AMANDA ROQUE MARTINS

**Efeitos da suplementação com óleo de peixe sobre a
resistência à insulina e a função mitocondrial no músculo
esquelético de camundongos alimentados com dieta
hiperlipídica**

Tese apresentada ao Programa
de Pós-Graduação Interunidades
em Biotecnologia USP/Instituto
Butantan/IPT, para obtenção do
Título de Doutor em
Biotecnologia.

São Paulo
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"Efeito da suplementação com ácidos graxos poliinsaturados ômega-3 e do exercício físico regular sobre a resistência à insulina induzida por dieta hiperlipídica"

Prezado Professor,

Sua licença para uso de animais em experimentação, constante no protocolo em epígrafe, **foi prorrogada até 05.04.2016.**

Reitero que havendo alteração de metodologia e inserção de novos alunos ao projeto de pesquisa vinculado à referida licença a CEUA/ICB deverá ser informada.

Cordialmente,

Prof. Dr. WOTHAN TAVARES DE LIMA
Coordenador - CEUA-ICB/ /USP

Ilmo. Sr.
Prof Dr. Rui Curi
Departamento de Fisiologia e Biofísica
Instituto de Ciências Biomédicas - USP

*Dedico este trabalho aos meus pais, Basílio e Gina, à minha irmã Bárbara e ao Ciro, meu
companheiro e melhor amigo.*

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"Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar.
Mas o mar seria menor se lhe faltasse uma gota".
(Madre Teresa de Calcutá)

RESUMO

Martins AR. Efeitos da suplementação com óleo de peixe sobre a resistência à insulina e a função mitocondrial no músculo esquelético de camundongos alimentados com dieta hiperlipídica. [tese (Doutorado em Biotecnologia)]. São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo; 2014.

A obesidade é associada ao desenvolvimento de diversas desordens caracterizadas por um quadro de resistência à insulina (RI), como é caso do diabetes tipo 2 e da síndrome metabólica. Apesar de ainda não serem completamente conhecidos os fatores que relacionam obesidade e RI, evidências sugerem que a concentração elevada de ácidos graxos (AG) livres observada nestas condições desempenha papel central no desenvolvimento da RI no músculo esquelético. Ácidos graxos poliinsaturados ômega-3 (AG ω -3) são conhecidos por melhorar a sensibilidade à insulina e a homeostase da glicose em modelos animais de RI. Esses efeitos positivos tem sido relacionados a diferentes mecanismos e estudos recentes são sugestivos de uma participação importante da mitocôndria, apesar de permanecer desconhecido. A produção de espécies reativas de oxigênio (EROs) exerce papel importante na associação entre disfunção mitocondrial e RI. Por isso, avaliamos a influência da suplementação com óleo de peixe, uma fonte rica de AG ω -3, sobre a fisiologia mitocondrial e a produção de peróxido de hidrogênio (H_2O_2) no músculo esquelético de camundongos alimentados com dieta hiperlipídica. Camundongos C57Bl/6 machos, com 8 semanas de idade, foram distribuídos em 4 grupos: dieta normolipídica (C), dieta hiperlipídica (H), C + óleo de peixe (CP) and H + óleo de peixe (HP). O óleo foi administrado por gavagem oral (2g/Kg de peso corpóreo), três vezes por semana durante 12 semanas. O grupo H apresentou maior produção de H_2O_2 e danos à função mitocondrial, com diminuição do consumo basal de O_2 , de intermediários do ciclo do ácido tricarboxílico (ICATs), da atividade da cadeia transportadora de elétrons e da biogênese mitocondrial. O menor consumo de O_2 induzido pela dieta hiperlipídica foi associado à elevada produção de EROs, à menor transcrição de PGC1 α , ATM, NRF1 e TFAM, e também à redução da fosforilação de Akt. Esses efeitos foram parcialmente prevenidos pelo óleo de peixe que elevou a respiração, bem como o conteúdo de ICATs, a ativação de Akt e AMPK, e também levou à maior expressão de ATM, catalase e proteínas envolvidas na biogênese e função mitocondrial (PGC1 α , PPAR α , CPT1). Os conteúdos de RNAm de GLUT4, PLIN5, UCP3, SOD1 e proteínas mitocondriais (NDUFB3, NDUFB5, SDHB, SLC25A12, CYC1 e SURF1) não foram alterados pela dieta, nem pelo óleo de peixe. A suplementação com óleo de peixe protegeu contra a RI no músculo esquelético induzida pela dieta hiperlipídica reduzindo a produção de EROs, que parece regular a atividade mitocondrial por mecanismo envolvendo a proteína ATM.

Palavras-chave: Resistência à insulina. Ácidos graxos ω -3. Metabolismo muscular. Mitocôndria.

ABSTRACT

Martins AR. Effects of fish oil supplementation on insulin resistance and mitochondrial function on skeletal muscle of mice fed a high-fat diet. [Ph. D. thesis (Biotechnology)]. São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo; 2014.

Obesity is associated with the development of several diseases characterized by a severe insulin resistance namely type 2 diabetes mellitus and metabolic syndrome. Although the factors linking obesity and insulin resistance were not precisely defined yet, evidence suggests that the elevated plasma free fatty acid (FFA) level observed in these conditions plays a central role in the development of skeletal muscle insulin resistance. Omega-3 polyunsaturated fatty acids (ω -3 PUFAs) have been shown to improve insulin sensitivity and glucose homeostasis in animal models of insulin resistance. These beneficial effects have been linked to different mechanisms and recent studies indicate that it may also involve changes in mitochondrial function. Reactive oxygen species (ROS) production plays a central role in the association between mitochondrial dysfunction and insulin resistance. So we investigated the effects of fish oil (FO), a dietary source of ω -3 PUFAs, on mitochondrial physiology and hydrogen peroxide production in skeletal muscle from high-fat fed mice. C57Bl/6 male mice, 8 weeks-old, were randomly divided into four groups: control diet (C), high-fat diet (H), C + FO (CP) and H + FO (HP). FO was given by oral gavage (2g/Kg b.w.), three times per week during 12 weeks. The H group showed increased hydrogen peroxide production and impaired mitochondrial function with lower basal oxygen consumption, tricarboxylic acid cycle intermediates (ICAT), oxidative phosphorylation activity and mitochondrial biogenesis. The lower O₂ consumption was associated with elevated ROS production, decreased transcription of PGC1 α , ATM, NRF1 and TFAM, and also reduced Akt phosphorylation. These effects were partially prevented by FO administration, increasing muscle O₂ consumption, as well as ICAT contents, activation of Akt, AMPK, and up-regulating mRNA expression of ATM, catalase and mitochondrial proteins (PGC1 α , PPAR α , CPT1). mRNA contents of GLUT4, PLIN5, UCP3, SOD1 and mitochondrial proteins involved in oxidative phosphorylation (NDUFB3, NDUFB5, SDHB, SLC25A12, CYC1 and SURF1) were not changed by H diet or FO. Dietary FO protected from diet induced insulin resistance in skeletal muscle by reducing ROS production, which may exert a key role in the regulation of mitochondrial function through a mechanism involving ATM.

Key-words: Insulin resistance. ω -3 fatty acids. Skeletal muscle metabolism. Mitochondria.

LISTA DE FIGURAS

Figura 1 – Modelo proposto de resistência à insulina induzida por dieta hiperlipídica.....	24
Figura 2 – Testes de tolerância à glicose (GTT) e à insulina (ITT).....	42
Figura 3 – Concentrações de insulina e glicose no sangue e cálculo de HOMA-IR.....	44
Figura 4 – Metabolismo da glicose em resposta à insulina no músculo sóleo isolado.....	46
Figura 5 – PA, EPA e DHA – Captação de glicose e fosforilação de Akt em C2C12.....	47
Figura 6 – Efeitos da DHL e do óleo de peixe sobre a via de sinalização da insulina.....	49
Figura 7 – DHL, ômega-3 e o estresse oxidativo no músculo esquelético.....	50
Figura 8 – Consumo de O ₂ no músculo isolado e concentrações de intermediários do Ciclo de Krebs.....	52
Figura 9 – Proteínas da cadeia mitocondrial transportadora de elétrons.....	53
Figura 10 – Função mitocondrial no músculo esquelético resistente à ação da insulina e efeitos do óleo de peixe sobre fatores relacionados com a biogênese mitocondrial.....	55
Figura 11 - Representação esquemática dos efeitos da dieta e do óleo de peixe sobre os parâmetros estudados.....	66

LISTA DE QUADROS

Quadro 1 - Sequências dos <i>primers</i> utilizados para determinação do conteúdo de RNAm para os genes de interesse no homogenato do músculo sóleo.....	37
Quadro 2 - Resumo dos efeitos da DHL e do óleo de peixe apresentados na seção de resultados.....	56

LISTA DE TABELAS

Tabela 1 - Composição das dietas experimentais.....	31
Tabela 2 - Características gerais dos camundongos alimentados com dieta normo (grupo C) ou hiperlipídica (grupo H), suplementados ou não com óleo de peixe.....	41
Tabela 3 - Parâmetros metabólicos séricos avaliados em camundongos alimentados com dieta normo (grupo C) ou hiperlipídica (grupo H), suplementados ou não com óleo de peixe.....	43
Tabela 4 - Atividade das enzimas hepáticas gama-GT, AST e ALT no soro de animais alimentados com DNL ou DHL, suplementados ou não com óleo de peixe.....	44
Tabela 5 - Efeitos da DHL e do óleo de peixe sobre parâmetros metabólicos e inflamatórios analisados no músculo esquelético de camundongos tratados por 12 semanas.....	45

SUMÁRIO

1 INTRODUÇÃO.....	16
1.1 Obesidade e resistência à insulina.....	18
1.2 Dieta hiperlipídica e mecanismos envolvidos no desenvolvimento da resistência à insulina.....	19
1.3 Aspectos gerais das mitocôndrias.....	23
1.4 Resistência à insulina no músculo esquelético e o envolvimento da mitocôndria.....	24
1.5 Ácidos graxos ω-3, resistência à insulina e função mitocondrial.....	26
2 OBJETIVOS.....	28
3 ESTRATÉGIAS EXPERIMENTAIS.....	29
4 MATERIAL E MÉTODOS.....	30
3.1 Animais.....	30
3.2 Dietas.....	29
3.3 Grupos experimentais.....	31
3.4 Teste de tolerância à glicose (GTT).....	31
3.5 Teste de tolerância à insulina (ITT).....	32
3.6 Avaliação de parâmetros séricos.....	32
3.7 Índice HOMA (<i>Homeostatic Model Assessment</i>).....	32
3.8 Responsividade à insulina em músculo sóleo isolado.....	33
3.9 Determinação da produção de H_2O_2 em músculo sóleo isolado.....	33
3.10 Consumo de oxigênio no músculo sóleo isolado.....	34
3.11 Atividade da citrato sintase (CS).....	34
3.12 Extração de lipídeos totais do músculo para avaliação de TG e NEFA.....	35
3.13 Análise de citocinas pró-inflamatórias no músculo.....	35
3.14 Densidade mitocondrial por microscopia eletrônica de transmissão (MET).....	35
3.15 Ativação de proteínas envolvidas na via de sinalização da insulina.....	36
3.16 Determinação do conteúdo de RNAm.....	36
3.17 Cultura de células musculares esqueléticas.....	38
<i>3.17.1 Determinação do efeito de ácidos graxos sobre o metabolismo de glicose em cultura de células musculares esqueléticas C2C12.....</i>	<i>38</i>
<i>3.17.2 Efeito dos ácidos graxos sobre a fosforilação de Akt (serina 473) em células</i>	

<i>musculares cultivadas</i>	38
3.18 Análise estatística	39
4 RESULTADOS	40
5 DISCUSSÃO	57
6 CONCLUSÃO	67
REFERÊNCIAS	68
APÊNDICE A – Martins AR et al. <i>Lipids Health Dis.</i> 2012 Feb 23;11:30. doi: 10.1186/1476-511X-11-30.....	80
APÊNDICE B – Vinolo MA et al. <i>Am J Physiol Endocrinol Metab.</i> 2012 Jul 15;303(2):E272-82. doi: 10.1152/ajpendo.00053.2012. Epub 2012 May 22.....	92
APÊNDICE C – Masi LN et al. <i>J Biomed Biotechnol.</i> 2012;2012:945131. doi: 10.1155/2012/945131. Epub 2012 Aug 26.....	105
APÊNDICE D – Hirabara SM et al. <i>J Nutr Biochem.</i> 2013 Jun;24(6):1136-45. doi: 10.1016/j.jnutbio.2012.08.014. Epub 2012 Dec 15.....	115
APÊNDICE E – Lima E et al. <i>Macadamia oil supplementation attenuates inflammation and adipocyte hypertrophy in obese mice.</i> Aceito para publicação em 20/07/2014, <i>Mediators of Inflammation</i>	126
APÊNDICE F – Amaral CL et al. <i>DNA methylation changes induced by a high-fat diet and fish oil supplementation.</i> Submetido em 13/08/2014. <i>British Journal of Nutrition</i>	138

1 INTRODUÇÃO

A resistência à insulina é definida como uma condição na qual a insulina não é capaz de manter a concentração normal de glicose no sangue devido à menor captação de glicose (músculo esquelético), inibição da gliconeogênese (fígado) e da lipólise (tecido adiposo) (Fedor et al., 2008; Schenk et al., 2008). Esta condição é frequentemente associada com doenças crônicas relacionadas à obesidade, como o *diabetes mellitus* tipo 2, doenças cardiovasculares e síndrome metabólica. Muitos fatores parecem contribuir para o desenvolvimento da resistência à insulina, incluindo predisposição genética, sedentarismo, hábitos alimentares, inflamação, estresse e defeitos na sinalização da insulina (Schulman, 2000).

Considerando a crescente epidemia mundial de obesidade (Finucane et al., 2011) e suas complicações patofisiológicas, é importante investigar os mecanismos envolvidos na resistência à insulina induzida pelo excesso de ácidos graxos, como é o caso das dietas ocidentais, ricas em gordura. Entre os diferentes tipos de ácidos graxos, os saturados de cadeia longa, como ácido palmítico e esteárico, são conhecidos por induzirem resistência à insulina (Griffin et al., 1999; Hirabara et al., 2010; Roden, 2004; Yu et al., 2002; Yuzefovych et al., 2010).

Diversos mecanismos tem sido demonstrados pelo nosso grupo (Hirabara et al., 2003, 2006, 2007, 2010) e também por outros pesquisadores (Brehm et al., 2006; Carvalho-Filho et al., 2005; Griffin et al., 1999; Roden et al., 1996) para explicar como concentrações elevadas de ácidos graxos podem levar à resistência à insulina. Randle et al. (1963) foram os primeiros a propor que a utilização da glicose e o fluxo glicolítico estão diminuídos quando há maior suprimento de ácidos graxos. Esta ideia foi explicada pela competição entre substratos, levando à inibição de enzimas, por exemplo, a piruvato desidrogenase, induzida pelo maior conteúdo de acetil-CoA decorrente da maior oxidação de ácidos graxos (Dresner et al., 1999; Randle et al., 1998), hipótese conhecida com ciclo glicose-ácido graxo.

O acúmulo de lipídeos em tecidos com capacidade limitada para estocar gordura, como o músculo esquelético e fígado, é associado com a resistência à insulina. Diversos estudos mostraram que o acúmulo de triacilgliceróis no músculo esquelético está associado com prejuízos na sinalização da insulina e também com alterações no estado redox, como elevada produção de espécies reativas de oxigênio (EROs), o que contribui para a resistência à insulina pela diminuição da β -oxidação e da atividade do ciclo do ácido tricarboxílico

(Anderson et al., 2009; Choi et al., 2007; Koves et al., 2008; Sabin et al., 2007; Savage et al., 2007).

A mitocôndria é o principal local de produção de EROs no músculo esquelético, expondo o DNA mitochondrial bem como proteínas e componentes lipídicos a concentrações elevadas destes metabólitos, o que leva a danos e modificações estruturais que, a longo prazo, podem resultar em prejuízos à função da organela (Indo et al., 2007; Schrauwen, Hesselink, 2004). Além disso, o aumento do conteúdo intramuscular de lipídeos tem sido associado à diminuição da expressão de PGC1- α e de genes que codificam proteínas dos complexos I, II, III e IV da cadeia respiratória mitocondrial (Sparks et al., 2005). Alguns estudos também mostraram menor expressão de carnitina palmitoiltransferase-1 (CPT-1) e outras enzimas mitocondriais no músculo esquelético de indivíduos obesos e diabéticos (Hulver et al., 2003; Kim et al., 2000; Simoneau et al., 1997).

De fato, o suprimento excessivo de nutrientes em modelos de DM2 acompanhado de menor taxa de respiração mitocondrial tem sido descrito como fator primário de indução de resistência à insulina em células musculares esqueléticas. Esta condição está de acordo com a proposição de que o estado redox mitocondrial tem papel fundamental na ligação entre a sobrecarga mitocondrial e a resistência à insulina (Koves et al., 2005). Apesar disso, o mecanismo através do qual EROs induzem RI no músculo esquelético ainda não é completamente elucidado.

Além de dieta balanceada e prática de atividades físicas, que são medidas efetivas na prevenção da resistência à insulina (Hamdy et al., 2001), estratégias terapêuticas alternativas tem sido muito utilizadas. Uma delas é a suplementação com fontes ricas em ácidos graxos poliinsaturados ômega-3 (AG ω -3), que aumentam a sensibilidade à insulina e a homeostase da glicose em modelos animais de resistência ao hormônio. Diversos estudos em roedores mostram que AG ω -3 revertem a resistência à insulina por diminuírem a inflamação (Oh et al., 2010; Serhan et al., 2008). Entretanto, há cada vez mais evidências que apontam a mitocôndria como principal alvo desses metabólitos (Jelenik et al., 2010; Neschen et al., 2007).

1.1 Obesidade e resistência à insulina

A maior prevalência de obesidade mundial nos últimos anos levou ao aumento dramático de suas comorbidades, incluindo resistência à insulina, *diabetes mellitus* tipo 2 (DM2), dislipidemias, doenças cardiovasculares, síndrome metabólica e câncer (Elks, Francis, 2010). Considerando a crescente prevalência de obesidade em crianças (Speiser et al., 2005), esse aumento é atualmente considerado um grave problema de saúde pública, tanto em países desenvolvidos quanto em países em desenvolvimento (WHO, 2010). Segundo a Pesquisa de Orçamentos Familiares 2008-2009, realizada pelo Instituto Brasileiro de Geografia e Estatística (IBGE, 2011), a prevalência de sobrepeso ($\text{IMC} \geq 25 \text{ kg.m}^{-2}$) na população adulta brasileira é de 50,1% em homens e 48% em mulheres, sendo que a obesidade ($\text{IMC} \geq 30 \text{ kg.m}^{-2}$), acomete 12,4% dos homens e 16,9% das mulheres do país. É importante destacar que, no período entre 1974 e 2009, a prevalência de sobrepeso e obesidade na população brasileira aumentou contínua e intensamente, principalmente nos indivíduos do sexo masculino (IBGE, 1975; 2004; 2011).

O excesso de peso e a obesidade resultam da interação entre diversos fatores, sendo eles genéticos, metabólicos, comportamentais e ambientais. O aumento rápido da prevalência de excesso de peso nas últimas décadas é sugestivo de que os componentes comportamentais e ambientais são os principais responsáveis pela epidemia (Amuna, Zotor, 2008; Stein, Colditz, 2004). Dentre esses, destacam-se o consumo de dieta hiperenergética (principalmente rica em lipídeos) e a inatividade física (Roberts, Barnard, 2005).

O aumento prolongado da concentração de AG livres no plasma causa redução nas ações da insulina em vários tecidos, principalmente em músculos estriados, fígado e adipócitos (Boden, 2003; Hunnicutt et al., 1994; Kim et al., 1996; Kraegen, Cooney, 2008; Oakes et al., 1997; Randle et al., 1963; Zhang et al., 2007). Entretanto, os mecanismos envolvidos ainda não são completamente conhecidos. Estudos recentes são sugestivos de que a resposta inflamatória desencadeada nessas condições participa no desenvolvimento da resistência à insulina.

Em camundongos e ratos, o aumento na oferta de lipídeos na dieta resulta em obesidade e DM2 (Schemmel et al., 1970; West et al., 1992). O camundongo C57Bl/6j somente apresenta DM2 após tornar-se obeso. Nesse animal, a obesidade induzida pela dieta é caracterizada por depósito seletivo de gordura no mesentério (Rebufft-Scrive et al., 1993), observação consistente com os achados de que a obesidade visceral é fator de risco para a doença (Carey et al., 1997). Ratos e camundongos alimentados com dieta hiperlipídica

apresentam maior acúmulo de gordura visceral, sendo que a resistência à insulina no músculo e hiperinsulinemia ocorrem a partir de 4 semanas (Han et al., 1997).

1.2 Dieta hiperlipídica e mecanismos envolvidos no desenvolvimento da resistência à insulina

Os AG são armazenados na forma de triglicerídeos nas células, principalmente nos adipócitos, sendo importante fonte de energia em diferentes condições, como jejum e exercício físico. A insulina é um potente inibidor da lipólise e, dessa forma reduzindo a liberação de AG dos adipócitos pela inibição da enzima lipase hormônio-sensível (Jansson et al., 1994). A maioria dos AG são potencializadores da secreção de insulina. Após algumas horas, entretanto, os AG passam a ser tóxicos, diminuindo a resposta secretória das ilhotas, levando ao quadro conhecido como lipotoxicidade (Curi, Procópio, 2009).

Além da função energética e moduladora da secreção de insulina, os AG também regulam outras funções celulares, como viabilidade e sinalização celulares, expressão de genes, respostas imunoinflamatórias, estado redox celular e sensibilidade à insulina (Hirabara et al., 2007, 2010; Lambertucci et al., 2008; Martins et al., 2012; Martins de Lima et al., 2007; Newsholme et al., 2007). Essas ações variam de acordo com o tipo e concentração de AG, tempo de exposição e tipo celular.

Em 1963, Randle et al. descreveram o ciclo AG-glicose ou ciclo de Randle. Esses autores mostraram que o aumento na disponibilidade de AG diminui a utilização de glicose em coração perfundido e músculo diafragmático incubado de rato. A explicação para esse fenômeno seria que o aumento na disponibilidade de AG para esses tecidos eleva a produção de acetil-CoA e NADH mitocondrial através da β -oxidação, os quais inibem a piruvato desidrogenase (PDH), diminuindo a conversão de piruvato em acetil-CoA. O aumento do conteúdo de acetil-CoA proveniente de AG também eleva a produção de citrato, o qual, juntamente com a razão ATP/ADP elevada, inibe a fosfofrutoquinase, resultando em redução da via glicolítica (Hirabara et al., 2003, 2007). Como consequência, há aumento no conteúdo de glicose-6-fosfato, que passa a inibir a hexoquinase II, causando acúmulo de glicose intracelular e, portanto, redução do seu transporte. Assim, os AG causariam inibição da captação e utilização de glicose, resultando em resistência à insulina (Randle et al., 1963).

A associação da infusão lipídica com outras técnicas, como o *clamp* de glicose e insulina e a ressonância magnética nuclear, permitiu a mensuração concomitante do conteúdo de metabólitos intracelulares e a metabolização da glicose. Foi verificado que os AG

diminuem o conteúdo intracelular de glicose-6-fosfato e glicose e que esses efeitos precedem a redução na captação de glicose estimulada por insulina (Perseghin et al., 2003; Roden, 2004; Roden et al., 1996; Savage et al., 2007; Thompson et al., 2000). Esses dados são sugestivos de que outros mecanismos estão envolvidos na resistência ao hormônio induzida por AG, além do ciclo de Randle. Em particular, o efeito de AG sobre a sinalização à insulina tem sido estudado em vários tipos celulares.

