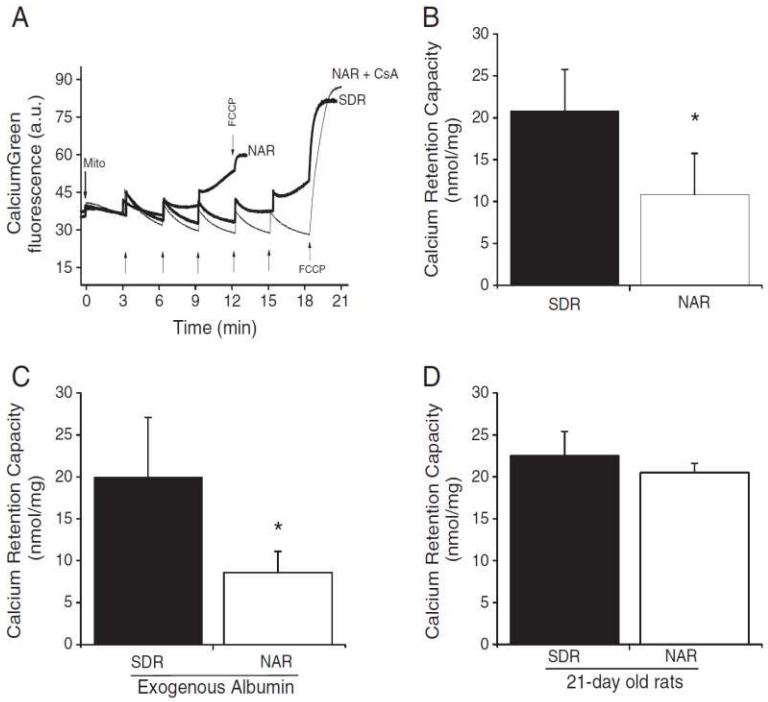

Fig. 3. Ca^{2+} retention capacity in liver mitochondria. A, representative traces showing the dynamics of the external Ca^{2+} concentration in response to sequential additions of Ca^{2+} (5 nmol every 3 min). The higher the fluorescence signal is, the higher the external Ca^{2+} concentration is. The sum of added Ca^{2+} over the steps before the spontaneous Ca^{2+} release from mitochondria was taken as the Ca^{2+} retention capacity and is shown as mean ± SD (panels B to D). B, Ca^{2+} retention capacity in mitochondria isolated from 3-month-old adult rats, N = 4. C, Ca^{2+} retention capacity in mitochondria from 3-month-old adult rats was assayed in the presence of exogenous albumin (0.2 mg/mL), N = 5. D, The Ca^{2+} retention capacity in mitochondria isolated from 21-day-old weaning rats, N = 5.

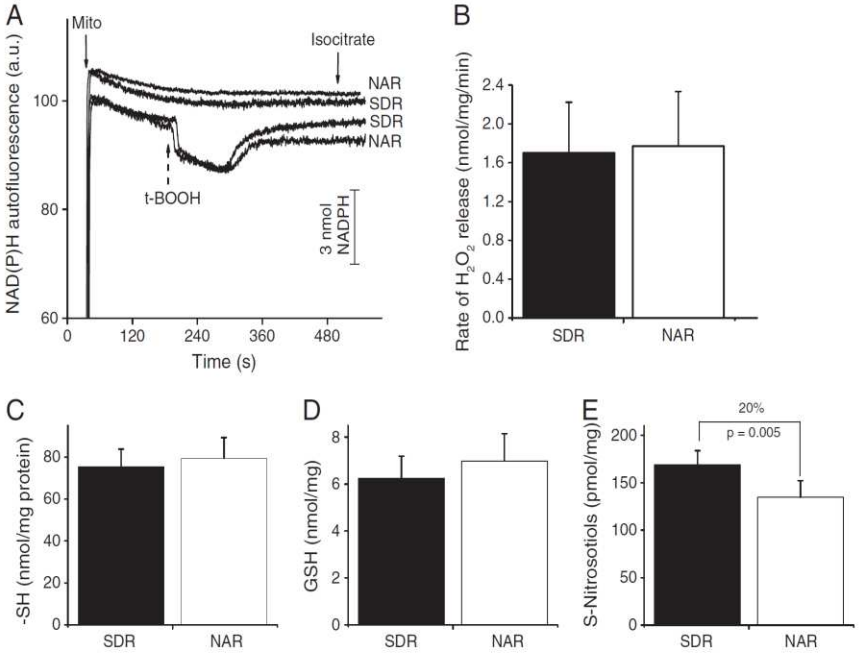

Fig. 4. Mitochondrial redox status. A, representative traces of reduced mitochondrial pyridines nucleotides (NAD(P)H) autofluorescence followed over time. Isocitrate was added at the end to feed isocitrate dehydrogenase and re-reduce NADP+ if oxidation had occurred. As an experimental control, the oxidant *tert*-butyl hydroperoxide (t-BOOH) was added to elicit NADPH oxidation. These experimental control curves were displaced downwards for better visualization. The addition of isocitrate did not evoke an increase in autofluorescence in any of the experiments. N = 6. B, mitochondrial release of hydrogen peroxide, N = 5. C, mitochondrial membrane proteins reduced thiol content, N = 5. D, mitochondrial content of total glutathione, N = 8; The GSH/GSSG ratio is not shown but was not different between the groups. E, mitochondrial content of S-nitrosothiols (S-NO), N = 6.

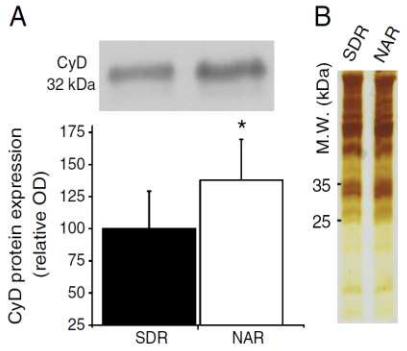

Fig. 5. Mitochondrial cyclophilin D (CyD) protein expression. Protein (10 μg) from isolated liver mitochondria was electrophoresed in SDS-PAGE gel and subsequently western blotted for CyD. A, means ± SD of relative optical densities of CyD bands, N = 4. B, as sample loading controls, samples were electrophoresed in SDS-PAGE gel. The gel was subsequently silver stained for total proteins (means ± SD of relative densities of the silver stained lanes were 100 ± 13 and 92 ± 21% for the SDR and NAR, respectively).

4. Discussion

In this work, we show that mitochondria from analbuminemic rats present a higher susceptibility to Ca^{2+}-induced permeability transition, which was associated with a nearly 20% depletion of nitrosothiols and overexpression of mitochondrial CyD in liver mitochondria (Figs. 4E and 5). A higher propensity for MPT opening was also observed in mitochondria isolated from NAR brain and heart (Figs. 1 and 2). Although MPT is a well-characterized process in *in vitro* systems (*e.g.*, isolated mitochondria and cells), its relevance in physiology and diseases has gained experimental support only in the last few years [49]. There is a growing body of evidence that MPT opening contributes to tissue degeneration/dysfunction in a variety of diseases [49–51]. However, little is known about the molecular events through which some physiological and pathological conditions (*e.g.*, heart failure or physical exercise) affect the Ca^{2+} threshold for MPT opening [52-55], which make mitochondria more or less susceptible to Ca^{2+}-induced MPT. CyD is a well- established component of MPT, and its genetic ablation or pharmacological inhibition (*e.g.*, cyclosporin A or analogs) greatly increases the Ca^{2+} threshold for MPT pore opening [48, 49]. Similar to our results, a higher CyD expression in experimental models for alcoholism [56], aromatase deficiency [55], heart failure [54] and in subpopulations of neuronal mitochondria [57, 58] correlates with a higher susceptibility to Ca^{2+}-induced MPT. Moreover, the depletion of the nitrosothiol content in NAR liver mitochondria (Fig. 4E) may also contribute to a higher susceptibility to Ca^{2+}-induced MPT in NAR. The status of mitochondrial nitrosothiols has been recently demonstrated to play a role in the MPT process and in mitochondrial and tissue integrity under challenging conditions [32, 59]. Indeed, a recent report indicates that S-nitrosylation of CyD on cysteine 203 partially inhibits MPT opening in H_2O_2-treated cells [33]. Therefore, an alteration of these two variables, known to be critical for MPT regulation, might explain mitochondrial dysfunction in NAR.

It is insightful that a lack of plasma albumin, an abnormality primarily confined to the extracellular compartment, affects mitochondria. This may comprise a novel finding in the sense that a benign extracellular disturbance (*i.e.*, elicits only mild clinical symptoms) can over time impair mitochondrial function. To note, 21-day-old weaning NAR do not present an increased susceptibility to MPT compared to their age-matched controls (Fig. 3D). In the case of NAR, it is known that several alterations occur secondarily to analbuminemia, as aforementioned. These alterations along with the primary defect (*i.e.*, lack of plasma albumin) may interact to predispose NAR tissues to degeneration, as evidenced by plasma oxidative stress [60], increased plasma liver enzymatic activities [4], and by microscopic liver pigmentation and glomerulosclerosis with aging [61]. It is important to highlight that albumin has antioxidant and cytoprotective properties [62, 63], and a lack of albumin by itself may comprise an important loss for redox homeostasis not only in plasma. Recent findings indicate that albumin plays a key role as a nitrosothiol trafficking protein, aiding in the transnitrosation of cellular proteins [64, 65] and conferring cytoprotection [64, 65]. Because albumin molecules comprise ~80% of the total pool of thiols in blood plasma [2], this finding may have important implications for NAR because these animals also present very low levels of plasma nitrosothiols [66]. Thus, the depletion of nitrosothiols in NAR mitochondria may be related to the likely compromised nitrosothiol trafficking in these animals.

The redox regulation of MPT sensitivity to Ca^{2+} load has long been studied by our group [26–30, 67] (for a comprehensive overview see Kowaltowski et al. [25]). The oxidation of membrane protein thiols by mitochondrial reactive oxygen species promotes disulfide bonds and protein aggregation in the inner mitochondrial membrane, which results in the assembling of the MPT pore [24, 25, 28, 29, 67]. The redox state of pyridine nucleotides is especially critical because they provide the reducing power for H_2O_2 detoxification in mitochondria [26, 27, 30, 47]. Therefore, we evaluated these and other important variables related to the mitochondrial redox state, but none of them differed between NAR and SDR (Fig. 4). The redox profile of NAR mitochondria greatly differs from that of hypercholesterolemic mice (LDL receptor knockout) mitochondria, which have been previously studied by our group [21, 22]. The mitochondria from the hypercholesterolemic mice present an inability to sustain a reduced state of mitochondrial NADPH, and consequently a higher generation rate of mitochondrial H_2O_2 and susceptibility to Ca^{2+}-induced MPT [21, 22].

With respect to hypertriglycerolemia in NAR, we previously showed that ApoCIII transgenic hypertriglyceridemic mice display an increased resting mitochondrial respiration (*i.e.*, State 4 respiration) and whole body metabolic rate due to mild mitochondrial uncoupling mediated by redox activation of mitochondrial ATP-sensitive K^+ channels [20]. Despite the hypertriglyceridemia of NAR (Table 1), we did not find significant differences for mitochondrial respiratory variables between the NAR and SDR (Table 2). Similar results were also observed for female NAR, which have nearly 5-fold higher plasma triglycerides levels than controls (data not shown). In this context, the main difference between NAR and ApoCIII transgenic hypertriglyceridemic mice is that the latter also present high levels of circulating FFA [18] while NAR instead display a FFA deficiency (Table I). It is important to note that triglycerides are not a substrate readily available for peripheral tissues, and their breakdown to glycerol and FFA is a critical step for the tissue uptake of plasma triglyceride-derived FFA [68]. Thus, rather than hypertriglyceridemia *per se*, plasma FFA seem to be the real cause of cellular lipotoxicity. In addition, we recently found that intravascular lipolysis is hampered in NAR [16], which presumably further limits FFA flux into peripheral tissues.

Overall, our data indicate that the higher susceptibility of NAR liver mitochondria to Ca^{2+}-induced MPT is associated with an increased expression of CyD and lower content of mitochondrial nitrosothiols. These alterations sum to the already described subclinical comorbidities in NAR. The association between the overexpression of cyclophilin-D and MPT opening propensity is an emerging paradigm in some diseases and deserves further investigation.

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4. Discussão Geral

Com base nas principais funções fisiológicas descritas para a albumina [1, 3, 5, 6, 57], a ausência desta no plasma sanguíneo imporia várias conseqüências biológicas que poderiam ser agrupadas em três categorias distintas: i) as relacionas à alteração de pressão coloidosmótica do plasma e distribuição de fluidos entre o compartimento circulatório e o extravascular; ii) das alterações metabólicas primariamente relacionadas ao metabolismo de gordura e, provavelmente, repercussões sobre o metabolismo de carboidratos, e também algumas alterações endócrinas; iii) e as relacionadas à integridade celular e tecidual, uma vez que a albumina tem propriedades antioxidantes e é também seqüestradoras de substâncias endógenas (ex. bilirubina) e exógenas que são potencialmente tóxicas [1, 58]. De fato, há várias alterações orgânicas descritas na analbuminemia humana e do rato [12, 14, 16, 18, 59, 60], sendo que muitas delas se enquadram nestas categorias acima

Os resultados apresentados nesta tese avançam o conhecimento a respeito das alterações secundárias à analbuminemia que se enquadram nas duas últimas categorias descritas acima. Primeiro demonstramos no capítulo I que a produção hepática de triglicérides é menor no NAR (**Fig. 1, pág. 31**); isto indica que a hipertrigliceridemia é provavelmente causada pela menor taxa de remoção de triglicérides do plasma. Também obtivemos evidências que a ausência de albumina no plasma inibe a atividade lipolítica neste compartimento (**Fig. 2, pág. 31; Fig. 3, pág. 32**). Tal inibição explica tanto o déficit de FFA plasmático quanto a hipertrigliceridemia, uma vez que a remoção de triglicérides plasmáticos pelos

tecidos periféricos é dependente da hidrólise dos triglicérides e da posterior mistura do FFA liberados ao *pool* de FFA ligados à albumina.

No capítulo II também mostramos importantes repercussões metabólicas da analbuminemia sobre o metabolismo de carboidratos. É sabido há bastante tempo que a disponibilidade de FFA regula a partição dos lipídios e carboidratos como substrato energético [61]. FFA plasmático cronicamente elevado promove resistência periférica a insulina e redução da utilização de glicose pelos tecidos periféricos [61, 62]. Os efeitos do FFA plasmático cronicamente ou agudamente elevado e do FFA plasmático agudamente diminuído sobre a secreção pancreática de insulina também são descritos na literatura. A secreção pancreática de insulina é prejudicada quando da diminuição aguda [63, 64] ou do aumento crônico de FFA plasmático [62]. Na condição de aumento agudo de FFA plasmático, se observa maior secreção pancreática de insulina [63, 65]. Uma das alterações secundárias a analbuminemia é o déficit crônico de FFA plasmático [18, 44]. No entanto, não há estudos que investigaram os efeitos do FFA cronicamente diminuídos sobre a secreção pancreática de insulina ou sobre o metabolismo de carboidratos, provavelmente pela ausência de um modelo experimental adequado. Assim, a repercussão desta menor disponibilidade de FFA plasmático sobre o metabolismo da glicose na analbuminemia era pouco previsível. Conjuntamente, os resultados mostrados no capítulo II indicam que o NAR apresenta metabolismo acelerado de carboidratos. A resposta do conteúdo de glicogênio hepático ao jejum (**Fig. 6, pág. 42**) e o maior fluxo neoglicogênico (**Fig. 7, pág. 42**) demonstram que o NAR é mais dependente de carboidrato como substrato energético do que seu controle.

Apesar de não podermos atribuir estas alterações exclusivamente ao déficit de FFA plasmático, o NAR apresentou maior secreção de insulina em resposta à glicose (**Fig. 1C, pág. 39; Fig. 3, pág. 40**) e conseqüentemente maior tolerância à glicose (**Fig. 1A, pág. 39**). Esta hipersecreção de insulina em relação ao rato controle SDR é um achado importante, pois somente outras poucas condições fisiopatológicas como o insulinoma, a síndrome da obesidade hipotalâmica e a síndrome hipoglicêmica estão associadas à hipersecreção de insulina pelo pâncreas [66-68]. No caso da analbuminemia, o provável significado fisiológico destas alterações no metabolismo de carboidrato é a compensação da menor disponibilidade dos lipídios (FFA) como substrato energético.