Além dos efeitos observados diretamente no metabolismo da glicose, foi demonstrado que os AG saturados afetam as vias intracelulares de sinalização à insulina no músculo esquelético e em miócitos (Hawley et al., 2000; Hirabara et al., 2010; Roden et al., 1996; Savage et al., 2007). Em estudos que utilizaram infusão lipídica associada ao *clamp* euglicêmico-hiperinsulinêmico, foi observada redução na fosforilação do substrato do receptor de insulina-1 (IRS-1) em resíduos de tirosina (Griffin et al., 1999; Kruszynska et al., 2002; Yu et al., 2002); da atividade da fosfatidilinositol 3-quinase (PI3-quinase) associada ao IRS-1 e -2 (Dresner et al., 1999; Griffin et al., 1999; Kim et al., 2002; Yu et al., 2002) e da fosforilação e conteúdo total da proteína quinase B (PKB ou Akt) (Kim et al., 2002) em músculo esquelético. Corroborando com o efeito direto de AG saturados sobre a sinalização à insulina no músculo esquelético, foi mostrado que o ácido palmítico diminuiu expressão e atividade do receptor de insulina (Dey et al., 2005) e a fosforilação de IRS-1 e -2 em resíduos de tirosina (Hirabara et al., 2007; Storz et al., 1999), redução de Akt (Cazzolli et al., 2001; Hajdуч et al., 2001; Hirabara et al., 2010; Storz et al., 1999; Thompson et al., 2000) e de glicogênio sintase quinase-3 beta (GSK-3 beta) em músculo sóleo isolado, cultura primária de miócitos de ratos, miotubos pmi28, C₂C₁₂ e miócitos L6, conforme revisado por Schmitz-Peiffer et al. (2000). Assim como o observado no músculo esquelético, o ácido palmítico também inibe a atividade e a fosforilação de Akt em coração perfundido de ratos e em células HL-1 (Soltys et al., 2002).

Diversos mecanismos parecem colaborar para a inibição da sinalização à insulina por AG saturados, entre eles a ativação de diferentes quinases, como PKCs, IKK β , JNK e p38 MAP quinase. Essas quinases atuam catalizando a fosforilação de IRS-1 em resíduos de serina, inibindo sua fosforilação em resíduos de tirosina pela insulina e direcionando essa proteína para a via de degradação pelo proteassoma (Paz et al., 1997; Zhande et al., 2002). Esses efeitos culminam com a redução do recrutamento de IRS-1 na sinalização à insulina, bloqueando sua ação (Bloch-Damti, Bashan, 2005; Evans et al., 2005; Tirosh et al., 1999).

Vários estudos também têm mostrado a ação de AG sobre a expressão de genes envolvidos no metabolismo de glicose e de lipídeos. O acúmulo intramuscular de lipídeos tem

sido associado à menor expressão de PGC-1 α e de outros genes que codificam proteínas dos complexos I, II, III e IV da cadeia transportadora de elétrons, o que resulta em prejuízos à biogênese e função mitocondriais (Sparks et al., 2005). O PGC-1 α é um coativador transcricional de genes envolvidos na fosforilação oxidativa mitocondrial, como o fator de respiração nuclear-1 (NRF-1), proteína ligante GA (GABP) e PPARs, o que indica a habilidade de PGC-1 α em integrar sinais fisiológicos e elevar a biogênese e função mitocondriais (Puigserver et al., 1998; Wu et al., 1999). Dessa forma, a redução na expressão de PGC-1 α em situações de altas concentrações de AG pode estar ligada a alterações negativas na função mitocondrial (Petersen et al., 2004).

Estudos realizados em pacientes com resistência à insulina mostraram menor expressão de genes mitocondriais, como citocromo c oxidase e subunidades dos complexos I e III da cadeia transportadora de elétrons (Heilbronn et al., 2007). Indivíduos obesos e diabéticos também apresentaram menor atividade de importantes enzimas mitocondriais, como carnitina palmitoiltransferase-1 (CPT-1), citrato sintase e β -hidroxiacil-CoA desidrogenase, analisadas no músculo esquelético (Hulver et al., 2003; Simoneau, Kelley, 1997).

A maior produção de espécies reativas de oxigênio (EROs) no músculo esquelético também está fortemente relacionada com situações como obesidade, DM2 e síndrome metabólica (Abdul-Ghani et al., 2008; Bonnard et al., 2008; Kumashiro et al., 2008). Quando produzidas de forma adequada, as EROs estão envolvidas em importantes processos fisiológicos que resultam em respostas celulares desejadas. Mas, se produzidas em excesso, estão negativamente associadas a diferentes vias de sinalização biológicas, podendo interagir com diversos componentes celulares, levando a modificações irreversíveis, quadro esse conhecido como estresse oxidativo (Bashan et al., 2009).

A elevação crônica da concentração plasmática de lipídeos e o acúmulo intramuscular excessivo de gorduras são acompanhados por aumento na produção de EROs (Carvalho-Filho et al., 2005; Duval et al., 2007; Lambertucci et al., 2008). Pacientes diabéticos apresentam maior produção de EROs em células endoteliais através de ativação de NADPH oxidase, mecanismo esse mediado por PKC (Inoguchi et al., 2000). O complexo NADPH oxidase também está presente no músculo esquelético, o que leva a sugerir a ocorrência de mecanismo similar em situações de alta disponibilidade de AG. Interessantemente, nosso grupo mostrou que exposição ao palmitato induz maior produção de superóxido em cultura de células musculares, via ativação de NADPH oxidase (Lambertucci et al., 2008).

Animais submetidos a dietas ricas em gordura ou a drogas com ação oxidante apresentaram maior produção de EROs no músculo esquelético, associado ao estresse oxidativo e resistência à insulina (Blouet et al., 2007; Ogihara et al., 2004). Por outro lado, estudos realizados com animais tratados com drogas de ação antioxidante, como N-acetil cisteína, ácido lipóico, vitamina E e taurina, mostraram melhora do estresse oxidativo e aumento da sensibilidade à insulina (Blouet et al., 2007; Haber et al, 2003; Maddux, Goldfine, 2000). O envolvimento do estresse oxidativo em situações de resistência à insulina também foi observado em estudos utilizando camundongos com superexpressão da enzima superóxido dismutase 2 (SOD2), os quais apresentaram maior sensibilidade à insulina no fígado e menores concentrações de glicose, insulina e EROs (Zhai et al., 2011). Estudos em células musculares L6, mostraram redução na captação de glicose estimulada por insulina quando expostas ao H_2O_2 , efeito prevenido através de pré-tratamento com o agente antioxidante ácido lipóico (Maddux et al., 2001). O envolvimento do estresse oxidativo no desenvolvimento da resistência à insulina induzida por dieta hiperlipídica pode ser resumido na figura 1.

Figura 1. Modelo proposto de resistência à insulina induzida por dieta hiperlipídica: A elevação crônica na emissão de H_2O_2 mitocondrial como resultado do consumo de DHL serve para reduzir a capacidade de reserva

dentro dos sistemas redox e induzir uma mudança oxidativa no ambiente redox celular. Essa alteração global na célula pode ser suficiente para inibir a atividade de Ser/Thr fosfatases (PP2A), promovendo a ativação de quinases sensíveis ao estresse (JNK, ERK, IKK β) e o acúmulo de fosfatos inibitórios em várias proteínas da via de sinalização da insulina. Essa mudança no ambiente celular induzida por DHL também compromete a capacidade dos sistemas redox em tamponar a produção de H₂O₂ pela NADPH oxidase ligada à membrana em resposta à insulina, resultando em alterações ainda maiores no ambiente redox celular que são suficientes para inativar tanto PTP quanto Ser/Thr fosfatases, que diminuem a propagação do sinal ao longo da via e prejudicam a captação da glicose. [Adaptado de Fisher-Wellman & Neuffer, 2012].

A mitocôndria é o principal local de produção de EROs na célula muscular. Dessa forma, o DNA mitocondrial, lipídeos e proteínas estão expostos a conteúdos elevados desses metabólitos, causando modificações estruturais que, a longo prazo, podem levar a alterações funcionais da organela (Indo et al., 2007; Schrauwen, Hesselink, 2004).

1.3 Aspectos gerais das mitocôndrias

As mitocôndrias são importantes organelas celulares que representam o principal sítio do metabolismo oxidativo dos eucariontes. Nelas estão presentes enzimas do ciclo tricarboxílico, enzimas da β -oxidação, elementos da cadeia transportadora de elétrons e da fosforilação oxidativa. Na matriz também são encontrados DNA, RNA e ribossomos que produzem alguns componentes da cadeia respiratória (Nicholls, Ferguson, 2002; Voet, Voet, 2000).

Estas organelas são envolvidas por duas membranas lipídicas: uma externa lisa e uma interna que apresenta quantidade muito maior de proteínas (Nicholls e Ferguson, 2002). Como esta membrana é a principal responsável pela respiração, a sua superfície, estimada pelo número de cristas mitocondriais, representa um indicador da taxa respiratória (Nicholls, Ferguson, 2002; Voet, Voet, 2000).

Nas células, as mitocôndrias podem apresentar estruturas morfológicas diferentes, variando desde filamentos alongados até retículos ramificados (Bach et al., 2003). A mitofusina 2 (Mfn2), uma proteína da membrana responsável pela fusão mitocondrial, foi, recentemente, correlacionada com o estado metabólico de células musculares. Foi mostrado que a inibição de Mfn2 diminuiu a velocidade respiratória, o potencial de membrana e a oxidação de glicose. As mitocôndrias fissionadas pela inibição de Mfn2 apresentavam estado metabólico menos ativo, então a morfologia mitocondrial representa um possível sítio de regulação do metabolismo (Bach et al., 2003). Além disso, a biogênese mitocondrial também apresenta importante papel na regulação metabólica e no desenvolvimento da obesidade (Nicolson, 2007; Nisoli et al., 2007).

1.4 Resistência à insulina no músculo esquelético e o envolvimento da mitocôndria

Estudos mostram diminuição do conteúdo e da função mitocondriais em indivíduos diabéticos e em obesos insulino-resistentes, sugerindo que a disfunção mitocondrial está fortemente associada ao desenvolvimento da resistência à insulina (Holloway et al., 2007; Schrauwen-Hinderling et al., 2007).

Devido à sua massa, que representa aproximadamente 40% do peso total do corpo, o músculo esquelético é um dos principais alvos que contribuem para o desenvolvimento de resistência à insulina, a qual tem sido relacionada à diminuição de massa e função mitocondrial neste tecido (Boushel et al., 2007; Kelley et al., 2002; Petersen et al., 2005). Apesar disso, não é possível concluir se os defeitos encontrados na mitocôndria são causa ou consequência do desenvolvimento da resistência ao hormônio.

Dentre outras funções, as mitocôndrias são a central energética das células, sendo as principais responsáveis pela produção de adenosina trifosfato (ATP) nas fibras musculares. Além de ser necessário para contração muscular, o ATP também é essencial a outras atividades celulares comuns, como síntese de proteínas, RNA e DNA, assim como a manutenção do transporte de íons através das membranas. Para isso, glicose e ácidos graxos são utilizados usualmente como substratos energéticos. O acetil-CoA, produzido a partir da glicólise (citossol) ou da β -oxidação de ácidos graxos (mitocôndria), entra no ciclo do ácido tricarboxílico na matriz mitocondrial, o que resulta na produção das coenzimas reduzidas nicotinamida adenina dinucleotídeo (NADH) e flavina adenina dinucleotídeo (FADH₂). A reoxidação dessas coenzimas na cadeia respiratória mitocondrial gera energia suficiente para os diferentes complexos respiratórios (exceto o complexo II) bombear os prótons da matriz para o espaço intermembranas. O gradiente de prótons é então convertido em energia química pela ATP-sintetase (na fosforilação de adenosina difosfato-ADP), que é alimentado pelo fluxo de retorno dos prótons à matriz mitocondrial. Em situações em que as mitocôndrias estão em menor número ou estão “disfuncionais”, os ácidos graxos não podem ser devidamente utilizados por essas organelas. Dessa forma, há acúmulo de intermediários lipídicos, o que pode levar à inibição da sinalização à insulina através de diferentes mecanismos, resultando em desenvolvimento de resistência à insulina no músculo (Griffin et al., 1999; Itani et al., 2002; Schmitz-Peiffer et al., 1999).

Conforme visto anteriormente, diversos estudos mostram grande envolvimento de AG saturados na gênese da resistência à insulina associada à obesidade e ao DM2. Dessa forma,

quando indivíduos ou animais saudáveis são submetidos à infusão lipídica ou à dieta rica em gordura, algumas desordens são observadas na mitocôndria, tais como redução na síntese de ATP, na respiração mitocondrial e na fosforilação oxidativa (Bonnard et al., 2008; Brehm et al., 2006; Chansemaume et al., 2006; Koves et al., 2008; Laurent et al., 2007; Lionetti et al., 2007; Newsholme et al., 2007; Sparks et al., 2005; Szendroedi et al., 2009). Além disso, quando células esqueléticas musculares em cultura são tratadas com ácido palmítico, apresentam redução na função mitocondrial e na expressão de PGC-1, fator de transcrição relacionado com a expressão de enzimas oxidativas e biogênese mitocondrial (Coll et al., 2006; Lambertucci et al., 2008; Muoio, Koves, 2007; Pimenta et al., 2008; Racheck et al., 2007). Nosso grupo demonstrou que AG saturados agem diretamente em células musculares esqueléticas em cultura, induzindo disfunção mitocondrial associada com resistência à insulina com relação ao metabolismo de glicose (Hirabara et al., 2010).

1.5 Ácidos graxos ω -3, resistência à insulina e função mitocondrial

Os compostos bioativos da dieta podem contribuir para reduzir os efeitos da inflamação sistêmica desencadeada pela ingestão crônica de dieta rica em gorduras. Os AG são ácidos carboxílicos com cadeia carbônica que pode conter de 4 a 36 carbonos. Eles podem ser saturados (nenhuma ligação dupla) ou insaturados (uma ou mais ligações duplas na configuração *cis* e não conjugadas). A denominação ômega (ω) é atribuída aos AG com instaurações a partir do grupamento metila da cadeia carbônica, sendo os mais conhecidos aqueles com insaturações na terceira (ω -3), na sexta (ω -6), na sétima (ω -7) ou na nona (ω -9) posição. Os seres humanos não sintetizam AG ω -3 em quantidades suficientes e precisam obtê-los da dieta, sendo, portanto, considerados AG essenciais. A razão recomendada por alguns autores de ingestão de AG ω -6 para AG ω -3 está entre 2:1 e 3:1 (Simopoulos et al., 1999). Entretanto, na dieta ocidental, essa razão ocorre entre 10:1 a 30:1 (Nelson, Cox, 2008). Assim, a suplementação com uma fonte rica em ω -3 é uma importante estratégia terapêutica para aumentar a ingestão desses ácidos graxos e diminuir a razão ω -6: ω -3.

Os AG poliinsaturados ω -3, encontrados em óleo de peixe, possuem efeitos antiinflamatórios bem estudados. Dentre os AG ω -3, estão os ácidos α -linolênico (C18:3, ALA), eicosapentaenóico (C20:5, EPA) e o docosa-hexaenóico (C22:6, DHA), que são precursores de outros eicosanóides (Pompeia et al., 2000). A suplementação com óleo de

peixe apresenta efeito benéfico no tratamento de doenças cardiovasculares, inflamatórias e autoimunes, incluindo artrite reumatóide e *diabetes mellitus*. Esses efeitos benéficos são atribuídos à alta concentração de AG poliinsaturados ω -3, especialmente EPA e DHA (Gorjão et al., 2009), que são os mais abundantes em peixes de origem marinha e água fria, sendo a razão entre ω -3 e ω -6 maior que 3,5 (Magalhães et al., 2010).

Os AG saturados favorecem maior deposição de gordura quando comparados aos AG poliinsaturados (Flachs et al., 2005). Esse efeito dos AG poliinsaturados não está relacionado com redução no consumo de alimentos, mas sim com mudança metabólica em diversos tecidos, incluindo inibição da lipogênese no fígado e estímulo da oxidação de AG no músculo esquelético.

Diversos estudos com modelos animais mostram que AG ω -3 previnem a resistência à insulina e melhoram a homeostase da glicose (Jucker et al., 1999; Neschen et al., 2007; Oh et al., 2010; Storlien et al., 1987, 1991), mas os mecanismos envolvidos ainda estão sendo avaliados. A hipótese mais largamente descrita na literatura defende que os AG ω -3 reverterem a resistência à insulina ao reduzirem a inflamação (Oh et al., 2010; Serhan e Chiang, 2007), mas um número crescente de evidências é sugestivo de que o efeito desses AG sobre o aumento da sensibilidade à insulina está relacionado com a melhora da atividade mitocondrial.

Mais especificamente, estudos mostraram que ácidos graxos ômega-3 foram capazes de prevenir o desenvolvimento de resistência à insulina em camundongos selvagens, mas não em uma linhagem de camundongos nocautes para a subunidade catalítica α -2 da proteína quinase ativada por AMP (AMPK) (Jelenik et al., 2010) ou para receptor ativado por proliferador de peroxissomo α (PPAR α) (Neschen et al., 2007). Tanto AMPK como PPAR α são conhecidos por regular a expressão de genes envolvidos na biogênese mitocondrial e na oxidação de ácidos graxos (Garcia-Roves et al., 2008; Serhan et al., 2008). Esses dados mostram que a mitocôndria parece exercer papel importante no efeito protetor dos AG ômega-3 sobre a resistência à insulina.

Estudos utilizando DHA são os mais comuns para analisar os mecanismos envolvidos com os efeitos benéficos dos AG ω -3 e, dentre eles, foram descritos aumento da β -oxidação e da sensibilidade à insulina no músculo esquelético através de ativação de AMPK (Lam et al., 2011). As combinações de AG ω -3, como a encontrada no óleo de peixe, são muito consumidas e são conhecidas por aumentar a oxidação de ácidos graxos, reduzir o peso corporal, além de prevenir o ganho de peso (Calder et al., 2011; Duda et al., 2008; Fedor &

Kelley, 2009; Flachs et al., 2005; 2009; Hull, 2011; Kopecky et al., 2009). Além disso, conforme já comentado anteriormente, o tratamento com AG ω -3 combinados leva ao aumento na expressão de genes que codificam fatores regulatórios que controlam a biogênese mitocondrial e o metabolismo oxidativo, incluindo PGC1- α , em adipócitos (Flachs et al., 2005).

Entretanto, os estudos que investigam a ação dos AG ômega-3 sobre a fisiologia mitocondrial atribuem os efeitos benéficos principalmente ao DHA e não ao EPA (Khairallah et al., 2010a, b). Além disso, ainda não há trabalhos mostrando a influência da suplementação rica em EPA sobre a atividade mitocondrial no músculo esquelético em situação de resistência à insulina.

Dessa forma, o presente estudo foi conduzido para determinar como a suplementação com óleo de peixe atua sobre a resistência à insulina induzida por dieta hiperlipídica, investigando os efeitos sobre a fisiologia mitocondrial no músculo esquelético de camundongos.

2 OBJETIVOS

O principal objetivo desse estudo foi investigar os efeitos da suplementação com uma fonte rica em ácidos graxos ômega-3 (um óleo de peixe contendo 3,7 EPA: 1 DHA) sobre a resistência à insulina no músculo esquelético de camundongos alimentados com dieta hiperlipídica. Além disso, investigar a participação da mitocôndria tanto no desenvolvimento da resistência à insulina quanto na ação dos AG ω -3.

3 ESTRATÉGIAS EXPERIMENTAIS

Nosso objetivo inicial foi saber se o modelo escolhido era eficaz na indução de obesidade/resistência à insulina e se a dieta ou o óleo não causavam quaisquer outros danos que poderiam influenciar esse quadro. Para isso, avaliamos parâmetros metabólicos gerais, como glicemia, insulinemia, perfil lipídico, atividade sérica de enzimas hepáticas, ganho de peso, consumo e eficiência alimentar e energética.

Em seguida, procuramos ver se o quadro de resistência à insulina reproduzia no músculo esquelético isolado e compreender os mecanismos pelos quais a dieta rica em gorduras e a suplementação com o óleo de peixe modulam o metabolismo de glicose e a função mitocondrial nesse tecido. Para isso, foram utilizadas técnicas de RT-PCR, Western Blotting, responsividade à insulina no músculo isolado utilizando radioisótopos, ensaios enzimáticos colorimétricos e fluorimétricos.

Finalmente, buscamos compreender a importância do envolvimento da mitocôndria no estabelecimento da resistência à insulina no músculo esquelético e, também, na ação dos AG ω -3. Para verificar o envolvimento da mitocôndria, utilizamos além de RT-PCR e Western Blotting, microscopia eletrônica de transmissão e respirometria no músculo isolado. Este aspecto é relevante porque o desenvolvimento da obesidade e da resistência à insulina altera a biogênese mitocondrial e seu estado metabólico. Apesar disso, há dados controversos em relação à fisiologia mitocondrial no músculo em situações de maior disponibilidade de lipídeos (como é o caso das dietas ricas em gordura). Observa-se tanto aumento quanto diminuição da função mitocondrial nessas situações, e alguns até defendem que a mitocôndria não está envolvida nas alterações observadas na RI. Além disso, não há estudos mostrando o envolvimento da mitocôndria nas ações de um óleo de peixe com maior proporção de EPA em relação a outro rico em DHA.

4 MATERIAL E MÉTODOS

4.1 Animais

Foram utilizados camundongos C57BL/6, com 8 semanas de idade, obtidos do biotério do Instituto de Ciências Biomédicas da Universidade de São Paulo (ICB-USP). Os animais foram adaptados durante uma semana em sala com ciclo claro-escuro de 12-12 h e temperatura de 23 ± 2 °C, mantidos com dieta normal (ração Nuvital da Nuvilab, Colombo, PR) e água *ad libitum*. Todos os procedimentos desse estudo seguiram os princípios éticos de experimentação animal e foram aprovados pelo Comitê de Ética em Experimentação Animal do ICB-USP, registro nº 32, pág. 85, livro 2, em 05/04/2010.

4.2 Dietas

O protocolo experimental foi conduzido utilizando uma dieta normolipídica (DNL, 9% de energia proveniente de lipídeos) ou uma dieta hiperlipídica (DHL, 59% de energia proveniente de lipídeos). Ambas foram preparadas de acordo com as recomendações do Instituto Americano de Nutrição para camundongos adultos (Reeves, 1997), diferindo somente na densidade energética (DNL = 3803 Kcal/Kg dieta, DHL = 5340 Kcal/Kg dieta) e no conteúdo de proteína (Tabela 1). A quantidade de proteína foi modificada na DHL a fim de corrigir a redução da ingestão desse nutriente pelos animais submetidos à dieta obesogênica. As fontes de proteínas e lipídeos na dieta foram caseína, óleo de soja e banha suína, respectivamente.

Tabela 1. Composição das dietas experimentais

COMPOSIÇÃO	DNL	DHL
	g/Kg	
Amido	465,7	115,5
Caseína	140	200
Amido dextrinizado	155	132
Sacarose	100	100
Óleo de soja	4	35
Banha suína	36	315
Fibra	50	50
Mix mineral	35	35
Mix vitamínico	10	10
L-cistina	1,8	3
Bitartarato de colina	2,5	2,5

4.3 Grupos experimentais

Após o período de adaptação ao biotério, os camundongos C57BL/6 foram aleatoriamente distribuídos em 4 grupos experimentais: (1) C - animais alimentados com DNL; (2) H - animais alimentados com DHL; (3) CP - animais alimentados com DNL e suplementados com AG ω -3 e (4) HP - animais alimentados com DHL e suplementados com ω -3. A suplementação com AG ω -3 foi realizada por meio de gavagem oral com óleo de peixe (EPA/DHA 3,7:1, proporção determinada através de análise cromatográfica), na dose de 2 g/Kg peso corpóreo, 3 vezes por semana. Os respectivos controles (animais não suplementados) receberam água por gavagem. O protocolo experimental teve duração de 12 semanas. Durante as 4 primeiras semanas de suplementação, todos os animais receberam DNL, que foi mantida ou substituída por DHL nas 8 semanas seguintes (dependendo do grupo ao qual pertenciam). Durante todo o período experimental foram avaliados o peso corporal e o consumo de ração dos animais, a cada 48 horas.

4.4 Teste de tolerância à glicose (GTT)

O teste de tolerância à glicose foi realizado durante a última semana de protocolo experimental com dietas e suplementação. Após 6 horas de jejum e uma coleta de sangue

inicial (tempo 0), os animais receberam injeção por via intraperitoneal (i.p.) de solução de glicose a 50% (2.0 g/Kg de peso corpóreo). Amostras de sangue foram coletadas pela veia caudal nos períodos de tempo 15, 30, 45, 60 e 90 minutos após a sobrecarga de glicose, para determinação da glicemia. No tempo 30 minutos, também foi coletada amostra para determinação da insulina plasmática, que foi realizada através de imunoensaio do tipo *Enzyme-linked Immunosorbent Assay* (ELISA) (Rat/Mouse Insulin ELISA kit, Millipore, Missouri, USA).