Outra alteração secundária encontrada no NAR é a disfunção mitocondrial, que foi caracteriza no capítulo III. A maior susceptibilidade à transição de permeabilidade mitocondrial induzida por Ca^{2+} (**Fig. 1 e 2, pág. 50**) soma-se as inúmeras comorbidades apresentadas pelo NAR e por indivíduos analbuminêmicos [9, 12, 14, 16, 18, 59, 60, 69-71]. O fato de a analbuminemia afetar negativamente o compartimento intracelular (*i.e*. mitocôndrias) é intrigante. Este achado e outros recentemente publicados na literatura [22, 72-76] compreendem um novo paradigma sobre fatores que podem afetar a susceptibilidade à transição de permeabilidade mitocondrial induzida por Ca^{2+}. No caso do NAR, há muitas outras alterações associadas que não permitem interpretarmos que tal disfunção resulta diretamente da ausência de albumina plasmática. Porém, pelo menos no que diz respeito à depleção de nitrosotiol nas mitocôndrias do NAR, a ausência de albumina plasmática pode ter um papel mais

direto [5]. É sabido que aproximadamente 80% do *pool* de tióis plasmáticos residem nas moléculas de albumina [7, 8]. O NAR, como não possui albumina plasmática, apresenta também um déficit bastante severo de nitrosotióis plasmático [57]. Em função da deficiência de albumina e de nitrosotióis no plasma, é possível que a depleção de nitrosotiol mitocondrial esteja relacionada a isto, uma vez que a albumina parece ter papel importante na transnitrosação de proteínas intracelulares por nitrosotióis extracelulares [4, 5]. A outra provável explicação para a maior susceptibilidade a transição de permeabilidade mitocondrial observada no NAR, é a maior expressão de ciclofilina D, proteína chave no processo de transição de permeabilidade mitocondrial [29, 38, 77]. O mecanismo pelo qual a expressão de ciclofilina D é afetada nesta condição fisiopatológica é totalmente desconhecido. No entanto, uma maior susceptibilidade à transição de permeabilidade mitocondrial também está associada à maior expressão de ciclofilina D em algumas outras alterações orgânicas [29, 73, 74, 76, 78, 79]. Esta associação entre expressão de ciclofilina D e transição de permeabilidade mitocondrial observada em algumas doenças é algo novo na literatura e merece atenção.

5. Conclusões

As conclusões apresentadas abaixo são, respectivamente, pertinentes aos objetivos específicos dos estudos apresentados nos capítulos I, II e III.

I. A taxa de produção hepática de lipídios e de liberação de triglicérides para a circulação não contribui para a hipertrigliceridemia do NAR. Contrariamente, uma menor remoção de triglicérides da circulação provavelmente contribui para tal alteração. Propomos que a falta da albumina para agir como um aceptor de FFA inibe a lipólise intravascular, que por sua vez prejudica a hidrólise de triglicérides e sua remoção do plasma.

II. O NAR apresenta maior tolerância à glicose e dependência nos estoques hepáticos de glicogênio. Esta maior tolerância à glicose é atribuída a hipersecreção de insulina quando a ilhota é estimulada pela glicose, enquanto que a sensibilidade periférica à insulina parece ser similar entre NAR e seu controle. Pode ser que o metabolismo de carboidrato acelerado no NAR represente uma adaptação compensatória à menor disponibilidade de FFA.

III. O NAR apresenta maior susceptibilidade à transição de permeabilidade mitocondrial induzida por Ca^{2+} que está associada à maior expressão de ciclofilina D e à depleção de nitrosotióis mitocondrial. Não encontramos evidências de outras alterações mitocondriais, sejam bioenergéticas ou do estado redox.

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Apêndice I

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Chapter 7

Safranine as a Fluorescent Probe for the Evaluation of Mitochondrial Membrane Potential in Isolated Organelles and Permeabilized Cells

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Abstract

The mitochondrial electrical membrane potential ($\Delta\psi$) is the main component of the proton motive force (Δp) generated across the inner mitochondrial membrane during electron flow through the respiratory chain. Among the techniques available to assess $\Delta\psi$, methods that rely on the spectrophotofluorometric responses of dyes are widely employed for whole suspensions of isolated mitochondria or permeabilized cells. Safranine is one of the dyes currently used most often for this purpose. Safranine is a lipophilic cationic dye that undergoes optical shifts upon its potential-dependent distribution between the external medium and the intramitochondrial compartment and on its stacking to inner mitochondrial membrane anionic sites. The association between the optical changes of safranine and the membrane potential allows unknown $\Delta\psi$ values to be estimated from an equation describing their relationship. Here, we describe the use of safranine as a fluorescent indicator of $\Delta\psi$ in isolated mitochondria and digitonin-permeabilized cells. We present suitable conditions to employ safranine as a $\Delta\psi$ indicator.

Key words: Bioenergetics, Digitonin, Energy metabolism, Membrane potential, Mitochondria, Oxidative phosphorylation, Safranine

1. Introduction

The protonmotive force (Δp) across the inner mitochondrial membrane is the energy transduction link between substrate oxidation and mitochondrial ADP phosphorylation (1). FAD and NAD coenzymes are reduced during the degradation of glucose and fatty acids through the glycolytic and β-oxidation pathways, respectively. These pathways also feed acetyl coenzyme A to the Krebs Cycle,

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which accomplishes the remaining degradation of the substrates and yields further amounts of reduced FAD and NAD (2). Amino acids are also broken down and fed into the Krebs cycle. The reduced coenzymes, NADH and $FADH_2$, deliver electrons to respiratory chain complexes I and II, respectively. Electrons are then carried from these entry complexes down to complex IV, where molecular oxygen accepts electrons and is reduced to water. Respiratory complexes I, III, and IV couple the electron transport to the pumping of H^+ ions from the matrix to the intermembrane space (3). This electron transport through the respiratory chain builds up a resting Δp of approximately −220 mV, which is composed of two thermodynamic potentials: an electrical gradient ($\Delta\psi$) and a pH gradient (ΔpH) across the inner mitochondrial membrane (4). The energy stored as Δp drives the re-entry of protons through the ATP synthase complex with concomitant ADP phosphorylation to ATP (5).

In energized nonphosphorylating mitochondria, a $\Delta\psi$ of nearly −170 mV is by far the largest component of the entire Δp; hence, the contribution of the ΔpH component is minor. In an experimental setup with isolated mitochondria, the ΔpH component of Δp can be further decreased by adding permeant anions, if desired, forcing the $\Delta\psi$ to increase its own contribution to the Δp (6). Due to the ease of measurement and the representativeness of $\Delta\psi$ to the entire Δp, $\Delta\psi$ is often the only component measured in cellular and mitochondrial studies that approach questions related to the Δp.

The assessment of $\Delta\psi$ will depend on the biological sample (i.e., isolated mitochondria, permeabilized cells, and intact cells) and the instrument employed, such as cation selective electrode, spectrophotometer, spectrofluorometer, microscope, and flow cytometer. For the evaluation of $\Delta\psi$ in intact cells, the influence of the plasma membrane potential on the measurements should also be taken into account (7). When evaluating whole suspensions of isolated mitochondria or permeabilized cells, the estimation of $\Delta\psi$ usually relies on the distribution of membrane permeant cations measured by selective electrodes (8, 9) or the spectrophotofluorometric responses of dyes (10). The dyes currently used most often for this purpose are rhodamine 123 (11) and safranine (12). In our laboratory, over the last two decades, we have employed safranine to estimate $\Delta\psi$ in mitochondria isolated from plants (13, 14) and several animal tissues (15–19), and in a variety of permeabilized cells (20–22).

Safranine is a lipophilic cationic dye that undergoes optical shifts upon its potential-dependent distribution between the external medium and the intramitochondrial compartment and on its stacking to inner mitochondrial membrane anionic sites (23). The spectral shifts of safranine elicited by the energized states of mitochondria were first described in mitochondrial suspensions by Colonna et al. (24). The linear association between safranine

absorbance changes and the membrane potential (as titrated by increasing extramitochondrial K^+ concentrations in the presence of valinomycin) allowed unknown $\Delta\psi$ values to be estimated by an equation describing their relationship (12). Later, the fluorescence quenching of safranine upon $\Delta\psi$ generation was investigated in vesicles and reconstituted proteoliposomes (25, 26). Monitoring $\Delta\psi$ through the fluorescence of safranine allows the use of a lower concentration of the dye, and this may represent the main advantage of fluorescence over absorbance measurements (see Notes 1 and 2). In addition, measurement of the safranine absorbance requires special spectrophotometers that are able to perform dual wavelength measurements simultaneously (12). Here, we describe the features of safranine as a fluorescent indicator of $\Delta\psi$ in isolated mitochondria and digitonin-permeabilized cells.

2. Materials

2.1. Isolated Rat Liver Mitochondria

1. Female *Wistar Unib Hannover* rats were housed at 23°C on a 12-h light–dark cycle with free access to a standard chow diet and water.
2. The following potassium-free isolation buffers were prepared on the day before mitochondrial isolation and stored at 4°C: Isolation buffer I (250 mM sucrose, 1 mM ethylene glycol-bis(2-amino-ethylether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid (EGTA), and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, pH 7.2); isolation buffer II (250 mM sucrose, 0.2 mM EGTA, and 10 mM HEPES buffer, pH 7.2); and isolation buffer III (250 mM sucrose and 10 mM HEPES buffer, pH 7.2).

2.2. PC12 Cell Culture

1. PC12, a cell line derived from a rat adrenal pheochromocytoma, was obtained from ATCC (Rockville, MD, USA).
2. Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose, with or without phenol red. This medium was supplemented with 10% horse serum, 5% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin.
3. Trypsin solution (0.25%) containing 1 mM ethylenediamine tetra-acetic acid (EDTA), osmolarity 295 ± 5 mOsm/L, and pH 7.0 ± 0.2.
4. Phosphate-buffered saline (PBS), pH 7.2, was prepared weekly and stored at 4°C.
5. A stock solution of digitonin (3.25 mM) was prepared in dimethyl sulfoxide (DMSO) and stored at −20°C.
6. The cell cultures were maintained in CO_2 incubator.

2.3. Determination of the Mitochondrial Membrane Potential in Isolated Organelles or Digitonin-Permeabilized Cells

1. Standard reaction medium: 250 mM sucrose, 10 mM HEPES buffer, 200 μM EGTA, 2 mM H_2PO_4, 1 mM $MgCl_2$, and complex I-linked substrate pool (3.4 mM malate, 1.86 mM α-ketoglutarate, 2.1 mM pyruvate, 2.1 mM glutamate), pH 7.2, at 28°C. For the experiments with PC12 permeabilized cells, this medium was supplemented with 0.1% bovine serum albumin.
2. Hyposmotic reaction medium: the same composition was used as for the standard reaction medium described above, except that 50 mM sucrose was used instead of 250 mM sucrose. These reaction mediums were prepared on the day before use and frozen until use.
3. Stock solutions: 2.5 mM safranine in water (see Note 3); 40 μg/mL valinomycin in DMSO; 1 M KCl in water; 50 mM ADP in water (the pH was corrected with NaOH to 7.2); 2 mg/mL oligomycin in ethanol; 1 mM carboxyatractyloside (CAT) in water; 1 μM carbonyl cyanide *p*-trifluorophenylhydrazone (FCCP) in ethanol; and 5 mg/mL alamethicin in ethanol. All of these reagents were stored at −20°C for up to 6 months.
4. A spectrofluorometer equipped with magnetic stirring and a temperature-controlled water bath was used. The following settings were used: high sensitivity, 2 Hz acquisition rate, excitation wavelength 495 nm, emission wavelength 586 nm, and both slits were set to 5 nm (see Note 4). The temperature was set at 28°C in all experiments.
5. A 3-mL cuvette was equipped with a magnetic stirring bar. All of the experiments were carried out with continuous stirring.

3. Methods

The cationic dye safranine is distributed across the inner mitochondrial membrane (negative inside) according to the $\Delta\psi$. The decay in intensity of the safranine fluorescence upon mitochondrial energization arises from the electrophoretic transportation of this dye from the extramitochondrial to the intramitochondrial compartment. The intramitochondrial accumulation of safranine in the millimolar range and its stacking to the inner mitochondrial membrane anionic sites are accompanied by spectral changes and fluorescence self-quenching due to the aggregation of safranine into dimers and polymers (23, 27). Therefore, the dye sample (protein) ratio may greatly affect the fluorescence response (26, 28). The purpose of the experiments shown below was to describe the response of the safranine fluorescence to different experimental conditions, as the safranine and mitochondrial concentrations were

varied in the suspension. In addition, the response of the safranine fluorescence to different densities of permeabilized cells was studied. In this way, suitable conditions to employ safranine as a $\Delta\psi$ indicator were determined.

3.1. Determination of the Mitochondrial Membrane Potential in Isolated Organelles

1. Mitochondrial Isolation: Mitochondria were isolated by differential centrifugation as described by Kaplan and Pedersen (29). All of the procedures after excision of the liver were performed either on ice or at 4°C. Briefly, 2-month-old fed rats were decapitated, and the livers were immediately removed and stored in isolation buffer I. After two washes with isolation buffer I, the liver was finely diced with scissors. Approximately 10 g of tissue was then transferred to the homogenizing potter (#23, Kontes Glass Co. Vineland, NJ), which was filled to 40 mL with isolation buffer I. The tissue was thoroughly homogenized (usually within about ten strokes) while the homogenizing potter was kept on ice. This homogenate was transferred to 40-mL-lidded tubes and centrifuged at $800 \times g$ for 10 min. The supernatant thus obtained was then transferred to a clean tube while avoiding contamination with the loose pellet. This tube was filled completely with isolation buffer I and centrifuged at $7{,}750 \times g$ for 10 min. After this, the supernatant was discarded and the pellet was suspended in isolation buffer II by gentle mixing with a small paintbrush and transferred to a clean tube. This tube was filled with isolation buffer II and centrifuged again at $7{,}750 \times g$ for 10 min. The supernatant was discarded and the pellet was washed by rinsing once with 400 µL of isolation buffer III. This pellet was then carefully resuspended in 400 µL of isolation buffer III by gently mixing with a small paintbrush. Finally, this suspension was transferred to a clean glass tube and kept on ice until use. The protein concentration of this suspension was determined using a modified Biuret assay.
2. Membrane Potential Experiments: The fluorescence reading (in the time course mode) was started with standard reaction buffer (2 mL) supplemented with 5 µM safranine in the cuvette (see Note 5). After a short base line reading was recorded, mitochondria were added to the cuvette to a final concentration of 0.5 mg/mL (Fig. 1a). After the initial fast fluorescence decay and the establishment of a steady-state signal, the potassium ionophore valinomycin (40 ng/mL) was added. Thereafter, KCl was consecutively added to clamp the $\Delta\psi$ at decreasing values. The first three KCl additions were 0.375 mM each, the next three were 0.5 mM each, and the following four were 0.75 mM each. Between the KCl additions, a steady-state of the fluorescence was waited to re-establish. After the last KCl addition, the protonophore FCCP was added to dissipate the $\Delta\psi$.

3. The sums of added potassium at each step (0.375, 0.750, 1.125, 1.625, 2.125, 2.625, 3.375, 4.125, 4.875, and 5.625 mM) were used to calculate the $\Delta\psi$ according to the Nernst equation: $\Delta\psi = 60 \times \log(K_{IN}^{+}/K_{OUT}^{+})$, where K_{IN}^{+} is the intramitochondrial potassium concentration and K_{OUT}^{+} is the sum of added potassium at each step. K_{IN}^{+} was assumed to be 120 mM (see Notes 6 and 7). Although a calibration curve can be built with the absolute fluorescence intensity of each step and its respective calculated $\Delta\psi$, comparisons among different conditions (as detailed below)

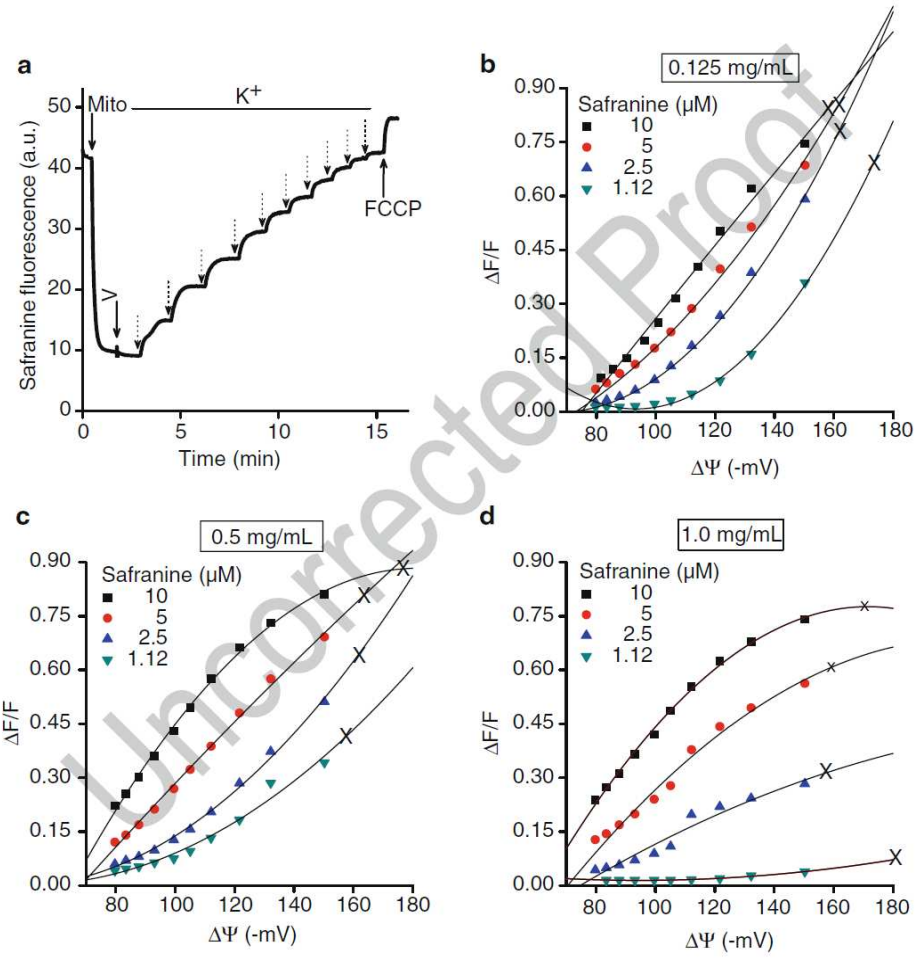

Fig. 1. Estimation of the mitochondrial transmembrane electrical potential and the effects of varying the safranine or mitochondrial protein concentration on the safranine fluorescence responses. (**a**) A representative trace of the changes in safranine fluorescence in response to titration of the electrical potential with KCl additions in the presence of 40 ng/mL valinomycin (V). The effect of 1.25, 2.5, 5, and 10 μM safranine on the fluorescence response was studied at different concentrations of mitochondrial protein (Mito; 0.125, 0.5, and 1.0 mg/mL, presented in *panels* **b**, **c**, and **d**, respectively). Calibration curves were plotted as "$\Delta F/F$" as a function of the calculated values of $\Delta\psi$, where "F" is the fluorescence after FCCP addition and "ΔF" is "F" minus the steady-state fluorescence after each KCl addition. The data was fitted to a second-order polynomial equation (*solid line*), and "X" represents the estimated resting membrane potential. The data shown in the figure is representative of three independent experiments. *a.u.* arbitrary units.