4.5 Teste de tolerância à insulina (ITT)

Realizado dois dias após o GTT. Os animais foram submetidos a 6 horas de jejum alimentar. Amostras de sangue foram coletadas antes (tempo 0) e 10, 20, 30, 40 e 50 minutos após injeção com solução de insulina (0.75 U/Kg, i.p.) para determinação da glicemia. A velocidade constante do decaimento da glicose (Kitt) foi calculada usando a fórmula $0,693/t_{1/2}$. O $t_{1/2}$ da glicose foi calculado a partir da curva da análise dos mínimos quadrados da concentração da glicose durante a fase de decaimento linear (Bonora et al., 1989).

4.6 Avaliação de parâmetros séricos

Ao final das 12 semanas de protocolo experimental, os animais foram mantidos em jejum por 3-4 horas, anestesiados e o sangue coletado através de punção do plexo orbital para a determinação dos parâmetros bioquímicos. As concentrações séricas de glicose, triglicérides, ácidos graxos livres, colesterol total e frações e atividades de alanina transaminase (ALA), aspartato transaminase (AST) e gama-GT foram determinadas por ensaios colorimétricos disponíveis comercialmente, seguindo as recomendações do fabricante (Labtest).

4.7 Índice HOMA (*Homeostatic Model Assessment*)

A determinação da resistência à insulina foi avaliada pelo cálculo do índice HOMA-IR descrito por Matthews et al. (1985), no qual $HOMA\ IR = \text{insulinemia de jejum (mU/L)} \times \text{glicemia de jejum (mmol/L)} / 22,5$.

4.8 Responsividade à insulina em músculo sóleo isolado

Após 12 semanas, os animais foram eutanasiados e os músculos sóleos isolados e incubados como previamente descrito (Challiss et al., 1983; Crettaz et al., 1980). Os camundongos foram sacrificados por decapitação, seus músculos sóleos cuidadosa e rapidamente isolados, mantidos sob tensão de repouso com *clips* de inóx, pré-incubados em tampão bicarbonato de Krebs-Ringer contendo 5,6 mM de glicose, pH 7,4 por 30 minutos, em banho aquecido a 37° C, 95% O₂ e 5% CO₂, com agitação contínua (100 oscilações/min). Após esse período, os músculos foram transferidos para outros frascos contendo o mesmo tampão, porém acrescido de 0,3 µCi/mL de [U-¹⁴C]-D-glicose e 0,2 µCi/mL de 2-deoxi-[2,6-³H]-D-glicose. NaOH (0,2 mL) a 1M foi adicionado em compartimento separado, para adsorção do ¹⁴CO₂. Os músculos foram incubados na ausência ou presença de 7 nM de insulina, por 1 hora, nas mesmas condições. A gaseificação foi mantida por mais 15 min e, então, interrompida. Ao final da incubação, os músculos foram brevemente lavados em tampão frio, a 4° C, secos em papel de filtro e congelados em nitrogênio líquido. Em seguida, as amostras foram processadas para as mensurações da captação de 2-deoxi-[2,6-³H]-D-glicose, incorporação de [U-¹⁴C]-D-glicose, síntese de [¹⁴C]-glicogênio e descarboxilação de [U-¹⁴C]-D-glicose, segundo métodos descritos por Challiss et al. (1986), Espinal et al. (1983), Engle e Jones (1978) e Leighton et al. (1985), respectivamente.

4.9 Determinação da produção de H₂O₂ em músculo sóleo isolado

Os animais foram anestesiados e os músculos sóleos removidos e incubados em tampão bicarbonato de Krebs-Ringer previamente oxigenado, a 37°C, na presença de 50 µM de Amplex® Red (Molecular Probes, Eugene, OR, USA) e peroxidase (0,1 UI/mL) por 1 hora. Na presença de peroxidase, o Amplex® reage com o peróxido de hidrogênio para formar um composto fluorescente estável, a resorufina. A estequiometria da reação de oxidação do Amplex Red é de 1:1 para o peróxido de hidrogênio. (Zhou et al., 1997). A fluorescência do composto formado foi avaliada com 530 nm de excitação e 590 nm de emissão. Os resultados foram normalizados pelo peso do músculo e pelo tempo de incubação.

4.10 Consumo de oxigênio no músculo sóleo isolado

Os músculos sóleos foram rapidamente isolados e mantidos tensionados com *clips* de inóx. As amostras foram acondicionadas em uma câmara contendo meio DMEM (pH 7,4), a 37° C. A taxa de consumo basal de oxigênio foi monitorada e, em seguida, oligomicina (2 µg/mL) foi adicionada ao meio para monitorar o estado 4 da respiração mitocondrial. Em seguida, foi adicionado o agente desacoplador CCCP (Carbonil cianeto P-triclorometoxifenilhidrazona) (2 µM) para medida de consumo máximo de oxigênio no músculo isolado. O consumo de oxigênio foi monitorado através de um eletrodo de oxigênio tipo Clark (Oxytherm, Hansatech Instruments, Kings Lynn, UK).

4.11 Atividade da citrato sintase (CS)

Para a determinação da atividade máxima da enzima citrato sintase (CS), utilizou-se músculo gastrocnêmio previamente armazenado a -80 °C, o qual foi homogeneizado na proporção 1:10 em tampão de extração, constituído de 50 mM Tris Base e 1 mM de EDTA, pH 7,4. Posteriormente, esse homogenato foi centrifugado a 1.300 g , por 15 min a 4°C. As alíquotas do sobrenadante foram separadas para dosagem de proteínas totais e para o ensaio enzimático. A atividade máxima da enzima CS foi determinada segundo protocolo modificado de Alp et al. (1976), a partir da quantificação do complexo amarelo formado entre a CoA liberada na reação (1) com o ácido 5,5-ditiobis(2-nitrobenzóico) (DTNB) presente no tampão de ensaio, conforme equação abaixo:

Citrato Sintase

O tampão de ensaio foi constituído por Tris base a 50 mM; EDTA a 1 mM; DTNB a 0,2 mM; acetil-CoA a 0,1 mM e Triton X-100 a 1% (v/v), pH 8,1. A reação foi iniciada com a adição de oxaloacetato a 1 mM no solução de ensaio. O método baseia-se na reatividade do DTNB (reagente de Ellman) com a CoA-SH, marcando o grupo Tiol (SH), resultando em um composto iônico que absorve luz a 412 nm. Assim, a taxa de formação de CoA-SH pode ser avaliada nesse comprimento de onda. Os resultados foram expressos em nmol/min x mg de proteína⁻¹.

4.12 Extração de lipídeos totais do músculo para avaliação de TG e NEFA

Os músculos gastrocnêmio previamente congelados foram homogeneizados em metanol (500 µL). Foram extraídos lipídeos totais após adição de clorofórmo (1 ml) e água deionizada (400 µL) seguida por agitação vigorosa e centrifugação. A fase orgânica foi evaporada utilizando um sistema à vácuo (Concentrator Plus, Eppendorf) e os lipídeos ressuspensos em etanol absoluto. Os triglicérides (TG) e ácidos graxos livres (NEFA) foram quantificados utilizando *kits* comerciais (*Triglicerídeos Liquiform*, Labtest, Brazil; *NEFA*, Wako Diagnostics, USA). Os resultados foram expressos em µmol/g peso do tecido.

4.13 Análise de citocinas pró-inflamatórias no músculo

O músculo gastrocnêmio foi homogeneizado em tampão de lise RIPA 1x (0.5 M Tris-HCl, pH 7.4, 1.5 M NaCl, 2.5 % ácido desoxicólico, 10% NP-40, 10 mM EDTA) (Millipore, Temecula, CA, USA, # 20-188) acrescido de inibidores de proteases (100 mM fluoreto de sódio, 10 mM EDTA, 10 mM ortovanadato de sódio, 10 µg/mL leupeptina, 10 µg/mL aprotinina, 10 mM pirofosfato de sódio e 1 mM PMSF – fluoreto de fenilmetilsulfonil). Após centrifugação, TNFα, IL-6 e IL-1β foram analisadas no sobrenadante pelo método de ELISA (Duo Set, R&D System, Minneapolis, MN, EUA). A concentração de proteínas foi determinada utilizando ácido bicinônico – método de BCA (Pierce ® BCA protein Assay kit, Thermo Scientific, Rockford, IL, USA, # 23227).

4.14 Densidade mitocondrial por microscopia eletrônica de transmissão (MET)

Após isolados, os músculos sóleos foram divididos em porções medindo 1 mm x 1 mm, aproximadamente. Os cortes foram fixados em solução tampão de glutaraldeído a 2,5% , na qual foram mantidos por 3 h, a 4 °C. Após esse período, a solução foi substituída por tampão cacodilato, contendo glutaraldeído a 0,1 M e mantida a 4 °C até processamento das amostras. Após o processo de lavagem, desidratação e inclusão em resina, o material foi cortado utilizando ultramicrotomo. Os cortes realizados pelo ultramicrotomo foram de algumas dezenas de nanômetros (1 nm= 10⁻³ mm) para permitir a passagem do feixe eletrônico e observação ao MET. O material cortado pelo ultramicrotomo foi coletado em microgrades e contrastado antes de ser observado. As grades foram observadas em microscópio Jeol JEM- 100 CXII, as imagens obtidas nos aumentos de 8, 14, 27 e 50.000x e

salvas para posterior análise. O processamento das amostras e aquisição das imagens foram realizados no Laboratório de Microscopia Eletrônica da Faculdade de Medicina da USP, campus de Ribeirão Preto.

4.15 Ativação de proteínas envolvidas na via de sinalização da insulina

A técnica de *Western blotting* foi realizada para avaliar o conteúdo e fosforilação de proteínas no músculo. Os músculos foram rapidamente isolados e incubados em meio bicarbonato de Krebs-Ringer previamente gaseificado, durante 20 min, na presença ou ausência de insulina (7 nM). Após incubação, os tecidos foram homogeneizados em tampão de extração (50 mM de Tris-HCl, pH 7,5; 150 mM de NaCl e 1 mM de ortovanadato de sódio) e, em seguida, as amostras foram centrifugadas a 13.000 g, a 4° C, por 20 min e os sobrenadantes submetidos à quantificação protéica pelo método de Bradford (1976), usando curva de albumina como padrão. As análises de foram realizadas segundo descrito por Towbin et al. (1979). Alíquotas de cada amostra, com a mesma concentração de proteínas totais (30 µg), foram tratadas de acordo com o método descrito por Laemmli (1970) e submetidas à eletroforese em *SDS*-gel de poliacrilamida (*SDS*-PAGE) (Shapiro et al., 1967). Em seguida, as proteínas do gel foram transferidas para uma membrana de nitrocelulose, por 80 min, a 100 V. As membranas foram incubadas, após o bloqueio, com anticorpos primários diluídos (1:1000) em solução basal acrescida de 3% de leite desnatado ou BSA, *overnight*, a 4°C. Foram utilizados anticorpos anti-fosfo-Akt (Ser⁴⁷³), Akt total, fosfo-GSK-3β, GSK-3β total, fosfo-AMPK, AMPK total, fosfo-CREB, CREB total, Mitofusina-2, PGC1-α e OXPHOS. As membranas foram lavadas e submetidas à incubação com os anticorpos secundários conjugados com peroxidase, por 1 hora, em solução basal contendo 1% de leite, à temperatura ambiente. Após nova sessão de lavagens (3 x 10 min), as membranas foram incubadas com o substrato para a peroxidase (*kit* ECL) por 1 min e imediatamente expostas a filmes de raio-X por períodos variáveis de tempo. Os filmes foram revelados de forma convencional. As intensidades das bandas das auto-radiografias foram quantificadas por densitometria óptica, pelo programa *Image J* (NIH, EUA).

4.16 Determinação do conteúdo de RNAm

A expressão de alguns genes envolvidos no metabolismo (glicose e ácidos graxos), sinalização à insulina, função mitocondrial e estresse oxidativo foi avaliada em músculo

esquelético (sóleo). Os tecidos foram homogeneizados em reagente TRIzol® (Life Technologies, USA) para extração do RNA total (Chomczynski, Sacchi, 1987). O RNA foi quantificado por leitura no aparelho Nano Drop 2000 (Thermo Scientific, Uniscience, São Paulo, Brasil) e o grau de pureza determinado pela razão 260/280 nm (razão igual a 2 indicando alto grau de pureza). O cDNA foi sintetizado a partir de 1 µg do RNA total extraído, utilizando a enzima transcriptase reversa. A expressão gênica foi quantificada por PCR em tempo real (Higuchi et al., 1992), utilizando o aparelho ROTOR GENE 3000 da Corbett Research (Mortlake, NSW, Austrália) e SYBR Green como marcador fluorescente, utilizando-se os primers de interesse, conforme especificados no Quadro 1. A quantificação da expressão dos genes foi determinada usando o método da Ct comparativa ($Ct = threshold\ cycle$; número de ciclo no qual o produto do PCR atinge o limiar de detecção), tendo a expressão da HRPT1 como padrão interno (Livak e Schmittgen, 2001).

Quadro 1. Sequências dos *primers* utilizados para determinação do conteúdo de RNAm para os genes de interesse no homogenato do músculo sóleo

Gene	Seq Forward	Seq Reverse
NDUFB3	TGGCCATGGTAAAATGGAAC	AAGCTTCTTCTGCACCGTTTCT
NDUFB5	GCATCCGATATCAAGATGGATCG	CTTTAACCGTAACTCAGCCTTTTCA
SDHB	CCCAGGAGGGCAAGCAA	GTACAGCCCGTCCAGCTTCT
SLC25A12	CACCACAGGACCGAGAGTCA	GGCTTTGGCACCCTTGTA
CYC1	CAGCTACCCATGGTCTCATCGT	CACCTGCTTGATACCTGGAAACC
SURF1	AAGTGAATCCTGAGACCAGACAGA	TGTGAGCCTCACTATGCCAACT
SOD1	AAAGCGGTGTGCGTGCTGAA	CAGGTCTCCAACATGCCTCT
SOD2	ACT GAA GTT CAA TGG TGG GG	GCT TGA TAG CCT CCA GCA AC
GPX	CCTCAAGTACGTCCGACCTG	CAATGTCGTTGCGGCACACC
CAT	GCAGATACCTGTGAACTGTC	GTAGAATGTCCGCACCTGAG
IRS1	CTCAGTCCCAACCATAACCAG	GAGTGTTTCATAGGCGAGATGG
UCP3	GACTATGGATGCCTACAGAACC	ACTCCAGCAACTTCTCCTTG
Plin5	CATGACTGAGGCTGAGCTAG	GAGTGTTTCATAGGCGAGATGG
CPT1	CCTCCGAAAAGCACCAAAAC	GCTCCAGGGTTCAGAAAGTAC
GLUT4	CATTCCCTGGTTCATTGTGG	GAAGACGTAAGGACCCATAGC
PGC1α	CACCAAACCCACAGAAAACAG	GGGTCAGAGGAAGAGATAAAGTTG

Descrição dos genes utilizados seguindo sua ordem no quadro: NADH dehydrogenase (ubiquinone) 1 beta subcomplex 3; NADH dehydrogenase (ubiquinone) 1 beta subcomplex 5; succinate dehydrogenase subunit B (iron-sulfur); solute carrier family 25 (aspartate/glutamate carrier), member 12; cytochrome c-1; surfeit 1; superoxide dismutase [Cu-Zn]; superoxide dismutase [Mn]; glutathione peroxidase 1; catalase; insulin receptor substrate 1; uncoupling protein 3 (mitochondrial, proton carrier); perilipin 5; carnitine palmitoyltransferase I; glucose transporter 4; peroxisome proliferator-activated receptor gamma, coactivator 1 alpha

4.17 Cultura de células musculares esqueléticas

Cultura de células C2C12 de camundongo foi realizada segundo o método descrito por Schmitz-Peiffer et al. (1999). Mioblastos de camundongo C2C12 foram mantidos em meio DMEM suplementado com 10% de soro fetal bovino, 2 mM de glutamina, 15 mM de HEPES e 500 U/mL de penicilina, pH 7,5, com 5% de CO₂. Para diferenciação dos mioblastos em células musculares, esses foram plaqueados em placas de 24 poços (20.000 células por poço) ou 6 poços (40.000 células por poço), crescidos até 80-90% de confluência em meio DMEM contendo 10% de soro fetal bovino e, após isso, cultivados em meio DMEM contendo 1% soro fetal bovino por 3-4 dias.

4.17.1 Determinação do efeito de ácidos graxos sobre o metabolismo de glicose em cultura de células musculares esqueléticas C2C12

Uma vez determinada a toxicidade dos ácidos palmítico, EPA e DHA, avaliou-se o efeito desses metabólitos nas células musculares C2C12 em concentrações não tóxicas. As células foram cultivadas por 72 h na ausência ou presença de EPA (50 μ M) ou DHA (50 μ M). Nas últimas 24 h, adicionou-se ou não o ácido palmítico (100 μ M). Após esse período, as células musculares esqueléticas foram incubadas por uma hora em meio DMEM contendo 5,6 mM de glicose, 0,2 μ Ci/mL de 2-desoxi-[2,6-³H]-D-glicose, na ausência ou presença de 7 nM de insulina. Ao término da incubação, as células foram lavadas com PBS a 4° C e congeladas em nitrogênio líquido. Em seguida, as células foram digeridas em 1 M de KOH, a 70° C, por 20 min. Procedeu-se, então, a mensuração da captação de 2-desoxi-[2,6-³H]-D-glicose segundo o método descrito por Challiss et al. (1986). Captação inespecífica de glicose foi determinada pelo uso de [1-¹⁴C]-L-glicose. Alíquotas de cada amostra foram utilizadas para a mensuração da quantidade de proteínas totais segundo método descrito por Bradford (1976) para a normalização dos resultados (nmol/mg de proteína).

4.17.2 Efeito dos ácidos graxos sobre a fosforilação de Akt (serina 473) em células musculares cultivadas

Uma vez determinado o tempo em que há fosforilação máxima da Akt, o efeito de AG sobre esta fosforilação foi avaliado. As células musculares foram cultivadas por 72 h na ausência ou presença de 50 μ M de EPA ou DHA. Nas últimas 24 h, adicionou-se ou não 100 μ M de ácido palmítico. Após este período, as células foram incubadas na ausência ou

presença de 7 nM de insulina por 15 min e a fosforilação da Akt em serina 473 avaliada pela técnica de western blotting como descrito anteriormente.

4.18 Análise Estatística

Os dados estão apresentados como média $\pm$ erro padrão da média (EPM) e foram analisados pelo teste ANOVA *two-way*, seguido pelo pós-teste de Bonferroni ($p < 0,05$).

5 RESULTADOS

Com o objetivo de induzir obesidade e resistência à insulina para estudar os efeitos da suplementação com óleo de peixe no músculo esquelético, foi necessário padronizar um modelo experimental que representasse quadros comumente vistos em clínica. Para isso, foram escolhidos os camundongos da linhagem C57Bl/6, posto que estes possuem propensão à obesidade e resistência à insulina e também por já serem utilizados em outros estudos em nosso laboratório, sendo possível estabelecer uma ligação entre as linhas de pesquisa do nosso grupo de trabalho. As dietas escolhidas também já são utilizadas em nosso grupo e também outros para caracterização deste modelo.

Durante as 12 semanas de protocolo experimental, foram monitorados o peso corporal dos animais e o consumo de ração. A partir destes dados, juntamente com a composição das diferentes dietas experimentais, foi possível calcular ingestão alimentar e energética, e também eficiência alimentar e energética.

Em relação ao acompanhamento do peso dos animais (Tabela 2), foi observada diferença no ganho de peso entre os grupos C e H, assim como entre CP e HP. Entre os grupos que receberam dieta normolipídica, não houve diferença significativa no ganho de peso. Já entre os animais que receberam dieta hiperlipídica, o ganho de peso foi menor nos suplementados com óleo de peixe.

O consumo alimentar foi monitorado durante as 12 semanas e não foi detectada diferença significativa entre os diferentes grupos que receberam dieta controle. Porém, essa diferença foi observada nos grupos que receberam dieta hiperlipídica em relação aos seus respectivos controles, assim como entre H e HP (Tabela 2).

Os dados de eficiência alimentar e eficiência energética seguem o mesmo padrão de resposta. Comparando esses dados com aqueles referentes à ingestão, pode-se observar que, apesar da menor ingestão alimentar pelos animais dos grupos que receberam dieta hiperlipídica, esses apresentaram maior eficiência alimentar e obtiveram mais energia ingerida em relação aos seus respectivos controles (Tabela 2).

Tabela 2. Características gerais dos camundongos alimentados com dieta normo (grupo C) ou hiperlipídica (grupo H), suplementados ou não com óleo de peixe.

	C	H	CP	HP
Ganho de peso (g)	3.08 ± 0.46 ^a	10.94 ± 0.7 ^b	3,46 ± 0.42 ^a	8.415 ± 0.76 ^c
Ingestão alimentar (g/dia/animal)	3.61 ± 0.09 ^a	2.44 ± 0.06 ^b	3.79 ± 0.06 ^a	2.18 ± 0.07 ^b
Eficiência alimentar (ganho de peso (g)/ consumo ração (g))	0.029 ± 0.05 ^a	0.45 ± 0.07 ^b	0.026 ± 0.04 ^a	0.39 ± 0.10 ^b
Eficiência enrgética (ganho de peso (g)/ kcal ração (g))	0.006 ± 0.036 ^a	0.098 ± 0.04 ^b	0.014 ± 0.016 ^a	0.077 ± 0.04 ^b
Índice de adiposidade *	1.353 ± 0.19 ^a	3.789 ± 0.16 ^b	1.729 ± 0.08 ^a	2.807 ± 0.23 ^c
Tecido adiposo retroperitoneal (g)	0.295 ± 0.05 ^a	0.749 ± 0.05 ^b	0.366 ± 0.02 ^a	0.693 ± 0.03 ^c
Tecido adiposo epididimal (g)	0.774 ± 0.11 ^a	2.147 ± 0.18 ^b	0.989 ± 0.06 ^a	1.825 ± 0.22 ^c
Tecido adipose mesentérico (g)	0.285 ± 0.04 ^a	0.69 ± 0.06 ^b	0.373 ± 0.06 ^a	0.48 ± 0.06 ^c

Suplementação com óleo de peixe (2 g/Kg peso corpóreo, 3x/semana, gavagem oral) ou água, iniciada quatro semanas antes da administração de dieta hiperlipídica e mantida até o final do protocolo experimental (12 semanas). Resultados expressos como Média ± EPM (n = 10). Os dados foram analisados por *two-way ANOVA* e pós-teste de Bonferroni. *O índice corresponde à soma dos depósitos de tecido adiposo. ^{a, b, c} Letras diferentes representam diferenças estatísticas entre os valores.

Para verificar a sensibilidade à insulina, foram realizados testes de tolerância à glicose e à insulina após 6 horas de jejum, na última semana do protocolo experimental, com intervalo de 3 dias entre os testes.

Os resultados obtidos com o GTT foram utilizados para construir a curva glicêmica e calcular a área incremental da glicose sérica. Houve diferenças entre os grupos que receberam o mesmo tipo de suplementação, mas não entre os animais que receberam o mesmo tipo de dieta. Apesar disto e embora sem diferença significativa, os animais do grupo CP apresentaram valores de glicemia menores que os do grupo C (Tabela 3 e Fig. 2B).

Os dados referentes ao ITT foram utilizados para montar a curva de decaimento da concentração plasmática de glicose, que foi posteriormente usada para o cálculo do K_{itt} . Em relação a esse parâmetro, foi observada diferença na taxa de decaimento de glicose do grupo H em relação ao C, o que é indicativo da resistência à insulina. A suplementação com óleo de

peixe reverteu parcialmente o efeito da DHL nesse parâmetro, mostrando melhora da sensibilidade à insulina (Tabela 3 e Figura 2D).

Figura 2. Testes de tolerância à glicose (GTT) e à insulina (ITT). Os testes foram realizados após 11 semanas do protocolo experimental. Curva com os valores de glicose sérica (A) e área incremental sob a curva da glicose sérica (B) estão representadas. Curva com valores de glicemia durante o ITT (C) e a taxa de desaparecimento da glicose sanguínea (Kitt; D). Resultados expressos como média $\pm$ EPM ($n= 12-15$ /grupo). ANOVA Two-way seguida por pós-teste de Bonferroni foi utilizada para a análise estatística. AUC, *area under the curve*.