require that the absolute fluorescence be normalized with respect to the amplitude of fluorescence response under each of these conditions. To do so, we calculated $\Delta F/F$, where F is the fluorescence intensity after the addition of FCCP and ΔF is F minus any given fluorescence intensity. Following this, $\Delta F/F$ was plotted as a function of the calculated $\Delta\psi$ and fitted to a second-order polynomial equation (Microcal Origin 8.0) (Fig. 1c, filled circle line).

3.2. Effects of Varying the Concentrations of Mitochondrial Protein and Safranine on the Fluorescence Response

The membrane potential calibration experiment was carried out exactly as described above, using varying concentrations of mitochondria (0.125, 0.5, and 1.0 mg/mL) and safranine (1.12, 2.5, 5, and 10 μM) in the suspension. The calibration curves obtained at each mitochondria and safranine concentration combination are plotted in Fig. 1b–d. The results indicate that the sample protein to safranine concentration ratio can greatly affect the safranine fluorescence response. For example, the fluorescence when using too little safranine (1.12 μM) was not responsive to changes in $\Delta\psi$ when the concentration of mitochondrial protein is high (1.0 mg/mL), but it may respond to changes in $\Delta\psi$ in the high range (−120 to −170 mV) if the concentration of mitochondrial protein is decreased to 0.12 mg/mL. The fluorescence response appears most reliable over the three mitochondrial protein concentrations studied (0.12, 0.5, and 1.0 mg/mL) at a safranine concentration at 5.0 μM, although 2.5 μM safranine may present reliable responses if the mitochondrial protein is ≤0.5 mg/mL. Using 5.0 μM safranine, the estimated resting $\Delta\psi$s are very close to each other, at −161, −163, and −159 mV, for mitochondrial protein concentrations at 0.12, 0.5, and 1.0 mg/mL, respectively.

3.3. Effects of Mitochondrial Volume on Membrane Potential Measurements

The commonly used technique of tetraphenylphosphonium (TPP^+) distribution across the inner mitochondrial membrane to follow the $\Delta\psi$ relies on the assumption of a given intramitochondrial volume (30). The volume is necessary to calculate the intramitochondrial TPP^+ concentration. However, under certain circumstances, mitochondria can undergo extensive changes in volume (31, 32). This issue regarding the mitochondrial volume precludes the use of the TPP^+ technique in some experimental setups. It is also known that changes in the mitochondrial volume affect light scattering (33), hence, the fluorescence intensity of the entire suspension might also be affected.

To investigate whether changes in mitochondrial volume affect the fluorescence of safranine, the following experiments were performed:

1. The extent of mitochondrial swelling induced by a hyposmotic medium (20% of the standard medium osmolarity) was assessed by light scattering (excitation wavelength 540 nm, emission

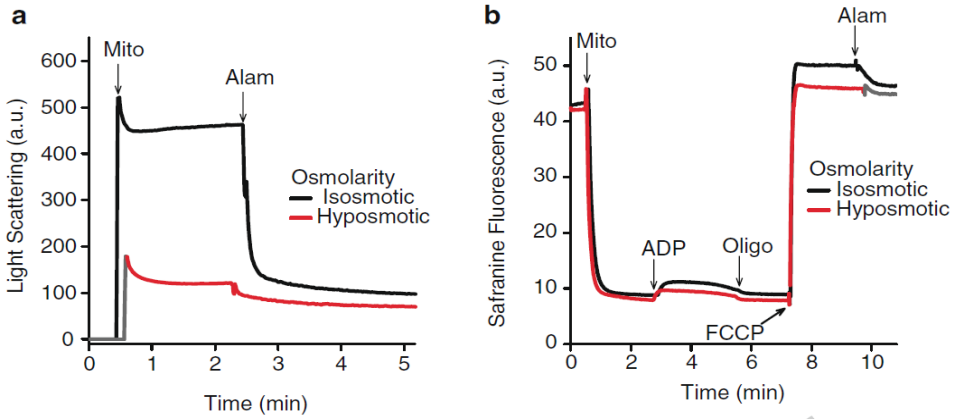

Fig. 2. The effects of mitochondrial volume on safranine fluorescence. (**a**) Mitochondria were added to hyposmotic or to isosmotic standard reaction mediums and the mitochondrial volume was estimated by light scattering. Alamethicin (Alam) was added where indicated to elicit maximal mitochondrial swelling. (**b**) Mitochondria were added to hyposmotic or to isosmotic standard reaction mediums supplemented with 5 μM safranine while the safranine fluorescence was monitored. ADP, oligomycin (Oligo), and FCCP were added where indicated to alter the mitochondrial energy state. The data shown in the figure is representative of three independent experiments. *a.u.* arbitrary units.

wavelength 540 nm, excitation slit 1.5 nm, and emission slit 3.0 nm; low sensitivity was used). After the mitochondria were added (0.5 mg/mL final concentration) and the light scattering leveling off, 10 μg/mL alamethicin was added to elicit maximal mitochondrial swelling (Fig. 2a).

2. The mitochondria were incubated in standard and hyposmotic reaction mediums supplemented with 5 μM safranine (Fig. 2b). After the mitochondria were added (0.5 mg/mL final concentration) and a new steady-state in fluorescence intensity had been established, the phosphorylation state was induced by adding ADP (300 μM) and later inhibited using oligomycin (2 μg/mL). FCCP (100 nM) was added at the end of the experiment to dissipate the $\Delta\psi$.
3. After the addition of FCCP, it is observed slightly higher safranine fluorescence values under isosmotic conditions than in an hyposmotic medium. To induce similar mitochondrial volumes under these experimental conditions, 10 μg/mL alamethicin was added to the suspensions incubated under both conditions. The data presented in Fig. 2 shows that the extensive mitochondrial swelling that occurs in response to the hyposmotic medium (Fig. 2a) only decreases by less than 10% the total amplitude of the safranine fluorescence response, i.e., the difference between the fluorescence measured before and after FCCP addition (Fig. 2b). After alamethicin was added to induce maximal mitochondrial swelling, the slight difference in safranine fluorescence between hyposmotic and isosmotic conditions was minimized (Fig. 2b).

3.4. Determination of the Mitochondrial Membrane Potential in Digitonin-Permeabilized Cells

1. PC12 cell culture: Cultured PC12 cells were maintained at 37°C and 5% CO_2 in DMEM containing phenol red and supplemented with 10% horse serum, 5% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Because PC12 cells present a doubling time of 48–72 h, the cells were passaged every 2 days and plated in 150-cm^2 tissue culture flasks at an initial density of 0.5×10^5 cells/cm^2.
2. PC12 cell suspensions: After 2 days in culture, PC12 cells from one flask were washed once in PBS and then incubated with 4 mL of trypsin-EDTA solution at 37°C for approximately 2 min. Supplemented DMEM (10 mL) was added and the cells were resuspended by pipetting them slowly up and down. The cell suspension was centrifuged at 2,800 × *g* for 3 min in a 15-mL conical tube at room temperature. The supernatant was discarded and the cell pellet was resuspended in 5 mL of supplemented DMEM without phenol red, but containing 20 mM HEPES (pH 7.2). The cell number was estimated using a *Neubauer* chamber (see Note 8). Thereafter, the cell suspension was centrifuged at 2,800 × *g* for 3 min; the resulting pellet was resuspended to a final density of 40×10^6cells/mL and aliquoted into 200 μL portions (8×10^6 cells each). These aliquots were maintained in 0.6 mL microcentrifuge tubes at room temperature for a maximum of 1.5 h.
3. PC12 cell permeabilization: For the experiments, each cell aliquot was centrifuged at 1,500 × *g* for 3 min at room temperature using a microcentrifuge, the supernatant was discarded and the pellet was washed once in standard reaction medium. The cell pellet was resuspended in 1 mL of standard reaction medium containing 40 μM digitonin, and incubated for 5 min at 28°C (see Note 9). Digitonin was used to selectively permeabilize the plasma membrane (34). Subsequently, the cell suspension was centrifuged at 1,500 × *g* for 3 min and resuspended in standard reaction medium to a final volume of 50 μL (see Note 10).
4. Mitochondrial membrane potential measurements in permeabilized PC12 cells: The fluorescence reading (in time course mode) was started with standard reaction medium containing 0.1% BSA, 40 μM digitonin, and 5 μM safranine (see Note 5). After a short base line reading was recorded (Fig. 3a), 50 μL of the cell suspension (PC12) was added to the cuvette to obtain 4×10^6 cells/mL in a final volume of 2 mL. After the fast fluorescence decay and the establishment of a steady-state signal, 40 ng/mL valinomycin was added. Thereafter, KCl was added consecutively. The first three KCl additions were 0.375 mM each, the next four were 0.5 mM each, and the final seven were 0.75 mM each. After the last KCl addition, the protonophore FCCP was added to dissipate the $\Delta\psi$.
5. Calibration curve: The sums of the added potassium at each step (0.375, 0.750, 1.125, 1.625, 2.125, 2.625, 3.125, 3.875,

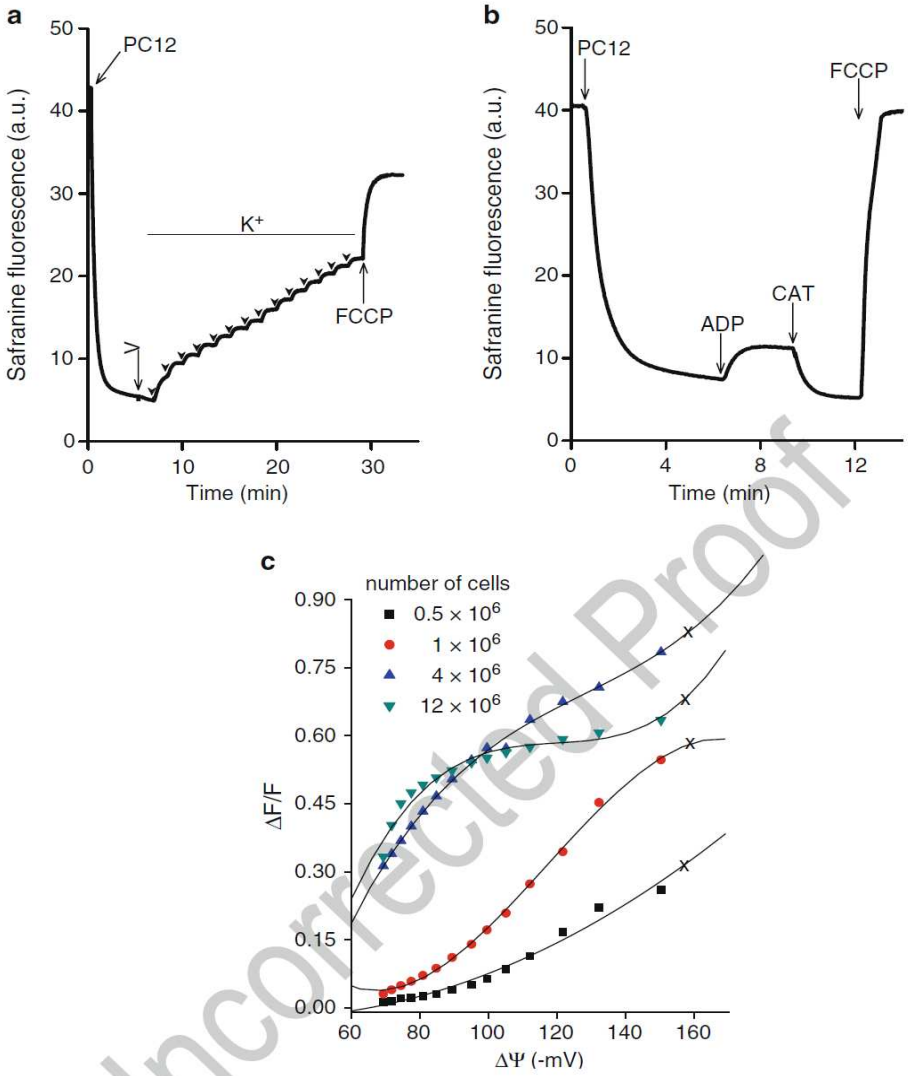

Fig. 3. Evaluation of the mitochondrial membrane potential in permeabilized PC12 cells using safranine: The effect of different cell densities. (**a**) A representative trace of the changes in safranine fluorescence in response to titration of the electrical potential with KCl additions in the presence of 40 ng/mL valinomycin. (**b**) Evaluation of the resulting changes in membrane potential due to oxidative phosphorylation. (**c**) The effects of different cell densities (0.5, 1, 4, or 12×10^6/mL) on the calibration curve. The data in *panel* **c** was fitted to a second- or third-order polynomial equation (*solid lines*), and "*X*" represents the estimated resting membrane potential. The data shown is representative of four independent experiments. For *panels* **a** and **b**, the cell density was 4×10^6/mL. *a.u.* arbitrary units.

4.625, 5.375, 6.125, 6.875, 7.625, and 8.375 mM) were used to calculate the $\Delta\psi$ according to the Nernst equation, assuming K_{IN}^+ to be 120 mM ((32), also see Notes 6 and 7). As described for isolated mitochondria (see Subheading 3.1, step 3), a calibration curve was built by plotting the $\Delta F/F$ as a function of the calculated $\Delta\psi$. Then, the curve was fitted to a polynomial equation (Fig. 3c, filled triangle line).

6. To evaluate the oxidative phosphorylation capability of permeabilized PC12 cells and the reliability of the methodology in detecting the expected changes in mitochondrial membrane potential, the experiment shown in Fig. 3b was conducted. After the cell suspension was added (to a final density of 4×10^6 cells/mL) and a new steady-state in fluorescence intensity had been established, the phosphorylation state was induced by adding 400 μM ADP and later inhibited using 10 μM CAT. FCCP (1 μM) was added at the end of the experiment to dissipate the $\Delta\psi$. The decrease in $\Delta\psi$ elicited by ADP (shown in Fig. 3b) is valuable as a measure of both: (1) proper cell permeabilization, as ADP is not permeable through the cell membrane; and (2) the ability of the cell mitochondria to phosphorylate ADP to ATP and therefore their functionality.

3.5. Effects of Varying the Concentrations of Permeabilized PC12 Cells on the Fluorescence Response

1. The membrane potential calibration experiment was carried out using 0.5×10^6, 1×10^6, 4×10^6, or 12×10^6 PC12 cells/mL. To obtain these cell densities, different volumes of permeabilized cell suspension (6.2, 12.5, 50, and 150 μL) were added to the standard reaction medium containing 0.1% BSA, 40 μM digitonin, and 5 μM safranine, to a final volume of 2 mL.
2. Membrane potential calibration experiments were carried out as described above (see Subheading 3.4, step 5) using consecutive additions of KCl. The calibration curves obtained at each density of permeabilized PC12 cells are plotted in Fig. 3c. The best fitting curve between second- or third-order polynomial equations was considered (Microcal Origin 8.0). The data presented in Fig. 3c shows that the pattern of safranine fluorescence responses to changes in $\Delta\psi$ is dependent on the density of cells in the suspension. Apart from the likely influence of mitochondrial concentration, as shown in Fig. 1b–d, the effects of cell density on the turbidity of the whole suspension may also contribute to the observed responses in permeabilized cells. The estimated resting $\Delta\psi$ was −157, −159, −158, and −158 mV at 0.5×10^6, 1.0×10^6, 4×10^6, and 12×10^6 cells/mL, respectively. Despite a very similar resting $\Delta\psi$ estimated among the conditions, the densities of 1×10^6 and 4×10^6 cells/mL presented better responses to changes in $\Delta\psi$ over the entire range evaluated.