Tabela 3. Parâmetros metabólicos avaliados em camundongos alimentados com dieta normo (grupo C) ou hiperlipídica (grupo H), suplementados ou não com óleo de peixe

	C	H	CP	HP
Glicemia jejum (mg/dL)	186.1 ± 5.66 ^a	221.0 ± 8.38 ^b	172.3 ± 7.22 ^a	201.6 ± 3.64 ^c
GTT (AUC)	7987 ± 779.3 ^a	13090 ± 643.9 ^b	6494 ± 696.8 ^a	13863 ± 946.9 ^b
ITT (% glicose/min)	3.01 ± 0.15 ^a	2.07 ± 0.16 ^b	3.00 ± 0.24 ^a	2.76 ± 0.22 ^a
Colesterol total (mg/dL)	148.2 ± 6.72 ^a	192.7 ± 11.96 ^b	101.3 ± 9.61 ^c	122.1 ± 9.32 ^a
Colesterol LDL (mg/dL)	127.4 ± 5.15 ^a	165.1 ± 14.49 ^b	73.2 ± 8.5 ^c	99.34 ± 4.25 ^{a,c}
Colesterol HDL (mg/dL)	14.73 ± 0.83	15.52 ± 1.86	17.58 ± 2.23	16.22 ± 1.50
Triglicérides (mg/dL)	83.65 ± 7.03	66.71 ± 4.34	79.51 ± 9.74	62.10 ± 3.15

Suplementação com óleo de peixe (2 g/Kg peso corpóreo, 3x/semana, gavagem oral) ou água, iniciada quatro semanas antes da administração de dieta hiperlipídica, e mantida até o final do protocolo experimental (12 semanas). GTT: teste de tolerância à glicose; ITT: teste de tolerância à insulina. Resultados expressos como Média ± EPM (n = 8-15). Os dados foram analisados por two-way ANOVA e pós-teste de Bonferroni. ^{a, b, c} Letras diferentes representam diferenças estatísticas entre os valores.

Ao final do protocolo experimental, os animais foram submetidos a jejum de 3-4 h e, antes do sacrifício, anestesiados e o sangue coletado para mensuração de parâmetros plasmáticos, utilizando *kits* específicos e seguindo as recomendações do fabricante.

A glicemia foi mais elevada nos grupos H e HP em relação aos seus respectivos controles. Além disso, também foi encontrada diferença significativa entre os dois grupos alimentados com dieta hiperlipídica, sendo observada diminuição da glicemia nos animais suplementados com óleo de peixe (Tabela 3).

Animais que receberam DHL apresentaram aumento das concentrações séricas do colesterol total e do colesterol LDL em relação aos animais alimentados com DNL. A suplementação com óleo de peixe levou à redução desses parâmetros em ambos os grupos (CP e HP). Triglicérides e colesterol HDL não foram alterados pela dieta nem pela suplementação com óleo (Tabela 3).

Não foi observada alteração da função hepática decorrente da dieta ou da suplementação com óleo de peixe, como pode ser verificado pela determinação da atividade das enzimas gama-GT, AST e ALT (Tabela 4).

Tabela 4. Atividades das enzimas hepáticas gama-GT, AST e ALT no soro de animais alimentados com DNL ou DHL, suplementados ou não com óleo de peixe

Grupos	γ GT (U/L)	AST (U/L)	ALT (U/L)
C	68,70 $\pm$ 19,09	62,06 $\pm$ 16,43	43,53 $\pm$ 23,92
H	70,51 $\pm$ 11,37	56,13 $\pm$ 14,99	52,39 $\pm$ 28,23
CP	67,64 $\pm$ 13,17	66,18 $\pm$ 12,70	56,41 $\pm$ 33,83
HP	76,31 $\pm$ 9,01	59,26 $\pm$ 22,53	52,39 $\pm$ 24,42

Resultados expressos em média $\pm$ DP (n = 9-16 animais). C = DNL + água; H = DHL + água; C+ ω -3 = DNL + óleo de peixe; H+ ω -3 = DHL + óleo de peixe.

A insulinemia de jejum dos animais alimentados com DHL foi superior à apresentada pelos animais alimentados com DNL, sendo que a associação com óleo de peixe promoveu reversão desse quadro (Figura 3A). A resistência à insulina apresentada pelos animais do grupo H (verificada pelos resultados obtidos nos testes de tolerância à glicose e à insulina) foi confirmada pelo índice HOMA-IR. A dieta hiperlipídica promoveu aumento no índice HOMA-IR em comparação aos animais alimentados com DNL. A suplementação com óleo de peixe levou à diminuição desse índice nos animais que receberam DHL (Figura 3C).

Figura 3. Concentrações de insulina e glicose no sangue e cálculo do HOMA-IR. Insulinemia de jejum (A), Glicemia de jejum (B) e índice HOMA-IR (C) de animais tratados com DHL, associada ao pré-tratamento com óleo de peixe. . Animais dos grupos CP e HP foram suplementados com óleo de peixe durante todo o período experimental. No período de 0 a 4 semanas todos os animais foram alimentados com DNL. No período de 4 a 12 semanas, os animais dos grupos H e HP foram alimentados com DHL. Resultados expressos em média $\pm$ EPM. A e C: * $p < 0,05$ vs grupo HP.

Uma vez confirmado o quadro de resistência à insulina induzido por dieta hiperlipídica, iniciamos os experimentos no músculo esquelético isolado. Assim, após as 12 semanas de tratamento, os animais foram sacrificados, os músculos gastrocnêmio foram congelados para posterior avaliação dos parâmetros inflamatórios e metabólicos apresentados

na Tabela 5. Não foram encontrados efeitos significativos induzidas pelo consumo de DHL nem pela suplementação com óleo de peixe.

Tabela 5. Efeitos da DHL e do óleo de peixe sobre parâmetros metabólicos e inflamatórios analisados no músculo esquelético de camundongos tratados por 12 semanas

	C	H	CP	HP
Triglicérides($\mu\text{mol/g}$ tecido)	1.44 ± 0.36	1.33 ± 0.24	1.69 ± 0.29	1.51 ± 0.27
NEFA ($\mu\text{mol/g}$ tecido)	1.37 ± 0.17	1.22 ± 0.20	1.14 ± 0.12	1.18 ± 0.13
Citrato sintase ($\mu\text{mol/min/mg}$ tecido)	0.107 ± 0.008	0.106 ± 0.008	0.103 ± 0.008	0.112 ± 0.007
Lactato (mg/dL)	2.363 ± 0.147	2.312 ± 0.094	2.820 ± 0.170	2.529 ± 0.125
TNF- α (pg/mg proteína)	9.983 ± 1.09	8.743 ± 1.16	9.850 ± 1.27	10.63 ± 1.07
IL-6 (pg/mg proteína)	6.822 ± 0.71	5.222 ± 0.38	6.017 ± 0.81	6.495 ± 0.53
IL-1 β (pg/mg proteína)	5.938 ± 0.79	4.360 ± 0.42	5.447 ± 0.95	5.196 ± 0.45

Suplementação com óleo de peixe (2 g/Kg peso corpóreo, 3x/semana, gavagem oral) ou água, iniciada quatro semanas antes da administração de dieta hiperlipídica, e mantida até o final do protocolo experimental (12 semanas). GTT: teste de tolerância à glicose; ITT: teste de tolerância à insulina. Resultados expressos como Média $\pm$ EPM (n = 8-15). Os dados foram analisados por ANOVA *Two-way* e pós-teste de Bonferroni. ^{a, b, c} Letras diferentes representam diferenças estatísticas entre os valores.

Os músculos sóleos foram rapidamente isolados e incubados seguindo protocolo previamente descrito. As amostras foram processadas e a síntese de glicogênio, captação, incorporação e oxidação de glicose avaliados.

Como esperado, os dados de captação de glicose (Figura 4A) mostram menor responsividade à insulina no músculo sóleo dos animais alimentados com dieta hiperlipídica. A suplementação com óleo de peixe reverteu essa alteração induzida por DHL. O mesmo foi observado para a análise de oxidação de glicose (Figura 4B) e para incorporação total de glicose (Figura 4D).

A síntese de glicogênio (Figura 4C) também foi alterada pelo consumo de dieta rica em gordura, estando menor no grupo H quando comparado ao grupo C. Embora o grupo HP apresente valores superiores aos encontrados para os animais do grupo H, não foi detectada diferença significativa entre os dois grupos.

Os dados referentes à captação e metabolismo de glicose apresentados confirmam o quadro de resistência à insulina no músculo esquelético, bem como o efeito protetor do óleo de peixe que aumentou a responsividade muscular à insulina.

Figura 4. Metabolismo da glicose em resposta à insulina no músculo sóleo isolado. O músculo foi rapidamente retirado e incubado em tampão Krebs Ringer com 5,6mM de glicose, 0,3 $\mu\text{Ci/mL}$ de $[\text{U-}^{14}\text{C}]$ -D-glicose e 0,2 $\mu\text{Ci/mL}$ de 2-desoxi-[2,6- ^3H]-D-glicose, na ausência ou presença de 7 nM de insulina durante uma hora. Ao final da incubação, os músculos foram lavados em tampão frio e digeridos em KOH 1M, a 70 °C, por 20 min. Após esse período, alíquotas das amostras foram coletadas e procederam-se as análises de captação e incorporação de 2-desoxi-[2,6- ^3H]-D-glicose, produção de $^{14}\text{CO}_2$ e síntese de ^{14}C -glicogênio. Os resultados estão apresentados como média $\pm$ erro padrão da média ($n = 10-15$). As linhas pontilhadas representam a média dos valores de captação de glicose (A), oxidação de glicose (B), síntese de glicogênio (C) e incorporação de glicose (D) no músculo sóleo isolado sem o estímulo com insulina.

A análise para verificar captação de glicose no músculo foi realizada em cultura de células para confirmar os efeitos encontrados. O efeito da dieta hiperlipídica sobre a sensibilidade à insulina foi reproduzido ao incubarmos células musculares C2C12 com ácido palmítico (PA), um ácido graxo saturado de cadeia longa que diminuiu a captação de glicose, quando comparado ao grupo controle (Figura 5A). E o efeito protetor do óleo de peixe utilizado para suplementar os animais também foi observado na cultura de células musculares após um pré-tratamento com EPA ou DHA purificados, que preveniram a resistência à insulina induzida por PA.

Figura 5. Captação de glicose e fosforilação de Akt em C2C12. Efeito do tratamento prévio com ácido eicosapentaenóico (epa) ou docosahexaenóico (dha) sobre as alterações na captação de glicose e na fosforilação de Akt induzidos pelo ácido palmítico (pa) em células C2C12. As células musculares foram cultivadas com epa ou dha (50 μ M), na presença ou ausência de insulina, por 48h. Em seguida, as células foram tratadas com ácido palmítico por mais 24h. **A:** Após esse período, as células receberam tratamento adequado com 0,3 μ Ci/mL de [U- 14 C]-D-glicose e 0,2 μ Ci/mL de 2-desoxi-[2,6- 3 H]-D-glicose (detalhadamente descrito em Materiais e Métodos) para assim, proceder com a mensuração da captação de 2-desoxi-[2,6- 3 H]-D-glicose. **B:** Após esse período, a fosforilação da Akt em serina 473 foi avaliada pela técnica de western blotting e a intensidade das bandas auto-radiografadas foram quantificadas por densitometria óptica. Co = grupo controle; Pa = grupo tratado com ácido palmítico e sem tratamento com epa ou dha. ⁺⁺⁺P<0,001 vs Co na ausência de insulina; ^{***}P<0,001 vs Co na presença de insulina; ^{&&}P<0,01 vs pa na presença de insulina; ^{###}P<0,001 vs pa + insulina; [#]P<0,05 vs Pa +insulina. Checar abreviaturas...

Dentre os mecanismos envolvidos com a sinalização à insulina, destaca-se a proteína distal da via molecular de ação deste hormônio, a proteína serina/treonina quinase B/Akt. Amplamente estudada nos últimos anos, a Akt é uma proteína-chave que controla diversas funções celulares, como crescimento, sobrevivência, proliferação e metabolismo. Foi encontrada diferença significativa na ativação da Akt entre os grupos C e H, havendo diminuição nos animais que receberam dieta hiperlipídica (Figura 6A). A suplementação com óleo de peixe preveniu essa diminuição de forma significativa. O mesmo foi observado

quando a análise foi realizada em cultura de células musculares C2C12 (Figura 5B), utilizando PA para comparar aos efeitos da DHL, EPA e DHA para relacionar com os efeitos decorrentes da suplementação com óleo de peixe.

Os resultados referentes à fosforilação de GSK-3 β seguiram o mesmo padrão de resposta daqueles referentes à fosforilação de Akt (Figura 6B). A suplementação com óleo de peixe causou fosforilação de Akt, que por sua vez aumentou a fosforilação de GSK-3 β em Ser9, o que resulta em sua inativação. Quando inativa, essa proteína não promove a fosforilação da glicogênio sintase que, quando menos fosforilada encontra-se na sua forma mais ativa, o que favorece a síntese de glicogênio no músculo esquelético.

Figura 6. Efeitos de DHL e óleo de peixe sobre a via de sinalização da insulina. Efeitos das dietas normo e hiperlipídica e da suplementação com óleo de peixe sobre a ativação das proteínas **A: Akt** e **B: GSK-3β** no músculo sóleo de camundongos C57Bl/6 tratados durante 12 semanas. Os músculos foram isolados, expostos ou não ao estímulo com insulina, foi realizada extração e quantificação de proteínas totais e, sem seguida Western Blotting com posterior análise de densitometria das bandas encontradas. **C:** expressão de IRS1 e GLUT4 no músculo sóleo, analisadas por RT-PCR. Resultados expressos em média $\pm$ EPM (n=6). +++P<0,001 vs respectivo na ausência de insulina.

Para a investigação dos mecanismos envolvidos no quadro metabólico apresentado, analisamos a produção de EROs e a expressão de algumas enzimas antioxidantes no músculo esquelético dos animais em estudo. O peróxido de hidrogênio (H_2O_2), por ser mais difusível e estável, é frequentemente utilizado como indicador da geração de EROs mitocondrial.

O consumo da dieta hiperlipídica aumentou a liberação de H_2O_2 pelo músculo sóleo isolado (Figura 7A). A suplementação com óleo de peixe aboliu esse efeito, sendo o valor obtido similar àquele encontrado nos animais do grupo C.

De maneira geral, a regulação da geração de EROs e a sua detoxificação são fundamentais para a manutenção da atividade das mitocôndrias. Na matriz mitocondrial e no espaço intermembranas, os radicais ânion superóxido ($O_2^{\cdot-}$), produzidos na cadeia respiratória, são transformados em H_2O_2 pela atividade das superóxidos dismutases (SODs) e, em seguida, eliminado pela catalase e peroxidases diversas. A não remoção dessa espécie reativa pode levar ao quadro de estresse oxidativo. O consumo de DHL reduziu a expressão de SOD2 e GPX1. A suplementação com óleo elevou a expressão de CAT (Figura 7B), o que é sugestivo de que o excesso de gordura na dieta compromete o sistema antioxidante na célula muscular e o óleo de peixe previne esse efeito.

Figura 7. DHL, ômega-3 e o estresse oxidativo no músculo esquelético. Efeitos das dietas normo e hiperlipídica e da suplementação com óleo de peixe sobre a liberação de H_2O_2 (A) pelo músculo sóleo de camundongos C57BL/6 mantidos em experimentação por 12 semanas. Os animais foram anestesiados e os

músculos sóleos removidos e incubados em solução Krebs contendo 50 μ M de Amplex® UltraRed e peroxidase (0,1 UI/mL) por 1 hora. A fluorescência foi avaliada com 530 nm de excitação e 590 nm de emissão. B: Expressão de mRNA das enzimas antioxidantes superóxido dismutase 1 e 2 (SOD 1 e 2), glutathione peroxidase 1 (GPX1) e catalase (CAT). Os resultados foram normalizados pelo peso do músculo e pelo tempo de incubação. Resultados expressos em média $\pm$ EPM (n=6).

A partir do indicativo de estresse oxidativo mitocondrial pelo aumento de EROs induzido pela DHL, avaliamos o consumo de O_2 no músculo isolado e, também, as concentrações de intermediários do ciclo do ácido tricarboxílico (ciclo de Krebs).

Os animais alimentados com dieta hiperlipídica apresentaram menor consumo de oxigênio no músculo sóleo, quando comparados aos que receberam dieta controle. A suplementação com óleo de peixe preveniu completamente esse efeito, sendo o valor similar àquele encontrado nos animais do grupo C (Figura 8A).

Também foram analisados os efeitos da dieta hiperlipídica e da suplementação com óleo de peixe sobre as concentrações de intermediários do ciclo de Krebs no músculo gastrocnêmio. A alimentação com dieta hiperlipídica levou à diminuição das concentrações de citrato, α -cetoglutarato, malato e oxaloacetato, sendo esse efeito significativo somente para α -cetoglutarato e oxaloacetato (Figuras 8B e 8D). A suplementação com ácidos graxos ω -3 elevou a concentração dos quatro substratos analisados, apresentando diferença estatística para citrato, α -cetoglutarato e malato (Figuras 8B, 8C e 8D).

Figura 8. Consumo de O₂ no músculo isolado e concentração de intermediários do Ciclo de Krebs. Efeitos das dietas controle e hiperlipídica e da suplementação com óleo de peixe sobre a taxa de consumo basal de oxigênio e sobre as concentrações de intermediários do ciclo do ácido tricarboxílico no músculo de camundongos C57Bl/6. Consumo basal de oxigênio no músculo sóleo de animais dos diferentes grupos experimentais; Controle do experimento de monitoramento do consumo de oxigênio no músculo, mostrando consumo basal, além de consumo após adição de oligomicina e CCCP (n=5) [A]. Concentrações dos intermediários citrato (B), alfa-cetoglutarato (C), malato (D) e oxaloacetato (E) no homogenato de músculo gastrocnêmio (n=5). Resultados expressos em média ± EPM. Análise estatística por ANOVA *Two-way*, com pós-teste de Bonferroni.

Para verificar a atividade da cadeia transportadora de elétrons e estimar a fosforilação oxidativa, foi analisado o conteúdo de proteínas dos diferentes complexos da cadeia e, também, a expressão de genes codificadores de algumas proteínas que integram esses complexos enzimáticos. Ambos o consumo de DHL e a suplementação com o óleo não causaram alterações no conteúdo protéico e na transcrição de mRNA dos complexos

analisados, com exceção de uma diminuição no complexo II induzida pela DHL (Figura 9). O complexo II possui um grupo heme que não participa do transporte de elétrons para a ubiquinona, mas parece ser necessário para reduzir a concentração de EROs. Dessa forma, uma diminuição no conteúdo de CII pode contribuir para o aumento do estresse oxidativo no músculo dos animais estudados.

Figura 9: Proteínas da cadeia transportadora de elétrons mitocondrial. Efeitos das dietas normo- e hiperlipídica e da suplementação com óleo de peixe sobre o conteúdo das proteínas dos complexos enzimáticos envolvidos na fosforilação oxidativa (OXPHOS) [A] e sobre a transcrição de mRNA de genes que codificam proteínas integrantes desses complexos no músculo sóleo de camundongos C57Bl/6 tratados durante 12 semanas. (B). Os resultados estão expressos como média $\pm$ EPM (n = 6).

Outros parâmetros da fisiologia mitocondrial foram analisados (Figura 10), incluindo o conteúdo de proteínas e a expressão de genes envolvidos na regulação do metabolismo mitocondrial. O consumo de DHL reduziu a transcrição e o conteúdo protéico de PGC1- α , um conhecido coativador de PPAR- α e NRF1, responsáveis pela indução de biogênese mitocondrial, que também tiveram sua transcrição reduzida pela DHL. O óleo de peixe

preveniu estas alterações para PGC1- α e PPAR- α , mas não para NRF1 (Figuras 10A, 10B e 10C).

AMPK e CREB, duas proteínas indutoras da ativação de PGC1- α , não foram alteradas pela DHL (Figuras 10D e 10E). Mas a suplementação com óleo aumentou a fosforilação de AMPK, quando comparada ao encontrado no grupo H. O mesmo foi observado para a transcrição de CPT-1 (Figura 10C). Não foi encontrada diferença significativa entre os grupos para a expressão da proteína desacopladora mitocondrial UCP3 (Figura 10C).

A proteína responsável pela fusão mitocondrial, mitofusina 2, que tem sido relacionada com o estado metabólico de células musculares, não foi alterada pelo consumo da DHL, mas foi observado aumento de seu conteúdo proteico no grupo HP (Figura 10F).

De fato, a análise qualitativa da morfologia mitocondrial não mostrou diferenças em relação ao número total de mitocôndrias entre os grupos. Entretanto, foram observadas diferenças na forma das mitocôndrias, presença de vesículas no interior das organelas e gotículas de gordura no músculo (Figura 10H). Os animais alimentados com DHL apresentaram, quando comparados aos do grupo controle, mitocôndrias pouco disformes, além de maior presença de gotículas lipídicas e vacuolização no interior das mitocôndrias. A suplementação com óleo de peixe nos animais alimentados com DHL modificou a forma das mitocôndrias, se comparadas às do grupo não suplementado. As organelas tiveram forma e distribuição alteradas. Já os animais que receberam óleo de peixe e DHL apresentaram mitocôndrias com cristas e membranas bem definidas. Não houve aparente variação de forma e distribuição. Além disso, quando comparadas às dos animais que receberam somente DHL, apresentam menor incidência de vacúolos no interior das organelas e de gotículas lipídicas nas proximidades das mitocôndrias.

Por último, foi analisada a transcrição do gene codificador da proteína ATM, que está diminuída no grupo H, efeito esse atenuado pela suplementação com óleo de peixe (Figura 10G).

Recentemente, foi demonstrada uma forte associação entre deficiência de ATM e estresse oxidativo, uma condição favorável à instalação de doenças neurodegenerativas, oncogênicas e metabólicas. Em animais nocautes para ATM^{-/-}, o tratamento com dieta hiperlipídica causou resistência à insulina, aterosclerose e intolerância à glicose, se comparado aos controles com elevado conteúdo de ATM. Evidências sugerem, portanto, que uma atividade reduzida da ATM está diretamente associada ao aumento da resistência à insulina nos tecidos periféricos incluindo o tecido muscular e que o papel dessa proteína é fortemente regulado por EROs.

Figura 10. Função mitocondrial no músculo esquelético resistente à ação da insulina e efeitos do óleo de peixe sobre fatores relacionados com a biogênese mitocondrial. Conteúdo protéico (A) e de RNAm (B) de PGC1- α . Conteúdo (RNAm) de CPT1, PPAR α , UCP3, TFAM e NRF1 (C). Fosforilação das proteínas AMPK (D) e CREB (E). Conteúdo da proteína mitofusina-2 (F). E (G): conteúdo de ATM (RNAm). Todas as análises foram realizadas no músculo sóleo, seguindo protocolos detalhadamente descritos na seção Material e Métodos. Os resultados estão expressos como média $\pm$ EPM (n = 5-6).

Os resultados descritos acima encontram-se resumidos no Quadro 2.

Quadro 2. Resumo dos efeitos da DHL e do óleo de peixe apresentados na seção de resultados.

	DHL	ω -3
Ganho de peso	↑	↓
Índice de adiposidade	↑	↓
Glicemia de jejum	↑	—
Insulinemia de jejum	↑	↓
HOMA-IR	↑	↓
Colesterol total e LDL	↑	↓
Triglicérides e HDL	—	—
AST, ALT e Gama-GT	—	—
Captação de glicose (músculo isolado)	↓	↑
Oxidação de glicose (músculo isolado)	↓	↑
Síntese de glicogênio (músculo isolado)	↓	—
Fosforilação de Akt e GSK3- β	↓	↑
IRS-1 e GLUT4 (RNAm)	↓	—
Produção de H ₂ O ₂ (músculo isolado)	↑	↓
SOD2 e GPX (RNAm)	↓	—
Catalase (RNAm)	—	↑
Consumo de O ₂ (músculo isolado)	↓	↑
[ICATs] (OAA e α -cetoglutarato)	↓	↑
[ICATs] (citrato e malato)	—	↑
OXPPOS - CII	↓	—
PGC1- α	↓	↑
PPAR- α (RNAm)	↓	↑
NRF1 e TFAM (RNAm)	↓	—
CPT1 (RNAm)	—	↑
Fosforilação de AMPK	—	↑
Mfn2	—	↑
ATM (RNAm)	↓	↑

6 DISCUSSÃO

Nesse trabalho, mostramos que o consumo de dieta hiperlipídica causa resistência à insulina no músculo esquelético, tendo envolvimento da mitocôndria para o desenvolvimento dessa desordem metabólica. Foi mostrado também que a suplementação com óleo de peixe leva à reversão desse quadro por melhora da função mitocondrial. Os dados apresentados serão discutidos com base na literatura atual.

A obesidade tem sido descrita como a principal consequência do consumo da dieta rica em lipídeos (Cha et al., 2011; Hariri e Thibault, 2010; Kendig et al., 2011). Em ratos e camundongos, pode ser estabelecida correlação positiva entre o conteúdo de lipídeos na dieta/porcentagem de energia proveniente desses lipídeos e o ganho de peso corporal e de tecido adiposo (Hariri, Thibault, 2010). Ratos alimentados com dietas ricas em lipídeos aumentam o peso corporal de modo mais rápido que aqueles alimentados com dietas normolipídicas (Deuel et al., 1944; 1947). Assim, em modelos de obesidade experimental, geralmente são usadas dietas que apresentam de 30% a 78% de seu valor energético total proveniente de lipídeos. Beuttner et al. (2007) realizaram estudos mostrando que o melhor protocolo experimental para induzir obesidade em animais é utilizar a dieta hiperlipídica semi-purificada, contendo, pelo menos, 40% da energia proveniente de gordura animal. Em concordância com os modelos de obesidade descritos na literatura, a dieta hiperlipídica utilizada nesse projeto apresentou 59% da energia proveniente de lipídeos, sendo que tanto na DNL quanto na DHL, a proporção energética entre banha suína e óleo de soja foi de 90% para 10%, respectivamente.

Aqui, mostramos que os animais que receberam DHL apresentaram ganho significativo de peso em comparação aos animais alimentados com DNL. Em concordância com estudos que mostram ajuste da ingestão alimentar de acordo com a densidade energética, foi observada redução do consumo de ração nos animais que foram alimentados com DHL em relação aos que consumiram DNL. Mesmo tendo o consumo de ração reduzido, a ingestão de proteína pelos animais alimentados com DHL foi semelhante ao apresentado pelos animais que receberam DNL. De fato, a concentração de proteína foi ajustada para compensar a diminuição da ingestão desse nutriente em decorrência da diminuição da ingestão alimentar no grupo DHL. Por outro lado, apesar da redução do consumo de ração, houve aumento da ingestão lipídica (especialmente banha suína, que é a principal fonte animal de lipídeos da DHL) pelos animais do grupo H, uma vez que, proporcionalmente, essa dieta apresenta maior concentração de banha do que a DNL. O aumento da eficiência energética observado nos

animais que consumiram DHL pode ser explicado pelo fato de que a energia decorrente dos lipídeos tem efeito mais pronunciado no ganho de peso corporal do que a energia decorrente de outros nutrientes. A termogênese induzida pela dieta é a energia necessária para digerir, absorver e armazenar nutrientes, produzindo perda de energia para o corpo que é de 2 a 3% para os lipídeos, de 25 a 30% para proteínas e 6 a 8% para carboidratos. Assim, a eficiência de utilização de um nutriente difere entre os macronutrientes, sendo que os lipídeos têm eficiência de 97 a 98%, proteínas de 70 a 75% e carboidratos de 92 a 94% (Hill et al., 2000; Warwick et al., 1992), o que explica, pelo menos parcialmente, a maior eficiência energética nos animais submetidos à DHL.

Os diferentes tipos de lipídeos da dieta podem modular de forma distinta o ganho de peso e a resistência à insulina em animais (Dobbins et al., 2002; McAuley et al., 2002). Assim, é importante ressaltar que a administração de óleo de peixe resulta em reversão da resistência à insulina induzida por dieta rica em lipídeos em ratos (Kim et al., 1996; Storlien et al., 1997). Tem sido demonstrado que a suplementação com EPA e/ou DHA previne o aumento da adiposidade provocado pela DHL (Kuda et al., 2009; Samame et al., 2009). Nascimento et al. (2010), em seu estudo com camundongos, utilizaram a dieta rica em gordura e sacarose e avaliaram os efeitos da suplementação com óleo de peixe. Observou-se menor ganho de peso, bem como redução da concentração plasmática de triglicérides e aumento da concentração de colesterol HDL dos animais que receberam dieta hiperlipídica rica em sacarose e foram suplementados com óleo de peixe. No presente estudo, foi observado menor ganho de peso dos animais do grupo HP em relação ao grupo H. No entanto, os primeiros continuaram apresentando aumento significativo de peso em relação ao seu respectivo controle (CP), mostrando que a suplementação com óleo de peixe apenas atenuou o efeito da DHL. O consumo alimentar, protéico e energético, bem com a eficiência energética dos animais do grupo HP permaneceram semelhantes aos apresentados pelos animais do grupo H.

Nossos dados corroboram estudos que mostraram redução no acúmulo de gordura corporal em animais suplementados com AG ω -3 (Hainault et al., 1993; Ruzickova et al., 2004; Takahashi et al., 2008). Baillie et al. (1999) mostraram redução de 25% no acúmulo de gordura epididimal em ratos, com 4 semanas de idade, alimentados durante 6 semanas com DHL (40%) enriquecida com óleo de peixe, sem observar diferença na ingestão calórica. Belzung et al. (1993) também observaram o mesmo em ratos Wistar machos, com 7 semanas de idade, alimentados por 4 semanas com DHL acrescida de AG ω -3. E mostraram, ainda, que

o efeito observado é dose-dependente, ou seja, quanto mais ω -3 na dieta, menor o acúmulo de gordura.

Os mecanismos pelos quais os AG ω -3 reduzem a gordura corporal não estão totalmente elucidados. Evidências em humanos (Parra et al., 2008) e roedores (Pérez-Matute et al., 2007; Takahashi et al., 2008) sugeriram que esses ácidos graxos podem contrubuir com a melhora da composição corporal pela supressão do apetite. Entretanto, outros estudos mostraram que esses efeitos são mediados por alterações na expressão de genes envolvidos na regulação do metabolismo lipídico em diferentes tecidos. A ingestão de AG ω -3 eleva a expressão de genes envolvidos na oxidação de lipídeos no fígado, músculos cardíaco e esquelético, além de reduzir a expressão de genes envolvidos na lipogênese no tecido adiposo, o que favorece a oxidação de lipídeos e reduz seu armazenamento (Buckley, Howe, 2009).

Os animais que receberam DHL durante 8 semanas apresentaram aumento nas concentrações de colesterol total e de LDL, sem, contudo, apresentar alteração nas concentrações de HDL e de triglicérides. Alterações no perfil lipídico em decorrência do consumo da dieta rica em lipídeos têm sido relatadas em estudo anterior (Wang et al., 2011) e acompanham o quadro de obesidade. A suplementação com óleo de peixe promoveu redução nas concentrações de colesterol total e LDL dos grupos CP e HP, sem alterar as concentrações de HDL e triglicérides. A diminuição de colesterol total e de triglicérides após suplementação com AG ômega-3 foi descrita por Hartweg et al. (2007), sendo que esse efeito está relacionado ao aumento da β -oxidação, reduzindo a síntese de triglicerídeos e sua secreção (Lombardo, Chicco, 2006). A determinação da atividade das enzimas hepáticas ALT, AST e gama-GT teve como objetivo avaliar o possível efeito hepatotóxico, decorrente do tratamento crônico tanto com DHL e/ou óleo de peixe. Os resultados obtidos mostraram que não houve alteração na atividade de enzimas hepáticas no plasma, sugerindo ausência de toxicidade.

Independente da obesidade, a DHL por si só contribui para o desenvolvimento de intolerância à glicose e resistência à insulina (Ferranti, Mozaffarian, 2008; Lichtenstein, Schwab, 2000). No entanto, a resistência à insulina aparece em estados pré-diabéticos. Na presença do comprometimento da resposta ao hormônio, as células β pancreáticas normais aumentam a produção de insulina e, enquanto a hiperinsulinemia for adequada para compensar a resistência à insulina, a tolerância à glicose permanece relativamente normal. Em indivíduos que desenvolvem DM2, ocorre falha na resposta compensatória das células β , resultando em intolerância à glicose (Dabelea et al., 2004; Olefsky et al., 2005). No presente projeto, os animais alimentados com DHL apresentaram intolerância à glicose e resistência à insulina, bem como aumento da glicemia e da insulinemia de jejum, além do índice HOMA-

IR, outro indicativo de resistência ao hormônio. A suplementação com óleo de peixe promoveu a redução da glicemia, da insulinemia de jejum, do índice HOMA-IR, bem como aumento do decaimento da concentração plasmática de glicose. Esses resultados estão de acordo com a literatura, que mostra melhora no metabolismo da glicose em decorrência da suplementação com óleo de peixe (Rossmeisl et al., 2009; Samame et al., 2009).

Vários grupos, incluindo o nosso, tem verificado que o tratamento com AG saturados em células musculares esqueléticas afeta o metabolismo de glicose, na presença de insulina (Hirabara et al., 2010; Lipina et al., 2013; Mazibuko et al., 2013). Foram observadas diminuições na síntese de glicogênio, produção de lactato, captação e oxidação de glicose. Os mesmos resultados foram observados no presente estudo: menor captação, incorporação e oxidação de glicose, além de síntese de glicogênio, na presença de insulina, nos músculos de animais alimentados com dieta rica em gordura. Alguns estudos são sugestivos do aumento de sensibilidade à insulina no músculo esquelético, causado por suplementação com AG poliinsaturados (Dimopoulos et al., 2007, Gringas et al., 2007), mas outros autores não encontraram essa mesma resposta (Lee et al., 2006; Hirabara et al., 2010). Apesar dos diferentes achados, há diversas evidências de que os AG poliinsaturados ω -3, principalmente EPA e DHA, contribuem com efeitos positivos para o aumento da sensibilidade à insulina no músculo esquelético (Lombardo et al., 2007; Roche et al., 2005), apesar de os mecanismos ainda não estarem bem descritos. Nos resultados aqui apresentados, pode-se observar prevenção da resistência à insulina no músculo esquelético através da suplementação com óleo de peixe, uma vez que a captação, a incorporação e a oxidação de glicose se mostraram mais elevadas nos animais tratados com óleo de peixe, quando comparados aos do grupo H. Apesar de estar de acordo com dados já descritos na literatura, nosso trabalho utiliza uma combinação de fatores que ainda não foram utilizados para estudar o efeito dos AG omega-3. Esses fatores são tempo de tratamento e composição das dietas, idade dos animais, linhagem utilizada, composição do óleo de peixe, via de administração e dose.

Diversos autores mostraram a ação de AG saturados alterando a sinalização intracelular à insulina no tecido muscular esquelético, conforme revisado por Schmitz-Peiffer (2000). A ativação das proteínas Akt e GSK-3 β está diminuída no músculo sóleo de animais que receberam DHL em relação aos alimentados com DNL. Vinolo et al. (2012) também encontraram menor ativação de Akt em camundongos submetidos ao mesmo protocolo de indução de obesidade/resistência à insulina utilizado nesse trabalho. Recentemente, Tishinsky et al. (2012) observaram maior ativação de Akt no músculo sóleo de ratos que receberam óleo de peixe incorporado à dieta, quando comparados à animais que receberam somente dieta rica

em gordura (55%), efeito semelhante ao encontrado no presente trabalho. Alguns estudos mostram menor ativação de GSK-3 β em músculos de ratos alimentados com dieta hiperlipídica (Cleasby et al., 2011; Tremblay et al., 2003), em decorrência da menor ativação da via da PI3-quinase. Entretanto, pouco se sabia sobre a ação dos AG ω -3 sobre a ativação dessa proteína no tecido muscular, mas aqui demonstramos que a suplementação com óleo de peixe foi capaz de prevenir essa menor ativação.

Alguns dos mecanismos que contribuem para a inibição da sinalização à insulina por DHL inclui a ativação de várias quinases, como PKCs e JNK, que levam à menor ativação de IRS-1. No nosso estudo, encontramos maior ativação de PKC θ e JNK além de menor expressão de IRS-1 após consumo de DHL, mas sem nenhum efeito do óleo de peixe. Dessa forma, o aumento da sensibilidade à insulina pelos AG ômega-3 no músculo parece envolver outros mecanismos.

Há muitos estudos envolvendo suplementação com AG ω -3 e redução da resistência à insulina, mas são escassos aqueles que relatam prevenção desse quadro através da suplementação. Além disso, são descritas suplementações com diferentes doses e tipos de óleo de peixe ou outras fontes de ω -3 e tempo total de tratamento diferentes dos desenvolvidos nesse estudo em modelos experimentais diferentes. Giacco et al. (2007) não encontraram efeitos sobre a sensibilidade à insulina em indivíduos saudáveis suplementados com 3,6 g de AG ω -3 por dia, durante 90 dias. Lombardo et al. (2007) utilizaram ratos *Wistar* com 2 meses de idade, alimentados com dieta rica em sacarose durante 8 meses e suplementados com óleo de peixe nos 2 últimos meses desse período. A partir desse protocolo experimental, foi observada diminuição nos valores de triglicérides no fígado e músculo esquelético, além de melhora da sensibilidade à insulina no músculo esquelético nos animais suplementados com óleo de peixe. Rossmeisl et al. (2009) utilizaram diferentes derivados de DHA para verificar efeitos preventivos e de reversão da obesidade e suas complicações induzidas por dieta hiperlipídica em camundongos (3 meses de idade). A dieta foi administrada durante 4 meses e os derivados de DHA foram utilizados juntamente com a dieta, no estudo preventivo, ou 2 meses após a indução de obesidade, para verificar o poder de reversão desses AG. Foram encontrados efeitos benéficos sobre a obesidade e desordens metabólicas associadas, em ambos os estudos.

Diversos autores sugerem o acúmulo de gordura ectópica, como resultado do consumo de dietas hiperlipídicas, como o principal mecanismo envolvido no desenvolvimento da resistência à insulina associada à obesidade, conforme revisado por Samuel e Shulman (2012). Alguns autores defendem a hipótese de que esse acúmulo indevido de gordura no

músculo esquelético é decorrente da menor função mitocondrial (Bonnard et al., 2008; Petersen et al., 2004; Roden, 2005), mostrando diminuição de conteúdo mitocondrial e menor expressão de genes envolvidos na biogênese mitocondrial (Sparks et al., 2005; Crunkhorn et al., 2007). Apesar disso, outros trabalhos mostram que a DHL aumenta a atividade de enzimas mitocondriais, função e biogênese mitocondrial (Garcia-Roves et al., 2007; Hancock et al., 2008; Turner et al., 2007).

No nosso estudo, não encontramos alterações nas concentrações de triglicérides nem de ácidos graxos livres no músculo. Apesar disso, a função mitocondrial mostrou-se prejudicada após consumo de DHL e o óleo de peixe apresentou efeitos benéficos sobre estas alterações. Como medida de metabolismo oxidativo e oxidação de AG, os dados de respirometria no músculo isolado mostram menor consumo de O_2 no grupo H, se comparado ao C. E o óleo de peixe atenuou esse efeito. De forma semelhante, Yokota et al. (2009) encontraram menor consumo de O_2 em mitocôndrias isoladas de músculo esquelético de camundongos alimentados com dieta hiperlipídica e Holmström et al. (2012) verificaram menor consumo de oxigênio no músculo sóleo de camundongos *db/db* obesos e resistentes à insulina, quando comparados a animais que receberam dieta controle. Lanza et al. (2013) encontraram maior consumo de O_2 em mitocôndrias isoladas de músculo de camundongos alimentados com DHL, sem nenhum efeito do óleo de peixe. Dados relativos ao efeito de AG ômega-3 sobre a função mitocondrial no músculo esquelético ainda são muito escassos e controversos. As diversas variáveis de um estudo experimental desse tipo torna difícil a compreensão dos mecanismos envolvidos, pois são modelos e condições experimentais diferentes, o que fortalece o caráter inovador do nosso estudo.

Além disso, a menor atividade do ciclo do ácido tricarboxílico (ciclo de Krebs) encontrada no músculo esquelético dos animais alimentados com DHL suporta o dado que mostra diminuição da capacidade mitocondrial, observada com o menor consumo de O_2 (Barbosa et al., 2013). A suplementação com óleo elevou as concentrações dos ICATs, sugerindo maior atividade do ciclo e, assim, melhora da função mitocondrial e mostrando o envolvimento da organela na ação desses ácidos graxos. Apesar disso, os parâmetros analisados para estimar os efeitos da DHL e do óleo rico em EPA sobre a fosforilação oxidativa não mostraram alterações.

A fosforilação oxidativa (OXPHOS) é a via metabólica através da qual a mitocôndria utiliza a estrutura celular, suas enzimas e a energia liberada pela oxidação de substratos para produção de ATP, envolvendo transferência de elétrons. Ao analisar a expressão de genes que codificam proteínas dos complexos enzimáticos avaliados, nenhuma

diferença foi encontrada. Outras proteínas envolvidas no processo de fosforilação oxidativa podem estar alteradas, uma vez que nossa análise restringiu-se a somente uma das proteínas de cada complexo. Sparks et al. (2005) encontraram menor expressão das mesmas proteínas aqui avaliadas, mas em músculos de humanos.

A maior expressão do gene que codifica PGC1- α , bem como seu conteúdo protéico no músculo foram elevados pelo consumo de ômega-3. A suplementação também levou a maior ativação de AMPK e maior conteúdo protéico de Mfn2. Esse último explica o menor número de alterações morfológicas nas mitocôndrias, que, provavelmente, deveu-se ao aumento do processo de fusão mitocondrial e, assim, melhora de sua funcionalidade. Estudos recentes mostram o impacto de alterações no conteúdo de Mfn2 sobre a função mitocondrial, o que não está associado somente remodelamento mitocondrial. Autores mostram que, quando Mfn2 está mais expressa no músculo, há aumento do consumo de oxigênio, maior potencial de membrana e aumento na oxidação de glicose (Pich et al., 2005). Evidências sugerem que PGC1- α também regula a dinâmica mitocondrial através da maior fusão mitocondrial (Soriano et al., 2006). Dessa forma, Mfn2 não é somente outro alvo do coativador nuclear PGC1- α , mas um componente de sinalização intracelular da maquinaria que regula o metabolismo no músculo esquelético. Ainda não há estudos mostrando os efeitos dos AG ω -3 sobre a expressão e regulação de Mfn2 no músculo esquelético.

A expressão de PGC-1 α , fator de transcrição essencial à biogênese mitocondrial, pode ser estimulada por estresse oxidativo (St-Pierre et al., 2006), ativação de AMPK e pelo elemento de ligação responsivo ao cAMP (CREB - *cAMP response element-binding*) (Handschin, Spiegelman, 2006). Esse último também pode ser ativado através de vias sensíveis ao cálcio e AMP cíclico (cAMP), como a proteína quinase II dependente da via cálcio/calmodulina (*CaMKII* - *calcium/calmodulin-dependent protein kinase II*) e proteína quinase A (PKA), que estão ativadas em condições de deficiência de insulina (Mayr, Montminy, 2001). Roberts-Wilson et al. (2010) demonstraram que a redução no conteúdo de PGC-1 α no músculo gastrocnêmio de ratos diabéticos induzidos por estreptozotocina, apesar da ativação de outras vias que superexpressam PGC-1 α , deve-se à redução da sinalização à calcineurina, uma fosfatase dependente de cálcio associada ao estímulo da expressão de PGC-1 α . Pode-se sugerir que o mesmo aconteceu no músculo sóleo dos animais analisados nesse trabalho.

Em concordância com os resultados observados para PGC-1 α , a expressão do RNAm de PPAR α no sóleo foi menor nos animais alimentados com DHL, efeito revertido pelo óleo de peixe. Os PPARs α e β são abundantes no músculo esquelético e sua ativação

resulta na indução de genes que participam da regulação da oxidação de ácidos graxos (Gilde, Van Bilsen, 2003; Leone et al., 1999; Muoio et al., 2002). O diafragma de camundongos nocaute para PPAR α apresenta comprometimento do metabolismo energético, sem alteração do perfil de tipos de fibras ou das cadeias pesadas de miosina (Lecarpentier et al., 2008). O estresse oxidativo do músculo resistente à insulina induzido por dieta hiperlipídica, portanto, parece reduzir a expressão de PPAR α no sóleo, sendo esse mais um fator que contribui para o comprometimento do metabolismo energético nesse músculo. A suplementação com óleo também aumentou a expressão de CPT1, enzima regulada por PPAR α e também envolvida na oxidação de AG.

A maior produção de EROs parece estar diretamente relacionada ao desenvolvimento de resistência à insulina induzida por dietas. Já foi demonstrado aumento da produção de EROs em células musculares tratadas com palmitato (Barbosa et al., 2013; Duval et al., 2007; Lambertucci et al., 2008), em mitocôndrias isoladas do fígado (Cardoso et al., 2010) e de músculo esquelético (Lanza et al., 2013) e no tecido adiposo (Kawasaki et al., 2012) de animais alimentados com DHL. No nosso trabalho, o menor consumo de O₂ levou ao aumento da produção de peróxido de hidrogênio pelo músculo sóleo de animais alimentados com DHL. Os animais suplementados com óleo de peixe apresentaram menor produção de peróxido de hidrogênio, estando de acordo com trabalhos que mostram redução da produção de EROs devido ao efeito antioxidante dos AGs ω -3 no tecido adiposo (Fan et al., 2013), em macrófagos (Wang et al., 2004) e no músculo esquelético (Dannenberger et al., 2013). Esse efeito confirma a importância da mitocôndria na ação dos AG ω -3 sobre a resistência à insulina no músculo esquelético.

Finalmente, mostramos aumento da expressão de ATM no grupo HP. Esta proteína, apesar de pouco explorada na literatura, já foi demonstrada como importante reguladora do metabolismo e sensor redox controlando as concentrações de EROs, conforme revisado por Ditch e Paull (2012). Recentemente, foi demonstrado que a proteína kinase ataxia-telangiectasia mutada (ATM), expressa na maioria dos tecidos, é membro da família de kinases relacionadas a fosfatidilinositol 3-quinase (PI3-K)-"like"-kinase (PI3KK) e está diretamente associada a proteínas da via de sinalização à insulina (Matsuoka et al., 2007).

Há evidências de que a instalação do quadro de resistência à insulina é desencadeada pelo aumento do estresse oxidativo intracelular, condição diretamente associada à disfunção mitocondrial. Embora a relação entre ATM e resistência à insulina seja pouco conhecida, nossa proposição é que a mitocôndria tem papel fundamental nos efeitos benéficos do óleo de peixe sobre a resistência à insulina induzida pela dieta hiperlipídica no músculo esquelético

(Figura 11). A melhora da função mitocondrial, nesse caso, foi associada com menor produção de EROs, que pode ter sido induzida pela maior expressão de ATM, a qual ativaria os sistemas antioxidantes e também a AMPK diretamente, levando à estimulação de PGC1- α e, conseqüentemente, aumento na expressão de genes envolvidos na capacidade oxidativa e biogênese mitocondrial, prevenindo os efeitos deletérios da dieta hiperlipídica sobre a função mitocondrial e, dessa forma, sobre a fosforilação de Akt e sensibilidade à insulina.

Dessa forma, o presente estudo vem contribuindo para a compreensão dos mecanismos envolvidos nos efeitos benéficos dos AG ω -3 na RI induzida por DHL, tendo como diferenciais o tempo e a forma de indução da resistência à insulina, análise do efeito preventivo através da suplementação com óleo de peixe em dose e composição distintas daquelas descritas na literatura.

Figura 11. Representação esquemática dos efeitos da dieta e do óleo de peixe sobre os parâmetros estudados. A mitocôndria tem um papel fundamental nos efeitos benéficos do óleo de peixe sobre a resistência à insulina induzida por dieta hiperlipídica no músculo esquelético. E a melhora da função mitocondrial, nesse caso, é decorrente da menor produção de EROs induzida pela maior expressão de ATM, que ativa os sistemas antioxidantes e também ativa diretamente AMPK, que leva à maior ativação de PGC1- α e consequente aumento de Mfn2, que favorece o remodelamento mitocondrial e, assim, favorece o funcionamento adequado da mitocôndria. O aumento de ATM também leva à maior fosforilação de Akt e, consequentemente, à maior captação de glicose pelo músculo esquelético. Com isso, há aumento da sensibilidade à insulina e da biogênese mitocondrial por ação dos AG ω -3 em situação de desafio metabólico por dieta hiperlipídica.