4. Notes

1. Our group previously showed that safranine affects mitochondrial responses to Ca^{2+}-induced mitochondrial permeability transition and to oxidative stress (15, 16). These effects are concentration-dependent and clearly evident when the concentration of safranine is >5 μM. Figure 4 presents data

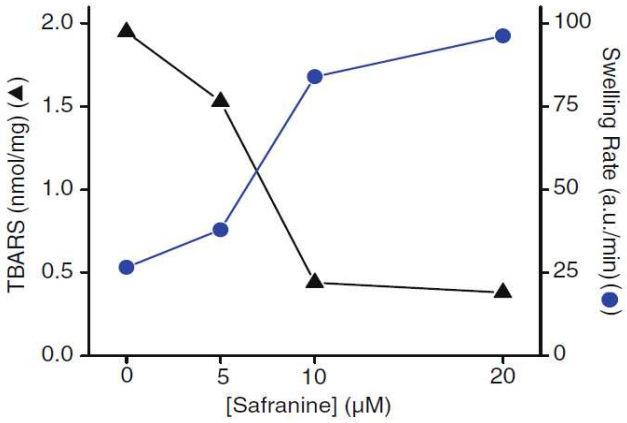

Fig. 4. The effects of safranine on Fe^{2+}-citrate-induced mitochondrial lipid peroxidation and on the Ca^{2+} plus phosphate-induced mitochondrial permeability transition (MPT). The data was derived from our previous studies (15, 16). Lipid peroxidation was assessed by thiobarbituric reactive substances (TBARS) and MPT was assessed from the initial rate of Ca^{2+}-induced mitochondrial swelling.

derived from our previous studies showing the stimulatory effects of safranine on Ca^{2+} plus phosphate-induced mitochondrial permeability transition – as monitored by the swelling rate (15). Conversely, safranine inhibits Fe^{2+}-citrate-induced mitochondrial lipid peroxidation (16).

2. Safranine at high concentrations (≥40 μM) was shown to inhibit rat liver mitochondrial respiration and energy transfer. ADP- and FCCP-stimulated respiration rates were progressively inhibited by safranine above 40 μM, whereas state IV respiration was slightly stimulated by safranine at concentrations higher than 70 μM (23). Safranine (≥10 μM) may also slow down the heart mitochondrial Ca^{2+} uptake rate to a small extent (35).
3. Safranine stock solutions should be protected from light. We usually store safranine stock solutions at −20°C for up to 6 months.
4. The use of a microplate reader to measure safranine fluorescence in the suspension could be considered. However, the need for efficient stirring of the suspension may preclude the use of this instrument.
5. Small variations in the fluorescence baseline, as are usually observed when safranine is added to the cuvette separately for each experiment, can be avoided by instead incorporating safranine into the stock reaction medium used for all the experiments. The reaction medium containing safranine should be protected from light. No change in safranine fluorescence was noted when the reaction medium was maintained at 28–37°C for up to 6 h.

6. The mitochondrial membrane potential is calculated according to the Nernst equation: $\Delta\psi = 60 \times \log(K_{IN}^{+}/K_{OUT}^{+})$, where K_{IN}^{+} is the intramitochondrial potassium concentration and K_{OUT}^{+} is equal to the added potassium. K_{IN}^{+} is usually assumed to be 120 mM (e.g., Figs. 1 and 3 and ref. 20). The advantage of this method is that an error in the estimate of K_{IN}^{+} would cause only a small error in the estimated membrane potential. If the K_{IN}^{+} were in fact 100 mM, $\Delta\psi$ would be overestimated by only 5 mV (12). K_{IN}^{+} can also be measured by accessible techniques if desired (32).
7. During the safranine-$\Delta\psi$ calibration experiment, some degree of mitochondrial swelling may occur due to K^{+} uptake. Nevertheless, this is not expected to result in an important variation in the intramitochondrial K^{+} concentration because one compensates for the other. Even though it might comprise a source of error in the estimation of $\Delta\psi$, the calibration protocol is rather valuable in assuring that the safranine fluorescence is responding properly to the changes in $\Delta\psi$.
8. If cell aggregation is observed, 0.01% DNAse may be added during the cell trypsinization and washing steps. Cell aggregation can result from the presence of dead cells in the suspensions.
9. The permeabilization protocol can differ among cell types. Different cells may require different concentrations of digitonin. Digitonin, when used below the optimal concentrations, may lead to an undersized initial safranine response due to incomplete cell membrane permeabilization. Cell staining with trypan blue may be useful in evaluating the extent of membrane permeabilization by digitonin. Digitonin should be avoided in excess as it may lead to a loss of $\Delta\psi$ over time due to some degree of mitochondrial membrane permeabilization (36).
10. For calibration purposes, the cells must be washed to remove the cytosolic K^{+} after digitonin permeabilization. In our experience, the higher the cell density, the greater the degree of unwashed cytosolic K^{+} remaining in the suspensions.

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Apêndice II

Resultados Suplementares

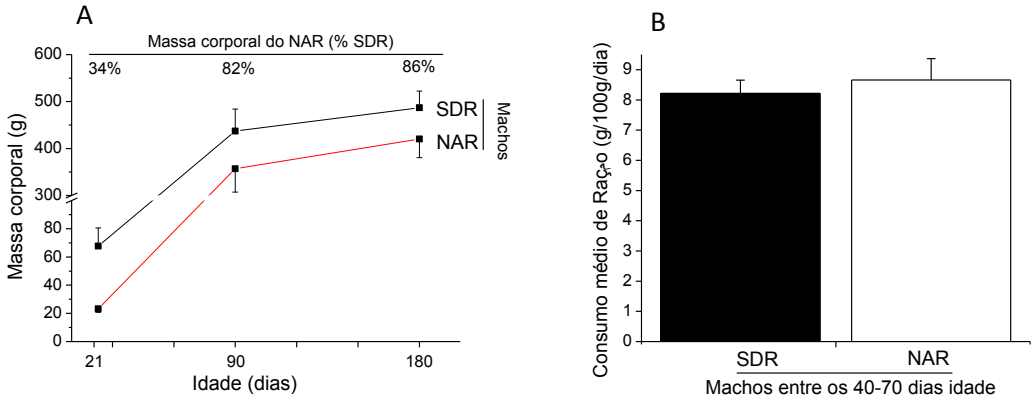
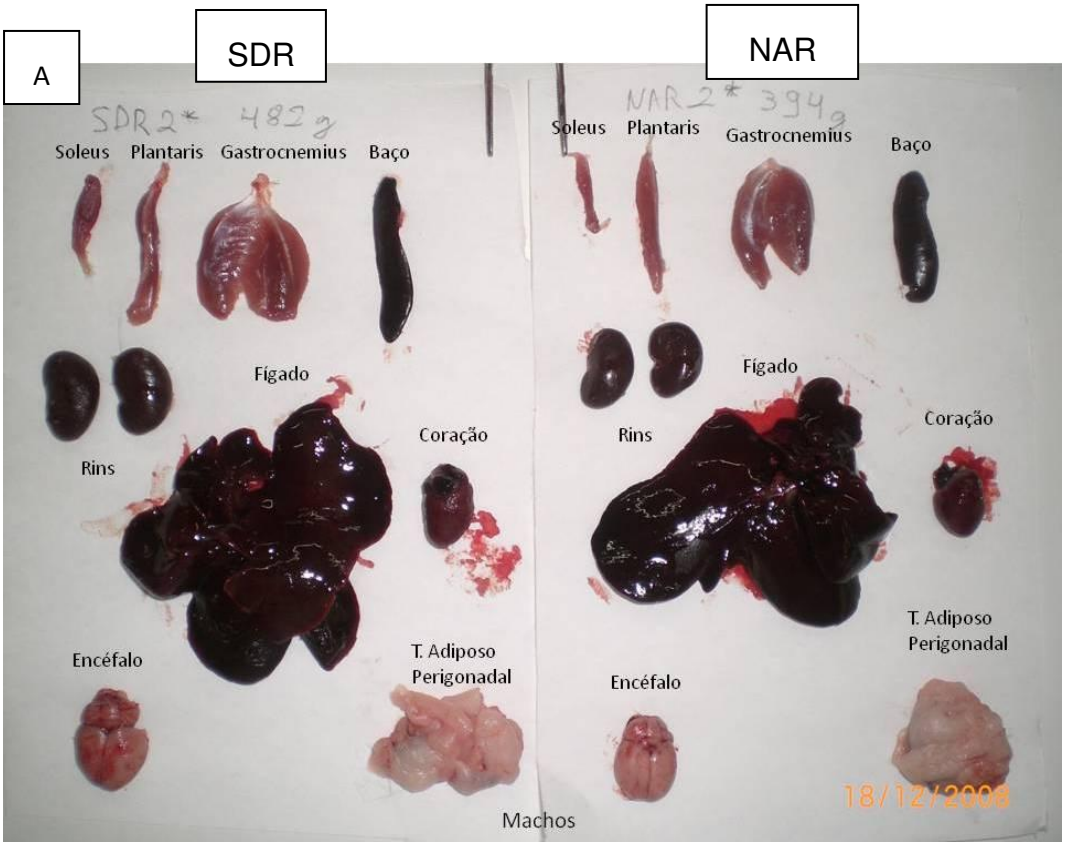


Figura 1. **Massa corporal total e consumo de ração por ratos SDR e NAR machos.** A, massa corporal aos 21, 90 e 180 dias de idade. A massa corporal do NAR foi significativamente menor que a do SDR em todas as idades, porém o déficit maior foi observado aos 21 dias de vida. B, consumo de ração (g) diário relativo a massa corporal do rato. O consumo foi mensurado durante o período de 40-70 dias de vida e não foi estatisticamente diferente entre os grupos.