7 CONCLUSÃO

O quadro de resistência à insulina foi induzido com o modelo proposto, tanto de forma sistêmica quanto periféricamente no músculo esquelético. E o óleo de peixe utilizado (EPA/DHA 3,7:1) se mostrou eficaz na reversão da resistência à insulina induzida por dieta hiperlipídica. Verificamos isso através das alterações encontradas em parâmetros sorológicos, metabólicos, pela análise de conteúdo e fosforilação de proteínas envolvidas na via de sinalização à insulina, pelo conteúdo de RNAm de genes relacionados com o metabolismo de glicose e ácidos graxos.

A mitocôndria está diretamente envolvida tanto no desenvolvimento da resistência à insulina quanto nos efeitos positivos dos ácidos graxos ω -3 e os mecanismos responsáveis não são conhecidos, mas envolvem o desbalanço redox. A suplementação com óleo de peixe causou menor produção de EROs, com maior expressão de ATM e PGC1- α , aumento da fosforilação de AMPK e do conteúdo de Mfn2.

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APÊNDICE A

REVIEW

Open Access

Mechanisms underlying skeletal muscle insulin resistance induced by fatty acids: importance of the mitochondrial function

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Abstract

Insulin resistance condition is associated to the development of several syndromes, such as obesity, type 2 *diabetes mellitus* and metabolic syndrome. Although the factors linking insulin resistance to these syndromes are not precisely defined yet, evidence suggests that the elevated plasma free fatty acid (FFA) level plays an important role in the development of skeletal muscle insulin resistance. Accordingly, *in vivo* and *in vitro* exposure of skeletal muscle and myocytes to physiological concentrations of saturated fatty acids is associated with insulin resistance condition. Several mechanisms have been postulated to account for fatty acids-induced muscle insulin resistance, including Randle cycle, oxidative stress, inflammation and mitochondrial dysfunction. Here we reviewed experimental evidence supporting the involvement of each of these propositions in the development of skeletal muscle insulin resistance induced by saturated fatty acids and propose an integrative model placing mitochondrial dysfunction as an important and common factor to the other mechanisms.

Keywords: Skeletal muscle, Insulin resistance, Saturated fatty acids, Mitochondrial dysfunction

Introduction

Insulin resistance is broadly defined as the reduction in insulin ability to stimulate glucose uptake from body peripheral tissues. At physiological conditions, insulin activates glucose uptake by stimulating the canonical IRS-PI3K-Akt pathway and by phosphorylating and inactivating Akt substrate 160 (AS160), a protein that, when activated, prevents glucose transporter (GLUT) 4 translocation to the membrane. Thus, by inhibiting AS160, insulin promotes the GLUT4 translocation from inner vesicles, promoting fusion to the plasma membrane and consequently glucose uptake [1].

Although insulin resistance is a key component of several chronic syndromes associated with obesity such as type 2 *diabetes mellitus* and metabolic syndrome, the involved factors and their underlying mechanisms linking excessive adiposity to insulin resistance were not

completely elucidated yet [2-5]. Evidence suggests that fatty acids, whose circulating levels are markedly increased in obesity and associated-diseases, might play a role in the development of skeletal muscle insulin resistance [6,7]. In this sense, prolonged exposure of skeletal muscle and myocytes to high levels of fatty acids leads to severe insulin resistance [8,9]. Among the different types of fatty acids, saturated long-chain fatty acids such as palmitic and stearic acids were demonstrated to be potent inducers of insulin resistance [5,10]. Several mechanisms have been suggested by us [2,5,11,12] and others [6,8,13-16] to explain how saturated fatty acids impair insulin actions such as the Randle cycle, accumulation of intracellular lipid derivatives (diacylglycerol and ceramides), oxidative stress, modulation of gene transcription, inflammation and mitochondrial dysfunction. In the present review, we discuss evidence supporting the involvement of these mechanisms in the regulation of insulin sensitivity by saturated fatty acids and propose the mitochondrial dysfunction found in conditions of elevated fatty acid levels has a central role in the pathogenesis of insulin resistance.

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Mechanisms underlying the insulin resistance induced by saturated fatty acids

Competition between fatty acids and glucose: the randle cycle

The first mechanistic explanation for the inverse relationship between fatty acids availability and glucose utilization was proposed by Randle *et al.* [13]. In this study, it was shown that an elevation in fatty acids supply to diaphragm and isolated heart is associated with an increase in fatty acid oxidation and an impairment in glycolytic flux and glucose utilization, such effect being mediated by allosteric inhibition of glycolytic enzymes. More specifically, the proposed hypothesis was that increased fatty acid oxidation raises the production of acetyl-CoA resulting in inhibition of pyruvate dehydrogenase activity and elevation of citrate levels at the tricarboxylic acid cycle. Citrate together with an increased ATP/ADP ratio reduce the activity of phosphofructokinase and consequently glucose flux through the glycolytic pathway, resulting in glucose 6-phosphate accumulation, hexokinase II inhibition, increase in intracellular glucose content and, consequently, reduction in glucose uptake [17,18].

In accordance with Randle's hypothesis, elevation in circulating fatty acids levels by either intralipid/heparin or lipid infusion in rats, humans and type 2 *diabetes mellitus* patients is associated with impairments in glucose uptake, utilization and oxidation in insulin-sensitive tissues (heart, skeletal muscle and adipose tissue) [19-21]. Acutely, fatty acids lead to Randle cycle effect, increasing intracellular content of citrate and glucose-6-phosphate and decreasing glycolytic pathway flux [2,11]. It has been also demonstrated that palmitate acutely increases glucose uptake in L6 myotubes by activating insulin signaling pathways (Akt and ERK1/2) [22].

However, in contrast to Randle's hypothesis, in which intracellular glucose accumulation must precede the inhibition of glucose uptake, further studies demonstrated that the insulin resistance induced by fatty acids is primarily associated with impaired glucose uptake rather than changes in hexose metabolism [8,18]. In studies involving lipid infusion associated with other techniques including glucose and insulin clamp and nuclear magnetic resonance a rapid reduction in glycolysis (previous to 2 hours) followed by impaired glucose disposal and glycogen synthesis (between 4-6 hours) was observed [7,14]. Roden *et al.* [14] demonstrated that the reduction in muscle glycogen synthesis is preceded by a decrease in intramuscular glucose 6-phosphate, suggesting that the increase in plasma fatty acid concentration initially induces insulin resistance by inhibiting glucose transport or its phosphorylation. Other studies also demonstrated that lipid infusion decreases intracellular glucose and glucose 6-phosphate content, due to

inhibition of glucose uptake by skeletal muscle [8,23]. These studies demonstrated that Randle cycle does not completely explain the effects of FFA on glucose metabolism indicating that other mechanisms are also involved in the FFA-induced insulin resistance.

Inhibition of skeletal muscle insulin signaling by saturated fatty acids

In addition to its important effects on glucose metabolism directly, saturated fatty acids were demonstrated to affect insulin intracellular signaling pathways in skeletal muscle and myocytes [5,14,24,25]. Studies have demonstrated a marked reduction in IRS-1 tyrosine phosphorylation [9], IRS-1 and -2-associated PI3-kinase activity [9,26], and Akt phosphorylation and activity [26] in skeletal muscle after lipid infusion in euglycemic-hyperinsulinemic clamp. Along with a direct effect of saturated fatty acids on skeletal muscle insulin signaling, palmitic acid was shown to decrease insulin receptor expression and activity [27] and phosphorylation of IRS-1 and -2 at tyrosine residues [2,28], Akt [5,28-31] and GSK-3, as reviewed by Schmitz-Peiffer *et al.* [32] in isolated soleus muscle, primary culture of rat myocytes, pm128 myotubes, C2C12 and L6 myocytes. Similarly to skeletal muscle, palmitic acid inhibits Akt phosphorylation and activity in rat perfused heart and in HL-1 cells, an immortalized cardiomyocyte like cell lineage [33].

Several mechanisms have been postulated to account for the inhibition of insulin signaling by saturated fatty acids, including the activation of various kinases such as PKCs, IKK β , JNK, and p38 MAP kinase. These kinases have been postulated to catalyze the phosphorylation of serine residues in IRS-1 inhibiting its activity and directing it for degradation by the proteasome [34,35]. Such effects culminate with a reduction in the phosphorylation of tyrosine residues of IRS-1 by insulin, blocking its downstream signal transduction [36-38]. Other serine/threonine kinase activated in high-fat diet-induced or palmitate-induced insulin resistance is mammalian target of rapamycin (mTOR) [39,40], but the mechanisms involved are unknown yet.

Lipotoxic intramyocellular lipid accumulation induced by fatty acids

When the amount of circulating lipids chronically exceeds white adipose tissue ability for uptake and storage, like obesity, fatty acids accumulate in other tissues with limited capacity for lipid storage such as liver and skeletal muscles. Such abnormal ectopic lipid accumulation (lipotoxicity) is strongly associated with insulin resistance [41,42].

Fatty acids accumulate intracellularly in myocytes mainly as long-chain fatty acyl-CoA, monoacylglycerol, diacylglycerol, phosphatidic acid, triacylglycerol and

ceramides [32,43-46]. Among these fatty acid derivatives, high intramyocellular levels of diacylglycerol, triacylglycerol, and ceramides are directly associated with insulin resistance. Corroborating with this hypothesis, high fat feeding is associated with an increase in intramyocellular content of diacylglycerol and triacylglycerol and insulin resistance, such effects being abolished by inhibition of muscle lipid accumulation due to genetic deletion of lipoprotein lipase, fatty acid transporters (CD36 and FATP1), and diacylglycerol acyl transferase-1 (DGAT-1) [47-49]. Diacylglycerol accumulation is associated with the activation of subgroup of novel kinases, members of the large protein kinase C (PKC) family. Among the novel kinases, diacylglycerol directly activates PKC θ that catalyzes the phosphorylation of serine-307 residue at IRS-1, reducing its tyrosine phosphorylation and activation by insulin.

Consistent with this, Schmitz-Peiffer *et al.* [50] reported increased concentration of DAG in rodent's muscle and activation of PKCs induced by high-fat diet. Similarly, infusion of lipid and heparin caused insulin resistance in muscles that was associated with accumulation of intracellular DAG and specific activation of PKC θ [8]. Insulin resistance in this model was due to lipid-induced defects in the insulin signaling pathway that was caused by a reduction in tyrosine phosphorylation of IRS1, increasing its phosphorylation in serine-307 residue [9]. However, there are still no evidence to explain how the activation of novel PKCs might relate to serine phosphorylation of IRS1, and which kinases might have a role in the pathway, as reviewed by Samuel *et al.* [51].

In addition to diacylglycerol accumulation, high-fat diet or palmitate treatment increases production of ceramide and sphingosines in skeletal muscle cells, which is associated with glucose intolerance and insulin resistance [52,53]. Evidence suggests that ceramide and phosphatidic acid mediate the deleterious effects of palmitic acid on insulin signaling in cultured myotubes and insulin mediated Akt and GSK phosphorylation in C2C12 myotubes [52,54]. Ceramides also affects insulin signaling by two distinct mechanisms involving the activation of Akt dephosphorylation at threonine 308 and inhibition of its translocation to the plasma membrane [55], such effects being dependent on ceramides activation of protein phosphatase 2A (PP2A) and PKC ζ , respectively [56,57]. In addition, glycosylceramide, a glycosyl derivative of ceramides was shown to inhibit insulin receptor activity inducing insulin resistance [56,57]. Recently, it has been demonstrated that increased lysophosphatidylcholine content, a phosphatidic acid, in L6 myotubes treated with palmitate also leads to JNK activation and IRS-1 Ser307 phosphorylation, contributing to the development of muscle insulin resistance [58].

Activation of inflammatory signaling pathways by saturated fatty acids

Saturated fatty acids activate inflammatory signaling pathways directly through interaction with members of Toll-like receptor (TLR) family and indirectly through the secretion of cytokines including TNF- α , IL-1 β and IL-6 [59-61]. TLRs are an evolutionarily ancient pattern-recognition class of receptors that facilitate the detection of microbes. Saturated fatty acids activate TLR-4 in skeletal muscle promoting c-Jun NH(2)-terminal kinase (JNK) and I κ b kinase (IKK) complex activation, which results in degradation of the inhibitor of κ B (I κ B α) and nuclear factor- κ B (NF κ B) activation. Activation of JNK and IKK β by saturated fatty acids is associated with a marked inhibition of insulin action due to the phosphorylation of serine residues on the insulin IRS-1 and inhibition of its stimulatory phosphorylation of tyrosine residues by the insulin receptor [62,63]. Corroborating with an important contribution of TLR-4 to muscle insulin resistance, mice containing a loss of function by mutation of this receptor are partially protected from fat-induced inflammation and insulin resistance [64]. In addition, diabetic and obese mice have increased skeletal muscle IKK and JNK activities, whose pharmacological and genetic inhibition leads to an improvement in insulin sensitivity and glucose tolerance [65-67].

Coletta and Mandarino [68] demonstrated that changes in genes and proteins from inflammatory pathway contribute to the mitochondrial dysfunction observed in insulin resistant muscle and it can lead to decreased fat oxidation, ectopic fat accumulation, insulin signaling abnormalities and finally insulin resistance. These authors indicate that this mechanism is compatible with and complementary to other hypotheses regarding the vicious cycle connecting inflammation, mitochondrial changes, lipid accumulation and insulin signaling defects. The novel aspect of this mechanism is that it connects inflammatory processes with changes in insulin sensitivity by means of altered mechanosignal transduction due to fibrotic changes.

Fatty acids were also demonstrated to reduce mitochondrial function through induction of pro-inflammatory cytokines. Saturated fatty acids, palmitic and stearic acids, stimulate the secretion of TNF- α , IL-1 β and IL-6 in human leukocytes [60,69]. Wen *et al.* [61] showed increased IL-1 β production and release by palmitic acid in macrophages through activation of the NLRP3-ASC inflammasome. The pro-inflammatory cytokines have been associated to impaired mitochondrial function, establishing a link between fatty acids and mitochondrial dysfunction. Indeed, some studies reported reduced mitochondrial function in cells exposed to TNF- α , IL-1 β or IL-6 [70,71].

Alteration in gene expression by saturated fatty acids

Evidence has been obtained that fatty acids modulate expression of genes involved in glucose and lipid metabolism. Schmid *et al.* [72] demonstrated that C57BL/6 mice submitted to high-fat diet present reduced expression of enolase, a glycolytic enzyme, and ATP synthase in skeletal muscle. In addition, other enzymes of the glycolytic pathway have been shown to be modulated by fatty acids, such as pyruvate dehydrogenase kinase isozyme 1 (PDK-1), whose expression is increased in pancreatic islets incubated with saturated fatty acids [73], and lactate dehydrogenase A (LDHA), which was down-regulated in white adipose tissue from high-fat-fed animals [74]. Moreover, increased intramyocellular lipid content has been associated with down-regulation of PGC-1 α and of other genes encoding protein mitochondrial respiratory complexes I, II, III, and IV [75], resulting in impaired mitochondrial biogenesis and function [76]. Some studies identified transcription factors that recognize conserved motifs at the promoters of mitochondrial oxidative phosphorylation genes, such as nuclear respiratory factor (NRF)-1 and GA-binding protein (GABP) (also known as NRF-2) [77]. Studies showed that the peroxisome proliferator activator receptors (PPARs) control mitochondrial gene subsets, modulating fatty acid oxidation (FAO) and uncoupling [77,78]. Later, studies showed that PGC-1 α is a transcriptional coactivator of NRF-1, GABP, and PPARs, demonstrating the ability of PGC-1 α to integrate physiological signals and to increase mitochondrial biogenesis and oxidative function [79,80]. Thus, a reduction of PGC-1 α content in conditions of high fatty acid levels might be associated with impairment of mitochondrial function [5,76]. In addition, insulin-resistant subjects have reduced expression of mitochondrial genes, such as cytochrome c oxidase and complexes I and III subunits of the electron transport chain [81]. Activities of carnitine palmitoyltransferase-1 (CPT-1) and other key mitochondrial enzymes, such as citrate synthase and β -hydroxyacyl-CoA dehydrogenase, have also been found decreased in skeletal muscle from obese and type 2 diabetic individuals [26,82,83]. These changes in gene expression and enzyme activities induced by fatty acids contribute to the reduced mitochondrial oxidative capacity consequently leading to mitochondrial dysfunction.

Increase in reactive oxygen species by saturated fatty acids

Type 2 *diabetes mellitus*, obesity and the metabolic syndrome are strongly correlated with increased skeletal muscle content of reactive oxygen species (ROS) [84-86]. All conditions cited above contribute to an oxidative environment, modulating insulin sensitivity either by increasing insulin signaling or impairing glucose

tolerance. The mechanisms by which this occurs are often multifactorial and complex, involving several cell signaling pathways [87].

Production of ROS can occur in response to diverse *stimuli* including: (1) intracellular factors, such as nutrient metabolism, endoplasmic reticulum stress, and detoxification of various xenobiotics; (2) extracellular factors like signaling through plasma membrane receptors, such as hormones and growth factors and by pro-inflammatory cytokines; and (3) physical-environmental factors (e.g. ultraviolet irradiation) [88-91]. When moderately produced, ROS are involved in important physiological processes that lead to desired cellular responses. However, high ROS production is negatively associated with different biological signaling pathways [87]. ROS can react with multiple cellular components, such as proteins, lipids and nucleic acids, generating reversible or irreversible oxidative modifications. Pathophysiological processes mediated by ROS are more likely to induce irreversible modifications in cellular components, a reasonable definition of the term oxidative stress [88].

Control of vascular tone, cell adhesion, immune responses, and growth factors and hormone action are examples of ROS participation in normal physiology [92,93]. Conversely, a negative role of ROS has been implicated in ageing-related diseases, malignant transformation, atherosclerosis, neurodegenerative diseases, obesity, and diabetes [88,94,95]. Insulin signaling can be also impaired by oxidative stress, but the mechanisms involved are not fully understood. Studies have been demonstrated that ROS lead to impaired insulin response by inducing IRS serine/threonine phosphorylation, decreasing GLUT4 gene transcription, and decreasing mitochondrial activity [37,96].

Chronic elevation in plasma lipids levels and excessive intramyocellular fatty acid disposal are characterized by increased ROS and reactive nitrogen species (RNS) production [15,97,98]. Diet-induced obese mice have an increased expression of inducible nitric oxide synthase (iNOS) and RNS generation in skeletal muscle such effects being associated with impaired insulin sensitivity [15,99], since mice with iNOS disruption in muscle are protected from insulin resistance induced by obesity [100,101]. Diabetic patients present elevated ROS production in endothelial cells through NADPH oxidase activation, a mechanism mediated by PKC [102]. NADPH-oxidase complex is also found in skeletal muscles, raising the possibility that a similar mechanism occurs under elevated FA availability. Our group recently demonstrated that palmitate induces superoxide production in cultured skeletal muscle cells via NADPH oxidase activation, at least in part [98]. Some evidence also links xanthine oxidase (XO) as a source of increased ROS generation in diabetes and obesity. XO

protein and activity is found increased in muscle arterioles and livers from animal models of type 1 diabetes and diet-induced obesity [88,103,104].

Animals treated either with high-fat diet or oxidant drugs such as buthionine sulfoximine (BSO), an inhibitor of glutathione synthase, have increased skeletal muscle ROS production, oxidative stress and are insulin-resistant [105,106]. On the other hand, animals food restricted or treated with antioxidant drugs such as N-acetyl-cysteine (NAC), lipoic acid, vitamin E, and taurine, have reduced oxidative stress and improved insulin sensitivity [106-108]. In addition, mice overexpressing SOD2 have decreased ROS levels, improved hepatic insulin sensitivity, normalization of blood glucose and insulin levels, and reduced activation of cellular stress signalling pathways [109]. These data suggest that mitochondrial ROS is important for the development of insulin resistance.

The involvement of oxidative stress in insulin resistance was also observed in studies performed in myocytes. In L6 muscle cells, H₂O₂ reduced insulin-stimulated glucose uptake and glutathione content, effects that were prevented by preincubation with the antioxidant lipoic acid [110]. Rat soleus muscle exposed to nitric oxide (NO) donors have decreased insulin-stimulated glucose uptake and glycogen synthesis, effects that were associated with reduced insulin-stimulated phosphorylation of IR, IRS-1, and Akt [15].

Since mitochondria is the main site of ROS production in skeletal muscle, mitochondrion DNA, protein and lipids components are exposed to high levels of these metabolites suffering structural modification and damage which at long term can result in impaired function of this organelle [111,112].

Mitochondrial dysfunction or reduced mitochondrial biogenesis and density can lead to a decrease in mitochondrial fatty acids oxidation, which results in increased levels of fatty acyl-CoA and DAG that can activate stress-related Ser/Thr kinase activity and inhibit glucose transport, as reviewed by Lowell and Shulman [113].

In the case of stress-activated kinases, oxidative stress also contributes to impair insulin signaling by increased uncoupling protein-2 (UCP-2) activity. When these proteins are activated, it results in a heat generation that does not contribute to ATP production [114,115]. UCP-2 negatively regulates glucose-stimulated insulin secretion by reducing the ATP production, which is key to provide energy for almost all cellular processes [113]. In addition, UCP-2^{-/-} mice demonstrate enhanced insulin secretory capacity after a high-fat diet due to improved β -cell functions in a type 2 diabetes animal model [116].

Mitochondrial uncoupling is a powerful tool to control ROS formation and consequently to preserve

mitochondrial function. It can be hypothesized that fatty-acid induced uncoupling decrease mitochondrial ROS production and thus it can prevent mitochondrial lipotoxicity. In fact, UCP-3 is upregulated using high-fat diets [117,118], fasting [119], etomoxir treatment (which inhibits the mitochondrial fatty acid oxidation) [120,121] and lipid infusion [122], all conditions being associated with excessive lipid accumulation in skeletal muscle. On the other hand, when fat oxidative capacity is improved, like with endurance training [123,124], weight loss [125], or lowering circulatory fatty acids [126,127] there is a decrease in UCP-3. Interestingly, UCPs are activated by fatty acids and/or its peroxidation products, reducing mitochondrial ROS production [128,129]. So, it can be suggested that UCP-3 is involved in the protection against mitochondrial lipotoxicity by decreasing ROS production when activated by FA, as reviewed by Schrauwen *et al.* [130].

In summary, oxidative stress seems to play an important role in mitochondrial dysfunction, which can further exacerbate stress signals and reduce ATP production. The pathways leading to insulin resistance may be synergistic and mitochondrial dysfunction can create a feedback loop, adding to the overall oxidative stress environment [87].

Impairment of skeletal muscle mitochondrial function by saturated fatty acids

Several studies have shown that mitochondrial content, mitochondrial function, and oxidative capacity are decreased in insulin-resistant obese and type 2 diabetic individuals [131,132] suggesting that mitochondrial dysfunction might play an important role in the pathophysiology of insulin resistance. Corroborating with this hypothesis, impaired mitochondrial function and reduced fatty acid oxidative capacity were found in isolated primary myocytes, isolated rectus abdominal muscle strips and muscle homogenates from insulin-resistant obese and type 2 diabetic patients [26,83,133]. Moreover, mitochondrial density is reduced in insulin-resistant skeletal muscle from children, offspring of people with type 2 diabetes, suggesting that impaired mitochondrial oxidative capacity can be an inherited defect and an early marker for the development of insulin resistance [96]. Some studies have also reported alterations (mutations, polymorphisms, and epigenetics) in the mitochondrial DNA in conditions of insulin resistance, such as obesity, type 2 diabetes, and metabolic syndrome [134-137].

Evidence points for an important role of fatty acids in the genesis of the mitochondrial dysfunction associated with obesity and type 2 *diabetes mellitus*. In this sense, lipid infusion or administration of high-fat diet to health human and rodents were associated with impaired

mitochondrial function characterized by a reduction in ATP synthesis, oxygen consumption and oxidative phosphorylation [16,75,138,139]. These findings were corroborated by *in vitro* studies in which treatment of cultured skeletal muscle cells with palmitic acid increased ROS production, impaired fatty acid oxidation and decreased PGC-1 expression [103,104,140,141]. Studies from our group demonstrated that saturated fatty acids directly induces mitochondrial dysfunction in C2C12 skeletal muscle cells, as evidenced by reduced ATP synthesis and mitochondrial polarization [5].