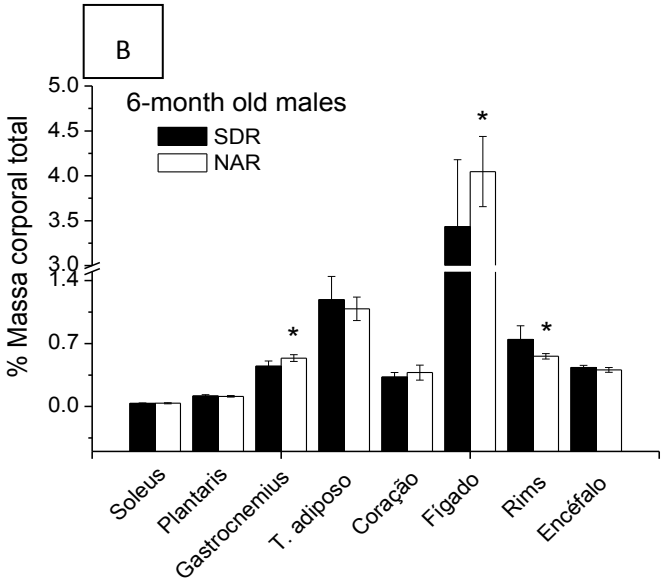


Figura 2. Massa de diversos órgãos do NAR e do SDR machos aos 6 meses de idade. A, fotos dos diversos órgãos retirados do SDR (painel da esquerda) e do NAR (painel da direita). Em B, média ± DP da massa relativa desses órgãos (% da massa corporal total). *p < 0,05)

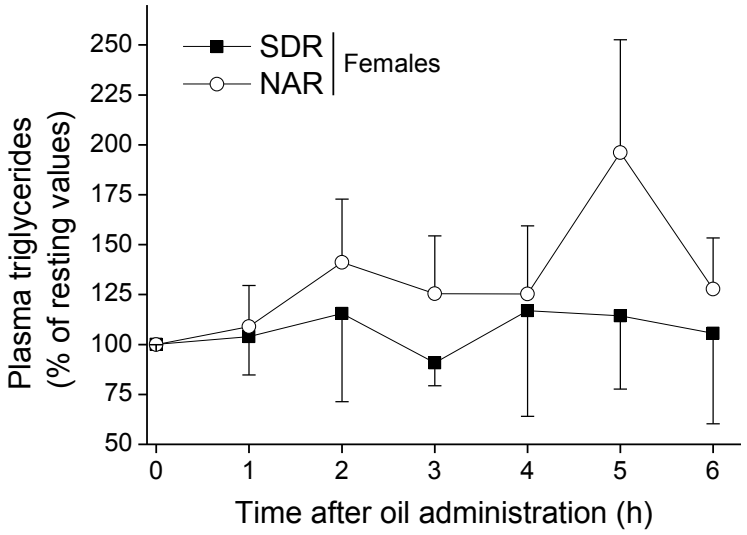


Figura 3. Teste de tolerância a gordura. Ratas fêmeas em jejum foram gavadas para a administração de óleo milho (10 mL/kg). Antes e após a gavagem, amostras de sangue foram coletadas da ponta da cauda para posterior dosagem de triglicérides plasmáticos. A área sob a curva de triglicérides foi significativamente (~37%, P≤ 0,05) maior nos NAR do que no SDR, N = 5 em cada grupo.

Tabela I. Variáveis bioenergéticas mitocondriais

	Macho		Fêmea	
	SDR	NAR	SDR	NAR
V4 (nmol O/mg/min)	9,8±3,6	8,9±3,2	14,5±4,9	12,8±3,1
V3 (nmol O/mg/min)	62,1±26,3	67,4±27,3	91,7±30,4	100,1±31,6
RCR	6,9±2,3	7,7±2,1	6,5±1,31	7,8±1,12
ADP/O	2,52±0,38	2,56±0,38	2,48±0,39	2,74±0,41

Média ± SD das variáveis bioenergéticas obtidas em mitocôndrias isoladas de fígado de ratos aos 3 meses de idade. O experimento foi conduzido como descrito no capítulo III. V4, respiração basal. V3, respiração estimulada por 300 µM de ADP. RCR, razão V3:V4. ADP/O, razão entre quantidade de ADP adicionado e quantidade de oxigênio consumido durante o estado V3. N = 7-14. Nenhuma variável se diferiu entre os grupos.

Tabela II. Índice de oxidação de ácidos graxos pela mitocôndria isolada

	SDR	NAR
V_Palmitoil-carnitina (nmol O_2/mg/min)	26,0 ± 2,01	26,7 ± 3,36
V_Piruvato (nmol O_2/mg/min)	45,6 ± 7,75	50,5 ± 6,08
Razão V_Palmitoil-carnitina: V_Piruvato	58,1 ± 9,03	53,4 ± 9,33

Média ± SD da respiração estimulada por 300 µM de ADP e suportada pelos substratos palmitoil carnitina (10 µM) ou Piruvato (5 mM) na presença de 2 mM de malato. O experimento foi conduzido com mitocôndrias isoladas de fígado de ratas fêmeas aos 3 meses de idade, essencialmente como descrito no Capítulo III. N = 4 em cada grupo.

Anexo I

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C E R T I F I C A D O

Certificamos que o Protocolo nº **1407-1**, sobre **"Função mitocondrial e estresse oxidativo em modelo de hiperlipidemia por aumento de pipogênese hepática em ratos analbuminêmicos"**, sob a responsabilidade de **Prof. Dr. Anibal Eugênio Vercesi / Tiago R. Figueira**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal – CEEA/Unicamp em **29 de novembro de 2007**.

C E R T I F I C A T E

We certify that the protocol nº **1407-1**, entitled **"Mitochondrial function and oxidative stress in an analbuminemic rat model of hyperlipidemia due to increase liver lipogenesis"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - Unicamp) on **November 29, 2007**.

Campinas, 29 de novembro de 2007.

Profa. Dra. Ana Maria A. Guaraldo
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Anexo II

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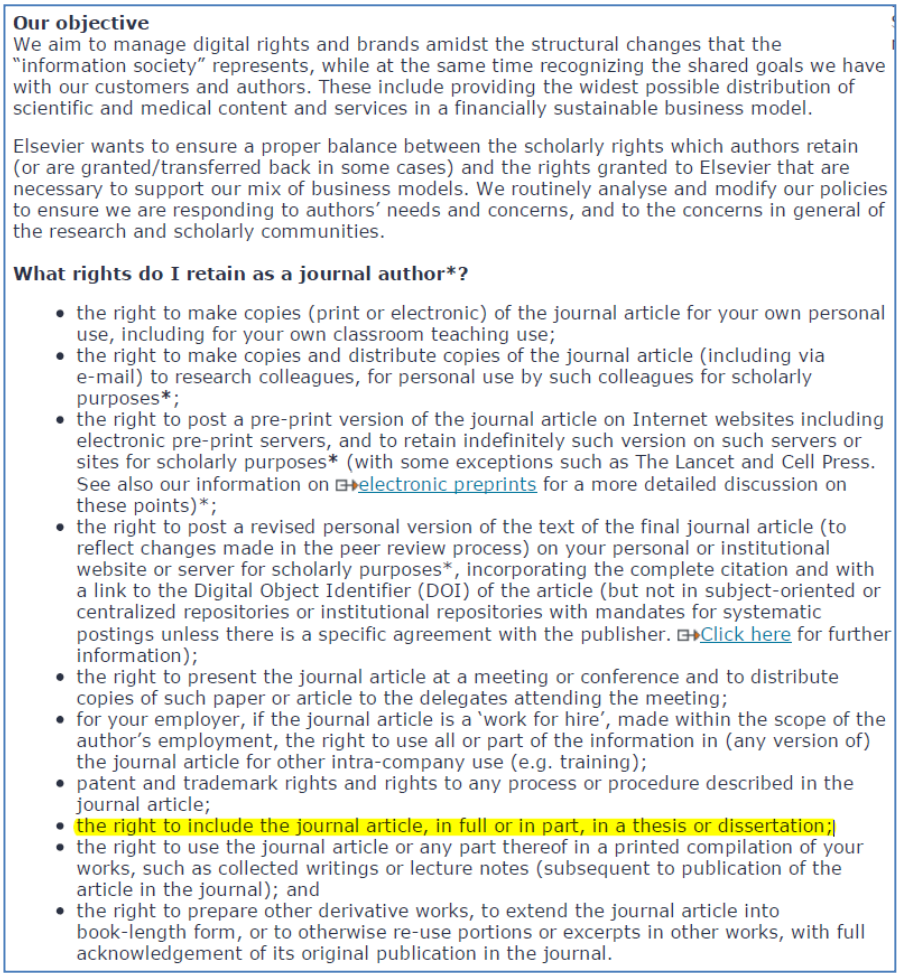

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