Mitochondrial dysfunction plays a central role in the fatty acid-induced insulin resistance

As discussed above, several mechanisms have been proposed to explain the insulin resistance induced by saturated fatty acids. All these mechanisms operate in coordinated, integrated manner linking fatty acids availability to skeletal muscle insulin resistance. To account for this multifactorial characteristic of saturated fatty acid actions, we propose herein an integrative model centered on mitochondrial dysfunction as an important factor in the genesis of insulin resistance induced by fatty acids (Figure 1).

In physiological conditions, fatty acids are normally and rapidly oxidized with low ROS production, little intracellular lipid accumulation and preservation of

insulin sensitivity (Figure 1A). In pathological conditions, chronic elevation in circulating fatty acid levels reduces the expression of genes involved in mitochondrial biogenesis oxidative capacity and increase production of ROS, impairing mitochondrial biogenesis and function (Figure 1B). As a consequence, oxidative capacity is impaired and mitochondrial mass is reduced, increasing still further ROS production, leading to accumulation of fatty acid-derived metabolites such as diacylglycerol and ceramides.

ROS and lipid metabolites have been positively associated with insulin resistance and activation of several kinases, such as NF κ B, p38 MAP kinase, JNK, and some novel and atypical PKC isoforms, as PKC- ζ and - ϵ , in skeletal muscle [27,36,50,141-144]. These kinases impair the insulin signaling pathway by inducing serine/threonine phosphorylation in IRS-1. Under this condition, insulin-stimulated tyrosine phosphorylation of IRS-1 is inhibited, impairing activation of downstream signaling pathways and decreasing glucose uptake and metabolism in response to the hormone [5,6,145,146] (Figure 2).

Concluding remarks

We discussed herein the mechanisms involved in insulin resistance in skeletal muscle cells induced by high availability of FFA. Several mechanisms have been proposed, such as Randle cycle, inhibition of insulin signaling

Figure 1 Role of mitochondria in the insulin resistance induced by free fatty acids (FFA). In the normal condition (A), mitochondrial function is normal and FFA are rapidly metabolized with low reactive oxygen species (ROS) production and without accumulation of lipid metabolites; normal insulin response is preserved in this condition. In pathological condition (B), the excess of plasma FFA levels induces high FFA uptake into the cell, modulating negatively the expression of genes related to mitochondrial biogenesis and oxidative capacity, and into the mitochondrion, increasing the electron flux through to electron transport chain and, consequently, ROS and RNS production. As a result, mitochondrial biogenesis and function are impaired, decreasing mitochondrial mass and oxidative capacity, leading to abnormal intracellular accumulation of lipid metabolites and ROS and RNS, which activate some protein kinases involved in the phosphorylation of IRS-1 on threonine and serine residues. When phosphorylated in threonine and serine residues, IRS-1 is not phosphorylated on tyrosine residues, preventing activation of downstream signalling pathways by insulin. In addition, RNS increases IRS-1 nitrosilation, resulting in high degradation of these protein, which can contribute to impaired insulin response

Figure 2 Mechanisms underlying insulin resistance by impaired mitochondrial function.

pathway, regulation of gene expression and enzymatic activities, increase in ROS and RNS production, and impairment in mitochondrial function. Although numerous studies have been performed in order to investigate each one of these mechanisms, the conclusive proposition has not been defined yet. Evidence suggests that these mechanisms are not exclusive and there are several data in the literature pointing out that more than one mechanism is involved in the insulin resistance induced by FFA. We proposed herein a unifying hypothesis that places the importance of mitochondria in the establishment of FFA-induced insulin resistance.

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Authors' contributions

ARM, RTN, and RHL participated of the acquisition, analysis, and interpretation of the data from literature. RG, MAV, and WTF organized the structure of the manuscript and had substantial contribution to the conception and design. MFCB and LRS were involved in the drafting of the manuscript and figures. SMH and RC critically revised the text and figures for the final version of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Tributylin attenuates obesity-associated inflammation and insulin resistance in high-fat-fed mice

Marco Aurélio Ramirez Vinolo,¹ Hosana G. Rodrigues,¹ William T. Festuccia,¹ Amanda R. Crisma,¹ Vitor S. Alves,¹ Amanda R. Martins,¹ Catia L. Amaral,¹ Jarlei Fiamoncini,¹ Sandro M. Hirabara,² Fabio T. Sato,¹ Ricardo A. Fock,³ Gabriella Malheiros,⁴ Marinilce F. dos Santos,⁴ and Rui Curi¹

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Vinolo MA, Rodrigues HG, Festuccia WT, Crisma AR, Alves VS, Martins AR, Amaral CL, Fiamoncini J, Hirabara SM, Sato FT, Fock RA, Malheiros G, dos Santos MF, Curi R. Tributyrin attenuates obesity-associated inflammation and insulin resistance in high-fat-fed mice. *Am J Physiol Endocrinol Metab* 303: E272–E282, 2012. First published May 22, 2012; doi:10.1152/ajpendo.00053.2012.—The aim of this study was to investigate whether treatment with tributyrin (Tb; a butyrate prodrug) results in protection against diet-induced obesity and associated insulin resistance. C57BL/6 male mice fed a standard chow or high-fat diet were treated with Tb (2 g/kg body wt, 10 wk) and evaluated for glucose homeostasis, plasma lipid profile, and inflammatory status. Tb protected mice against obesity and obesity-associated insulin resistance and dyslipidemia without food consumption being affected. Tb attenuated the production of TNF α and IL-1 β by peritoneal macrophages and their expression in adipose tissue. Furthermore, in the adipose tissue, Tb reduced the expression of MCP-1 and infiltration by leukocytes and restored the production of adiponectin. These effects were associated with a partial reversion of hepatic steatosis, reduction in liver and skeletal muscle content of phosphorylated JNK, and an improvement in muscle insulin-stimulated glucose uptake and Akt signaling. Although part of the beneficial effects of Tb are likely to be secondary to the reduction in body weight, we also found direct protective actions of butyrate reducing TNF α production after LPS injection and in vitro by LPS- or palmitic acid-stimulated macrophages and attenuating lipolysis in vitro and in vivo. The results, reported herein, suggest that Tb may be useful for the treatment and prevention of obesity-related metabolic disorders.

butyrate; macrophages; diabetes; cytokines; white adipose tissue

THE CHRONIC LOW-GRADE INFLAMMATION associated with obesity plays a central role as a link between excessive fat accumulation and the development of pathologies (20). In obesity, adipose tissue is markedly infiltrated by proinflammatory macrophages and other leukocytes that secrete proinflammatory cytokines and chemokines (20, 41, 50). The overproduction of these inflammatory mediators together with changes in adipokine production and nonesterified fatty acid (NEFA) levels leads to the development of insulin resistance. In addition to adipose tissue, several organs, including the liver (1) and hypothalamus (28), develop a proinflammatory profile in response to the excessive nutrient supply. This systemic inflam-

matory state is associated with the activation of intracellular signaling pathways such as JUN NH₂-terminal kinase (JNK) and I κ B kinase- β (IKK β) that in turn phosphorylate serine residues in the insulin receptor substrate, inhibiting tyrosine phosphorylation and the interaction with phosphatidylinositol-3 kinase (19, 21).

In accord with an important role of inflammation in the development of obesity-associated diseases, whole body deletion of intracellular kinases activated upon inflammation, namely JNK (18) and IKK β (1), and pharmacological treatment with anti-inflammatory agents such as ω -3 fatty acids and salsalate, a salicylate derivative, were demonstrated to protect mice from the deleterious effects of high-fat feeding and obesity on insulin sensitivity (16, 32).

Butyrate is a short-chain fatty acid (SCFA) produced during fermentation of fibers and other substrates by an anaerobic bacteria resident in the gastrointestinal tract (36). In addition to its role as an energetic substrate, butyrate regulates leukocyte functions, including production of proinflammatory mediators and leukocyte recruitment (33, 38, 45, 46, 48). At least two different mechanisms seem to be involved in these effects, inhibition of histone deacetylase (HDAC) activity and activation of a G protein-coupled receptor (GPR), namely GPR43 (47). Regarding the production of inflammatory mediators, most of the studies demonstrated that butyrate attenuates their production by leukocytes. In line with this anti-inflammatory action, promising results have been obtained using butyrate in inflammatory conditions such as inflammatory bowel disease (47).

Elevation of SCFA availability by increasing dietary fiber intake or diet supplementation with butyrate may prevent the development of metabolic disarrangements and the insulin resistance associated with obesity (12, 13, 31, 43). Dietary supplementation with butyrate (at 5% wt/wt) prevented the development of insulin resistance and obesity. These effects have been related to the promotion of energy expenditure through enhanced mitochondrial expression of PGC-1 α and uncoupling protein 1 (UCP1) (13). A recent study has shown that butyrate supplementation of milk formula given to preweaning calves improved insulin sensitivity (23).

Tributylin (Tb) is a triglyceride containing three butyrate moieties that has been evaluated with the aim of overcoming pharmacokinetic problems associated with butyrate administration, including its rapid metabolization. After oral administration, Tb is rapidly absorbed and hydrolyzed to butyrate by

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plasmatic esterases; the peak of Tb and butyrate concentrations after oral administration of Tb is at 25 and 30 min, respectively (42). This triglyceride presents more favorable pharmacokinetics compared with butyrate and low toxicity (8). In addition, Tb also presents direct effects on cells and is more potent than butyrate (14).

In the present study, we investigated whether treatment with Tb results in protection against diet-induced obesity and associated insulin resistance. For this, mice fed a high-fat diet treated with Tb were evaluated for glucose homeostasis, plasma lipid profile, and inflammatory status. To further characterize the effects of Tb/butyrate, experiments with RAW 264.7 macrophages and *in vivo* in models of endotoxemia and lipolysis-induced leukocyte recruitment to adipose tissue were also performed.

MATERIALS AND METHODS

Animals. All experimental protocols were approved by the Animal Care Committee of the Institute of Biomedical Sciences, University of Sao Paulo. Male 8-wk-old C57BL/6 mice were kept individually in cages at 23°C on a 12:12-h light-dark cycle with food and water *ad libitum*. After adaptation (1–2 wk), mice were randomly assigned in one of four groups: control (C) and tributyrin (Tb) received a standard rodent chow diet (total energy: 75.8% carbohydrates, 9.5% fat, and 14.7% protein), and high-fat diet (HFD) and HFD + Tb groups were fed a diet rich in lard (total energy: 26.0% carbohydrates, 59.1% fat, and 14.9% protein). Animals were treated with Tb at 2.0 g/kg body wt (Tb and HFD + Tb groups) or water (C and HFD groups) by gavage three times/wk at intervals of 48 h during the entire protocol (10 wk). The Tb dose was chosen on the basis of preliminary observations, indicating its effectiveness to prevent insulin resistance induced by palmitate in isolated skeletal muscle of rats (data not shown) and on previous study (42).

Body weight and food intake were recorded three times/wk. At the end of the protocol, 6-h-fasted mice were euthanized by cervical dislocation after an intramuscular injection of ketamine (100 mg/kg body wt) and xylazine (50 mg/kg body wt) for tissue harvesting. Energy efficiency was calculated by the following formula: energy efficiency = body weight gain/energy intake. The energy intake per mouse was determined using the following conversions: standard chow = 3.80 kcal/g; HFD = 5.34 kcal/g.

Oxygen consumption/carbon dioxide production and respiratory quotient determination. Oxygen consumption/carbon dioxide production was measured in fed animals through a computer-controlled, open-circuit calorimeter system LE405 Gas Analyzer (Panlab-Harvard Apparatus, Holliston, MA). Mice were singly housed in clear respiratory chambers, and room air was passed through chambers at a flow rate of 0.8 l/min. The air flow within each chamber was monitored by an Air Supply and Switching sensor (Panlab-Harvard Apparatus). Gas sensors were calibrated prior to the onset of experiments. The analyses were performed during a period of 24 h. Sample air was passed sequentially through O₂ and CO₂ sensors for determination of O₂ and CO₂ content, from which measurements of oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) were estimated. $\dot{V}O_2$ and $\dot{V}CO_2$ were calculated by Metabolism Version 2.2 software. The day and night values of $\dot{V}O_2$ and $\dot{V}CO_2$ were calculated as the sum of the values of 6 h of measurement in each period. Respiratory quotient (RQ) was calculated using $\dot{V}CO_2/\dot{V}O_2$.

Blood analyses. Blood glucose, triglycerides, total cholesterol, HDL, and the activities of alanine (ALT) and aspartate (AST) transaminases were determined using a commercial kit (Labtest Diagnóstica). NEFA were measured using the HR NEFA series kit (Wako diagnostic, Richmond, VA) according to the manufacturer's instructions. The LDL cholesterol concentration was determined using the formula of Friedewald et al. (11). Resistin, leptin, macrophage

chemoattractant chemokine-1 (MCP-1), and TNF α were measured by Luminex-based bead array method using a LINCoplex simultaneous multianalyte detection system (Linco Research) according to the manufacturer's instruction. Insulin was determined using rat/mouse insulin ELISA (Millipore).

Glucose tolerance test. Glucose tolerance test (GTT) was performed on the 8th wk of treatment. After 6 h of fasting and blood collection (*time 0*), mice were injected intraperitoneally (ip) with a 50% glucose solution (2.0 g/kg body wt). Blood samples were collected at 15, 30, 45, 60, and 90 min after glucose injection for determination of glycemia.

Insulin tolerance test. Blood samples were collected before or 10, 20, 30, 40, and 50 min after ip insulin injection (0.75 U/kg) for serum glucose determination. The constant for glucose disappearance rate during the test (K_{it}) was calculated using the $0.693/t_{1/2}$ formula. The glucose $t_{1/2}$ was calculated from the slope of the least-square analysis of the plasma glucose concentrations during the linear decay phase.

Peritoneal macrophage isolation and culture. Cells were obtained by washing the peritoneal cavity with 5 ml of RPMI-1640 culture medium (Cultilab, Sao Paulo, Brazil). Macrophage-rich cultures (>90% of the cells were F4/80⁺) were obtained by incubating peritoneal cells in 24-well polystyrene culture plates for 2 h at 37°C in a 5% CO₂ humidified air environment (37). Nonadherent cells were removed by washing with RPMI. Adherent cells were then incubated with 1 μ g/ml lipopolysaccharide (LPS; *E. coli*, serotype 0111:B4; Sigma Chemical) for 24 h. The supernatant was collected to determine the contents of the IL-1 β , IL-6, IL-10, TNF α , and keratinocyte-derived chemokine (KC) by ELISA and nitric oxide (NO) by Griess assay (7).

In vitro evaluation of adipokine production. The epididymal adipose depot was removed, cut in pieces of ~100 mg, and washed two times with sterile PBS. The explants were further cut into pieces of 10–15 mg, washed more two times with PBS, and then incubated in DMEM (Sigma Chemical) containing 10% fetal bovine serum at 37°C for 24 h. Culture medium was collected, and IL-1 β , IL-6, and TNF α adiponectin and leptin contents were determined by ELISA.

Measurement of lipolysis in isolated adipocytes. Epididymal fat pads were minced with fine scissors and added to sterile polypropylene containers containing 10 ml of sterile DMEM with HEPES (25 mmol/l), collagenase type II (1.25 mg/ml), and 4% BSA. The mixture was incubated for 30 min at 37°C in an orbital shaker at 150 oscillations/min. The isolated adipocytes were then filtered through a fine plastic mesh (150 μ m), transferred to a conical (50 ml) plastic tube, and washed three times with the same buffer without collagenase (25 ml/wash). After the last wash, the medium was totally aspirated. Lipolysis was determined by incubating isolated adipocytes (aliquot of 40 μ l) at 37°C in a final volume of 0.2 ml of DMEM with 2% fatty acid-free BSA for 120 min under constant shaking with or without isoproterenol (1 μ mol/l). NEFA concentration in the infranatant was determined using the HR NEFA kit. Aliquots of adipocyte suspension were fixed with 10% formaldehyde for subsequent analyses of cell diameter. Ten photos of each sample were taken using an optical microscope, and the diameter of the cells (500 in total) was determined using the Image-Pro Plus program. NEFA levels were expressed in relation to the adipocyte number.

Liver histology and triacylglycerol measurements. Liver fragments (100 mg) were subjected to extraction for 16 h at 4°C with 4 ml of CHCl₃/methanol (2:1, vol/vol). Two milliliters of 0.6% NaCl were added to the extract, and the mixture was centrifuged at 2,000 g for 20 min (22). The organic layer was collected and dried, and the residue was dissolved in isopropanol and assayed for triglyceride content. For histological analysis, a fragment of the liver of each animal was removed, fixed with 3.7% buffered formaldehyde solution for 8 h at room temperature, dehydrated, processed, and embedded in Paraplast (Sigma Chemical). Sections (7 μ m) were stained with hematoxylin and eosin to evaluate tissue morphology as well as the degree of liver steatosis.

Quantitative RT-PCR. Total RNA from the epididymal adipose depot was extracted with Trizol reagent (Invitrogen Life Technologies) (5) and reverse transcribed to cDNA using the High-Capacity cDNA kit (Applied Biosystems). Gene expression was evaluated by real-time PCR (17) using a Rotor Gene (Qiagen) and SYBR Green as fluorescent dye. Primer sequences are shown in Table 1. Quantification of gene expression was carried out using a previously described method (27), with ubiquitin C gene as an internal control.

Glucose metabolism in isolated soleus muscle. Soleus muscles were rapidly and carefully isolated, weighed, preincubated at 35°C in Krebs-Ringer bicarbonate buffer, pH 7.4, and pregassed for 30 min with 95% O₂-5% CO₂ containing 5.6 mmol/l glucose with agitation at 120 oscillations/min. After 30 min, muscles were transferred to vials containing Krebs buffer supplemented with 0.3 µCi/ml D-[U-¹⁴C]glucose and 0.2 µCi/ml 2-deoxy-D-[2,6-³H]glucose in the presence or absence of insulin (7 nmol/l). Gasification was maintained for >15 min and then removed. After 1 h of incubation, muscles were washed in cold buffer, briefly dried on filter paper, and frozen in liquid N₂. Muscles were processed for 2-deoxy-D-[2,6-³H]glucose uptake, D-[U-¹⁴C]glucose incorporation, and [¹⁴C]glycogen synthesis (6). Extracellular space was determined by incubating some muscles in the presence of 0.1 µCi/ml L-[1-¹⁴C]glucose and 5.6 mmol/l L-glucose. Glucose uptake was calculated by subtracting extracellular L-[1-¹⁴C]glucose from total 2-deoxy-D-[2,6-³H]glucose.

Western blotting analysis. Isolated soleus muscles and liver were homogenized in lysis buffer containing protease and phosphatase inhibitors and centrifuged (12,000 g, 10 min, 4°C). The supernatants were mixed with 4× SDS-PAGE sample buffer, and the protein content of homogenates was determined by the Bradford method (2). Equal amounts of protein were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes. Detection was carried out using antibodies against phospho-Akt (Ser⁴⁷³), phospho-JNK (T183/Y185), Akt, and JNK from Cell Signaling Technology (Boston, MA).

LPS-induced endotoxemia. Male adult Balb/c (*Mus domesticus*) mice were treated by gavage with water (control) or Tb (2.0 g/kg) 1 h before being challenged with an intravenous injection of LPS (1.25 µg, *E. Coli* B55:O5; Sigma Chemical) (9, 10). Mice were anesthetized, and blood was collected 1 h after LPS injection.

Lipolysis activation. Two protocols were used to activate lipolysis, namely pharmacological β-adrenergic activation and food restriction. In the former, mice fed a standard diet were treated with either amide solution (3.34 g/kg body wt) or Tb (2.0 g/kg body wt) three times. Isoproterenol (0.1 mg/kg) or saline was injected ip twice (4 h between each injection) (24, 49). Mice received amide or Tb 1 h before each adrenergic administration and 1 h before euthanasia. Mice were euthanized 14 h after the second isoproterenol injection. Food restriction was carried out as described previously (24). Briefly, high-fat-fed mice (~40 g body wt) treated with either an amide solution or Tb were calorie-restricted for 3 days (70% of their ad libitum food intake). The amount of energy provided by either Tb or the amide solution through gavage was equivalent and discounted from the total calories provided to the mice in the food restriction protocol. In these experiments, mice were not pretreated with Tb or amide solution.

Statistical analysis. Comparisons of the results were performed using Student's *t*-test or one-way ANOVA and the Tukey multiple comparison posttest. The statistical test used in each of the analyses is described in the figure and table legends. Significance was set at *P* < 0.05. Results were obtained from at least three separate experiments and expressed as means ± SE.

RESULTS

Tb reduced body weight gain in high-fat-fed mice. Mice fed a HFD were treated with Tb or water during the period of 10 wk. As depicted in Fig. 1, Tb treatment significantly attenuated the increase in body weight gain (−40%; Fig. 1, A and B) and visceral and subcutaneous adipose tissue masses (−20%; Fig. 1, C and D). HFD intake also resulted in a significant increase in liver weight (25%, from 1.20 ± 0.03 g in the control group to 1.50 ± 0.04 g in the HFD group), which was partially reversed by Tb (1.38 ± 0.04 g). No effect of Tb on the weight of skeletal muscle, heart, or brown adipose tissue (BAT) was observed (data not shown). The reduction in adiposity induced by Tb was associated with a significant attenuation in adipocyte hypertrophy (data not shown). The daily food intake of the experimental groups was 4.57 ± 0.17 (C), 4.51 ± 0.16 (Tb), 2.87 ± 0.17 (HFD), and 2.89 ± 0.22 (HFD + Tb) g·day^{−1}·animal^{−1}, and no change in the caloric intake was observed (Fig. 1E). Despite similar caloric intake, a significant reduction in energy efficiency (−31.6%; Fig. 1F) was observed in high-fat-fed mice after treatment with Tb. The mice in this group (HFD + Tb) also presented an increment on oxygen consumption/carbon dioxide production compared with HFD (Fig. 1, G and H), suggesting an increased energy expenditure. These changes were accompanied by a reduction in the RQ (Fig. 1I).

Tb treatment partially prevented the changes in plasma leptin and resistin levels and reverted the increase in NEFA concentrations induced by high-fat diet (Table 2). Indeed, Tb partially reverted the hypertriglyceridemia and hypercholesterolemia induced by high-fat feeding (Table 2). Taken together, these results indicate that Tb improves the dyslipidemia associated with high-fat feeding.

Tb attenuated high-fat diet-induced insulin resistance. Tb treatment reduced fasting hyperglycemia, hyperinsulinemia, and insulin resistance, as evaluated by the homeostasis model assessment (HOMA) of insulin resistance (Table 2), in high-fat-fed mice. Tb also ameliorated glucose intolerance (Fig. 2, A and B) and partially reverted the insulin intolerance (Fig. 2, C and D) induced by high-fat feeding. Corroborating with these beneficial effects on glucose homeostasis, Tb prevented the reduction in insulin-stimulated skeletal muscle glucose uptake and incorporation into glycogen associated with diet-

Table 1. The annealing temperature and sequences of the primers used

Gene	Primer Sense	Primer Antisense	Annealing Temperature (°C)
UBC	AGCCAGTGTACCAACCAAG	ACCCAAGAACCAAGCACAAGG	57
IL-1β	GGCAGTACCTGTGTCTTTCCC	ATATGGGTCCGACAGCAGGAG	59
IL-6	CCTTCTGGGACTGATGTTGTGAC	GGGTGGTATCCTCTGTGAAGTCTCC	59
MCP-1	ACCTGGATCGGAACCAAAATGG	GAAGTGCTTGAGGTGGTGTGG	57
TNFα	TCTTCTCATTCCTGCTGTGGC	CACTTGGTGGTTTGCTACGACG	57
F4/80	CCTGAACATGCAACCTGCCAC	GGGCATGAGCAGCTGTAGGATC	59
CD11c	AGCAGGTGGCATTGTGGGAC	ACCTCTGTCTCCTCCTCTCCG	59

UBC, ubiquitin C; MCP-1, macrophage chemoattractant chemokine-1.

Fig. 1. Body weight, food intake, and adiposity of high-fat-fed mice treated [high-fat diet (HFD) + tributyrin (Tb)] or not (HFD) with Tb and the control groups (C and Tb). Body weight (A) was measured during the experimental protocol. Body weight gain (B) was calculated considering the initial and final body weights. Visceral (epididymal and retroperitoneal) and subcutaneous (inguinal) adipose tissue weights (C and D) were measured at the end of the protocol. Food intake (E) was monitored during experimental period, and energy efficiency (F) of the chow was calculated as described in MATERIALS AND METHODS. Results are means $\pm$ SE ($n = 10$ – 12 /group). In A–F: * $P < 0.05$ vs. C; # $P < 0.05$ vs. Tb; † $P < 0.05$ vs. HFD + Tb (ANOVA with Tukey's posttest). G–I: on the 10th wk of treatment, indirect calorimetry was performed to determine oxygen consumption (O₂; G), carbon dioxide production (CO₂; H), and respiratory quotient (RQ; I). Results are means $\pm$ SE ($n = 5$ – 6 /group). * $P < 0.05$ vs. HFD (Student's *t*-test).

induced obesity (Fig. 3, A–C) or caused by palmitate (500 μ M) treatment in vitro in skeletal muscle of lean mice (data not shown). In line with an amelioration of muscle/insulin resistance, Tb abolished the impairment in Akt activation by insulin found in high-fat-fed mice, as evaluated by the content of phosphorylated Akt (Ser⁴⁷³; Fig. 3, D and E).

Tb attenuated macrophage and white adipose tissue production of inflammatory mediators. Tb treatment significantly prevented the increase in the 24-h production of TNF α and IL-6 by peritoneal macrophages isolated from high-fat-fed mice under basal, nonstimulated conditions (Fig. 4, A and C). Upon LPS stimulation, Tb attenuated the higher production of TNF α , IL-1 β , and IL-6, but not KC, by HFD macrophages (Fig. 4, A–D). Tb had no significant effect on macrophage production of IL-10 and NO (data not shown). In line with the reduced production of TNF α by peritoneal macrophages, Tb treatment significantly attenuated the increase in circulating TNF α (Fig. 4E) and IL-6 (data not shown) levels induced by LPS administration. To test whether the effects of Tb/butyrate on TNF α production by macrophages were due to a direct

action, we evaluated the cytokine production in RAW 264.7 (a murine macrophage cell line) after incubation with various concentrations of butyrate. Palmitic acid- and LPS-stimulated production of TNF α by RAW 264.7 cells was attenuated partially by butyrate at 1 mM, suggesting a direct action of this SCFA. This effect of butyrate was mimicked by trichostatin A (TSA), a potent inhibitor of HDAC activity, indicating that this pathway may be involved (Fig. 4, F and G).

Tb treatment prevented the reduction, induced by the high-fat diet, in adiponectin production by fat explants (Fig. 5B), but it had no effect on leptin and IL-6 release (Fig. 5, A and C). The cytokines whose concentrations were below the limit of detection (TNF α , IL-1 β , IL-10, and MCP-1) had their mRNA levels measured by quantitative PCR. In line with its anti-inflammatory actions, Tb treatment markedly attenuated the increase in white adipose tissue (WAT) TNF α , IL-1 β , and MCP-1 mRNA levels in high-fat-fed mice (Fig. 5, D–F). The IL-10 expression was similar between experimental groups (data not shown). Tb treatment was associated with a marked effect on macrophage recruitment to WAT induced by the high-fat diet; reduction of

Table 2. Serum determinations in high-fat-fed mice treated (HFD + Tb) or not (HFD) with Tb and the control groups (C and Tb)

	C	Tb	HFD	HFD+Tb
Triglycerides, mg/dl	43.2 ± 2.6	45.3 ± 3.7	56.7 ± 3.8*#	50.4 ± 4.5
Cholesterol, mg/dl	132.2 ± 6.8	129.8 ± 4.8	183.0 ± 8.3*#	161.2 ± 6.8
LDL cholesterol, mg/dl	67.3 ± 5.5	63.6 ± 6.1	111.3 ± 6.4*#	97.1 ± 9.4
HDL cholesterol, mg/dl	63.3 ± 3.7	58.1 ± 2.1	62.1 ± 3.8	58.2 ± 3.1
NEFA, mM	0.30 ± 0.03	0.32 ± 0.03	0.45 ± 0.05*#†	0.27 ± 0.03
AST, U/ml	111.8 ± 15.9	114.3 ± 9.9	103.8 ± 15.0	102.9 ± 15.1
ALT, U/ml	30.2 ± 10.0	36.5 ± 9.2	34.0 ± 6.5	29.9 ± 4.4
Leptin, pg/ml	4,061 ± 744	6,512 ± 786	20,944 ± 2145*#†	15,642 ± 1,178*#
Resistin, pg/ml	3,788 ± 391	3,950 ± 353	7871 ± 595*#†	6,024 ± 447*#
Fasting glucose, mM	9.48 ± 0.40	8.86 ± 0.34	10.86 ± 0.35*#†	9.48 ± 0.35
Insulin, ng/ml	0.37 ± 0.04	0.47 ± 0.07	1.01 ± 0.08*#†	0.75 ± 0.09*#
HOMA-IR	21.92 ± 3.38	26.84 ± 3.85	75.70 ± 5.80*#†	45.77 ± 6.31

Results are means ± SE; $n = 8-10$ /group. HFD, high-fat diet; C, control; Tb, tributyrin; NEFA, nonesterified fatty acids; ALT, alanine transaminase; AST, aspartate transaminase; HOMA-IR, homeostasis model assessment of insulin resistance. * $P < 0.05$ vs. C; # $P < 0.05$ vs. Tb; † $P < 0.05$ vs. HFD + Tb (ANOVA with Tukey's posttest). Glucose and insulin were measured in 6-h-fasted mice after 6 wk of treatment. Insulin sensitivity was determined through HOMA-IR, which was calculated according to the following formula: $\text{HOMA-IR} = \{[\text{fasting insulin (mU/ml)} - \text{fasting glucose (mg/dl)}]/405\}$.

M1 macrophage marker mRNA levels (F4/80 and CD11c) was observed in mice treated with Tb (Fig. 5, *G* and *H*). However, no effects of Tb were seen on mRNA levels of the M2 macrophage markers (MGL-2 and arginase 1; data not shown).

Tb treatment suppressed lipolysis and macrophage recruitment to the WAT. Rates of lipolysis are directly related to macrophage recruitment to adipose tissue (24). Therefore, we tested whether Tb affected leukocyte recruitment by modulating lipolysis. Tb treatment markedly attenuated the increase in basal and isoproterenol-stimulated lipolysis induced by high-fat feeding (Fig. 6A). In vitro, the treatment of adipocytes with butyrate or the GPR43 agonist (phenylacetamide-1) reduced isoproterenol-stimulated lipolysis (Fig. 6B). Corroborating with the data obtained in vitro, Tb treatment reduced circulating NEFA levels in response to isoproterenol administration in vivo (Fig. 6C).

Next, we investigated whether inhibition of adipose tissue lipolysis by Tb would affect macrophage recruitment in vivo. Tb prevented the increase in expression of the macrophage marker F4/80 in food-restricted and β -adrenergically stimulated mice, suggesting that it may affect macrophage recruitment by reducing WAT lipolysis (Fig. 6, *D* and *E*).

Tb attenuated hepatic steatosis induced by high-fat diet. Varying degrees of liver steatosis were observed in all high-fat-fed mice. Cells containing large fat inclusions were observed throughout the hepatic lobules; centrilobular hepatocytes, however, were more strongly affected, showing many fat vacuoles of different sizes. Infiltration of inflammatory cells was observed frequently. Tb significantly attenuated liver steatosis, although inflammatory cells and cells containing large fat inclusions, mostly in the centrilobular region, were still observed to a much lesser degree (data not shown). In accordance with these findings, a significant reduction in hepatic triglyceride accumulation was observed in mice treated with Tb (Fig. 7A). Tb treatment also reduced IL-1 β and F4/80 expression in the livers of high-fat-fed mice (Fig. 7, *B* and *C*). No difference in TNF α , IL-6, or CD11c expression was observed between the experimental groups (data not shown).

Tb suppressed JNK activation by high-fat feeding. In accord with our initial hypothesis that the improvement in insulin sensitivity in high-fat-fed mice induced by Tb is due to an

attenuation in the activity of intracellular kinases activated upon proinflammatory stimuli, we found that Tb treatment significantly reduced the activation of JNK in the liver and skeletal muscle induced by high fat diet, as indicated by the content of phosphorylated JNK (T183/Y185) (Fig. 8).

DISCUSSION

Butyrate is a SCFA with relevant effects on cancer and inflammation and on the development of obesity. The major problem of using this compound in vivo has been the difficulty in achieving and maintaining its concentrations in blood since this fatty acid is rapidly metabolized by colonocytes. To overcome this problem, we have used Tb, a prodrug of butyrate that presents low toxicity and has been shown to increase plasmatic butyrate levels up to 2.4 mM and to sustain butyrate concentrations above 0.1 mM for more than 120 min after oral administration (8, 29).

In the present study, Tb protected mice against obesity and obesity-associated insulin resistance and dyslipidemia. Data indicate that these effects are, at least in part, due to the anti-inflammatory actions of this compound. Accordingly, Tb reduced the expression of proinflammatory cytokines in adipose tissue and liver and attenuated the elevation in circulating levels of TNF α in response to LPS and the production of TNF α , IL-1 β , and IL-6 by peritoneal macrophages isolated from high-fat-fed mice. In vitro, butyrate attenuated the production of TNF α stimulated by LPS and palmitic acid. Furthermore, Tb also reduced the expression of MCP-1 and macrophage infiltration in the WAT and restored the production of the anti-inflammatory adipokine adiponectin in high-fat-fed mice. These effects were associated with a reduction in hepatic steatosis, liver, and skeletal muscle content of phosphorylated JNK and an improvement in muscle insulin-stimulated glucose uptake and Akt signaling. In addition to the attenuation of inflammation, Tb may also protect mice from insulin resistance by reducing body weight gain and regulating the production of gut hormones, as demonstrated recently (26).

Tb treatment reduced body weight gain and fat accumulation induced by high-fat feeding, an effect that may be related to the increase in energy expenditure and oxygen consumption ob-

Fig. 2. Glucose (GTT) and insulin tolerance tests (ITT) were performed after 8 wk of treatment. Glucose curve (A) and incremental area of blood glucose (B) are presented. Glucose curve during ITT (C) and the rate of blood glucose disappearance (K_{itt} ; D) are shown. Results are means $\pm$ SE ($n = 5-8$ /group). * $P < 0.05$ vs. C; # $P < 0.05$ vs. Tb; † $P < 0.05$ vs. HFD + Tb (ANOVA with Tukey's posttest). AUC, area under the curve.

served in these animals. A similar effect was also described in experimental models, using diets supplemented with butyrate or high contents of fibers (12, 13). In mice receiving the butyrate-supplemented diet, these effects seemed to be the result of a combination of increased spontaneous motor activity and BAT thermogenesis (13). In our study, no changes in BAT mRNA levels of the thermogenic markers UCP1, PGC-1 α , and deiodinase-2 (data not shown) were observed, suggesting that Tb administration enhances energy expenditure by mechanisms different from the butyrate-supplemented diet.

The reduction in adiposity induced by Tb was associated with slight changes in circulating triglyceride and cholesterol levels and attenuation in the elevation in serum leptin and resistin levels associated with diet-induced obesity. Tb treatment also protected mice from glucose intolerance and insulin resistance induced by high-fat diet as evaluated by GTT, insulin tolerance test, HOMA, and muscle glucose uptake and insulin signaling. Extending these findings, Tb prevented skeletal muscle insulin resistance induced in vivo by high-fat diet and in vitro by prolonged incubation with palmitate. This latter

Fig. 3. Glucose uptake, metabolism, and insulin signaling in isolated soleus muscle. Glucose uptake (A), total glucose incorporation (B), and glycogen synthesis (C) were evaluated in soleus muscle. The dashed lines indicate the basal values (without insulin stimulation). Results are means $\pm$ SE ($n = 5-6$ /group). * $P < 0.05$ vs. C; # $P < 0.05$ vs. Tb; † $P < 0.05$ vs. HFD + Tb (ANOVA with Tukey's posttest). Immunoblot analysis of Akt phosphorylation (Ser⁴⁷³) was performed in soleus muscle extracts. D: the relative levels of phosphorylated (p)-Akt/AKT ratio of muscle incubated or not with insulin (INS) are shown in the bar graph. E: representative Western blot showing Akt activation ($n = 4-5$ /group). Results are means $\pm$ SE. * $P < 0.05$ vs. without INS (Student's *t*-test).

Fig. 4. Production of inflammatory mediators in vitro by macrophages and in vivo in lipopolysaccharide (LPS)-treated mice. Peritoneal macrophages were incubated for 24 h. The culture supernatants were then assayed for the production of cytokines. A–D: TNF α (A), IL-1 β (B), IL-6 (C), and keratinocyte-derived chemokine (KC; D) quantification in the supernatants of nonstimulated (black bars) and LPS-stimulated macrophages (open bars). Results are means $\pm$ SE ($n = 8$ –10/group). * $P < 0.05$ vs. C; # $P < 0.05$ vs. Tb; † $P < 0.05$ vs. HFD + Tb (ANOVA with Tukey's posttest). E: serum concentration of TNF α was determined 1 h after intravenous LPS administration. Results are means $\pm$ SE ($n = 6$ –7/group). * $P < 0.05$ vs. LPS condition (Student's t -test). RAW 264.7 cells were preincubated for 1 h with butyrate or trichostatin A (TSA) and then stimulated with palmitic acid (PA) or LPS for 23 h. F and G: TNF α was then determined in the supernatant of LPS- (F) and PA-stimulated cells (G). Results are means $\pm$ SE ($n = 5$). * $P < 0.05$ vs. PA- or LPS-stimulated cells without treatment (ANOVA with Tukey's posttest).

result indicates that Tb treatment protects skeletal muscle from insulin resistance independently of its effect on body weight. However, the precise mechanisms underlying this effect are still under investigation. Activation of AMP-activated protein kinase and PGC-1 α directly by butyrate (13) or indirectly by attenuation of the production of proinflammatory cytokines (40) may be involved. Butyrate and other SCFAs may also contribute to the improvement of glucose metabolism by stim-

ulating the secretion of glucagon-like peptide-1 through the receptors GPR41 and -43 (26, 44).

Excessive fat accumulation is associated with the recruitment and accumulation of leukocytes with different phenotypic and functional characteristics, such as the M1 proinflammatory macrophages in the adipose depot, which contribute to the low-grade inflammation and insulin resistance found in obesity (34). Tb attenuated M1 accumulation and production of pro-

Fig. 5. Production of adipokines and cytokines and expression of leukocyte markers in epididymal white adipose tissue (WAT). Leptin (A), adiponectin (B), and IL-6 (C) were measured in the 24-h culture supernatant of WAT explants ($n = 9$ –10/group). mRNA expression of TNF α (D), macrophage chemoattractant chemokine (MCP-1; E), IL-1 β (F), F4/80 (G), and CD11c (H) was analyzed in WAT of high-fat-fed mice treated (HFD + Tb) or not (HFD) with Tb and their lean controls (C and Tb). Results are means $\pm$ SE ($n = 6$ –8 animals/group). * $P < 0.05$ vs. C; # $P < 0.05$ vs. Tb; † $P < 0.05$ vs. HFD + Tb (ANOVA with Tukey's posttest).

Fig. 6. WAT lipolysis and macrophage recruitment. Nonesterified fatty acid (NEFA) released by adipocytes isolated from the experimental groups (A) or adipocytes incubated with butyrate or G protein-coupled receptor 43 agonist [phenylacetamide (phenyl); B] were measured after stimulation with isoproterenol in vitro. Results are means $\pm$ SE ($n = 5-6$). * $P < 0.05$ vs. control; # $P < 0.05$ vs. Tb; † $P < 0.05$ vs. HFD + Tb (ANOVA with Tukey's post test). C: plasma NEFA levels after administration of isoproterenol ($n = 6$ animals/group). Results are means $\pm$ SE. * $P < 0.05$ vs. C (Student's t -test). D and E: F4/80 expression in WAT of obese mice submitted to 30% food restriction (restr) for 3 days (D) or lean mice after isoproterenol (isop) administration (E) ($n = 5-6$ animals). Results are means $\pm$ SE. * $P < 0.05$ vs. C (ANOVA with Tukey's posttest).

inflammatory mediators in the adipose tissue and liver of high-fat-fed mice. Although the molecular mechanisms involved in the initial activation of inflammatory cells in a high-fat diet are not totally understood, evidence exists in favor of a role of Toll-like receptor agonists such as saturated fatty acids (e.g., palmitic acid) (39) and LPS (3) in this process. Butyrate suppressed the response of LPS- and palmitate-stim-

ulated macrophages and attenuated cytokine production after LPS administration. These results were also obtained with TSA, a potent inhibitor of HDAC, indicating that this pathway may be involved in the anti-inflammatory effect of butyrate. Indeed, previous studies have shown that butyrate and other inhibitors of HDAC reduce the activation of Toll-like receptor signaling in cells (e.g., neutrophils and macrophages) through

Fig. 7. Measurement of triglyceride levels and inflammatory marker expression in liver. A: hepatic triglyceride contents were determined at the end of the experimental protocol. B and C: the mRNA expression of IL-1 β (B) and F4/80 (C) was analyzed in livers of high-fat-fed mice treated (HFD + Tb) or not (HFD) with Tb and their lean controls (C and Tb). Results are means $\pm$ SE ($n = 5-7$ animals/group). * $P < 0.05$ vs. C; # $P < 0.05$ vs. Tb; † $P < 0.05$ vs. HFD + Tb according to ANOVA with Tukey's posttest statistical analysis.

Fig. 8. Activation of JNK pathway in the liver and skeletal muscle. Immunoblot analysis of JNK phosphorylation (T183/Y185) was performed in liver and soleus muscle extracts. The relative levels of p-JNK/JNK ratio in liver (A) and skeletal muscle (B) are shown in the bar graphs. Representative Western blots showing JNK activation are shown. Results are means $\pm$ SE ($n = 4/\text{group}$). * $P < 0.05$ vs. HFD mice (Student's t -test).

the modulation of intracellular pathways, including mitogen-activated protein kinase and NF- κ B (4, 48).

SCFAs inhibit lipolysis through fatty acid-activated GPR43 (15). Although butyrate is a less potent agonist of GPR43 than acetate and propionate, the results of the present study and the data obtained by others (52) suggest that this fatty acid can also inhibit the release of fatty acids from WAT (lipolysis). The relevance of this effect is that the fatty acids released by WAT not only act directly or indirectly through stimulation of cytokine production (39) on other tissues inducing insulin resistance but also play a role in the recruitment of leukocytes to this tissue (24). The increased rate of NEFA efflux from adipose tissue, induced by fasting or by pharmacological intervention, leads to macrophage accumulation in this tissue (24), an effect that was abolished by Tb. These results are suggestive of a role of the antilipolytic effect of butyrate in the reduction of inflammation in WAT. Tb treatment also reduced MCP-1, an important chemokine for macrophage infiltration of WAT (22). Tb treatment also attenuated the hepatic steatosis induced by a high-fat diet. This protective effect of Tb has also been demonstrated in endotoxemic rats and seems to involve inhibition of NF- κ B (29).

Inflammatory mediators, including TNF α and IL-1 β derived from adipose tissue, liver, and leukocytes and fatty acids released from the WAT, promote insulin resistance. Evidence that activation of the stress/inflammatory JNK and IKK/NF- κ B pathways is the link between inflammation and insulin resistance has been presented (30, 51). The attenuation of JNK activation in the liver and skeletal muscle of Tb-treated mice may be related to the reduction in inflammatory mediators and NEFA levels. Another possibility is that Tb effects are secondary to its actions on colon since butyrate has been shown to modulate gene expression in colonocytes and to enhance the intestinal barrier (25, 35). Although further studies are necessary to characterize in detail the effect of Tb and butyrate in the colon and their consequences in the whole organism, the fact that Tb did not change plasmatic levels of LPS (data not shown) found in high-fat feeding mice indicates that the beneficial effects of Tb are not due to change in the intestinal permeability.

In the present study, we showed that Tb attenuates several metabolic changes observed in mice fed a high-fat diet, including high plasma lipid (cholesterol, triglycerides, and NEFA) levels and increased production of inflammatory mediators (e.g., TNF α , IL-1 β , and MCP-1). In addition to the effects of Tb secondary to the reduction in body weight, we demonstrated that Tb/butyrate has direct effects, such as the inhibition of proinflammatory mediator production by macrophages, protection of skeletal muscle against insulin resistance induced by palmitate, and inhibition of lipolysis and leukocyte accumulation in WAT. Unlike other studies performed using diets supplemented with butyrate or with high contents of fibers, we present data showing a protective effect of butyrate in obesity and insulin resistance after oral administration of Tb (3 times/wk). Despite the fact that the dose of Tb used in the present study is high, this triglyceride could be incorporated in food products or used in lower concentrations together with other compounds to achieve these beneficial effects in humans. The results reported herein and by others suggest that butyrate and its derivatives may be used therapeutically for the treatment and prevention of obesity-related metabolic disorders.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

M.A.V., H.R., W.T.F., S.M.H., and R.C. did the conception and design of the research; M.A.V., H.R., W.T.F., A.C., V.A., A.M., C.A., J.F., S.M.H., F.S., R.F., G.M., M.F.d.S., and R.C. performed the experiments; M.A.V., H.R., W.T.F., A.C., V.A., A.M., C.A., J.F., S.M.H., F.S., R.F., G.M., M.F.d.S., and R.C. analyzed the data; M.A.V., H.R., W.T.F., A.C., V.A., A.M., C.A., J.F., S.M.H., F.S., R.F., G.M., M.F.d.S., and R.C. interpreted the results of the experiments; M.A.V., H.R., W.T.F., S.M.H., M.F.d.S., and R.C. prepared the figures; M.A.V., H.R., W.T.F., S.M.H., and R.C. drafted the manuscript; M.A.V., H.R., W.T.F., S.M.H., M.F.d.S., and R.C. edited and revised the manuscript; M.A.V., H.R., W.T.F., A.C., V.A., A.M., C.A., J.F., S.M.H., F.S., R.F., G.M., M.F.d.S., and R.C. approved the final version of the manuscript.

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APÊNDICE C

Research Article

Sunflower Oil Supplementation Has Proinflammatory Effects and Does Not Reverse Insulin Resistance in Obesity Induced by High-Fat Diet in C57BL/6 Mice

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High consumption of polyunsaturated fatty acids, such as sunflower oil has been associated to beneficial effects in plasma lipid profile, but its role on inflammation and insulin resistance is not fully elucidated yet. We evaluated the effect of sunflower oil supplementation on inflammatory state and insulin resistance condition in HFD-induced obese mice. C57BL/6 male mice (8 weeks) were divided in four groups: (a) control diet (CD), (b) HFD, (c) CD supplemented with n-6 (CD + n-6), and (d) HFD supplemented with n-6 (HFD + n-6). CD + n-6 and HFD + n-6 were supplemented with sunflower oil by oral gavage at 2 g/Kg of body weight, three times per week. CD and HFD were supplemented with water instead at the same dose. HFD induced whole and muscle-specific insulin resistance associated with increased inflammatory markers in insulin-sensitive tissues and macrophage cells. Sunflower oil supplementation was not efficient in preventing or reducing these parameters. In addition, the supplementation increased pro-inflammatory cytokine production by macrophages and tissues. Lipid profile, on the other hand, was improved with the sunflower oil supplementation in animals fed HFD. In conclusion, sunflower oil supplementation improves lipid profile, but it does not prevent or attenuate insulin resistance and inflammation induced by HFD in C57BL/6 mice.

1. Introduction

Occidental diet is characterized by high caloric intake, mainly saturated fatty acids and glucose consumption, contributing to the development of obesity and insulin resistance. In the past 15 years, obesity has been associated to chronic inflammation in several tissues and cells, such as liver, adipose tissue, skeletal muscle, and immune cells.

In fact, chronic and subclinical “low-grade” inflammatory state is a hallmark of obesity, and this condition has been proposed to play a central role in the development of insulin resistance, type 2 *diabetes mellitus*, and steatosis [1]. During the past decades, the prevalence of obesity has

enormously increased worldwide [2]. A possible interaction factor between metabolic disorders and inflammation is the disruption in lipid metabolism caused by high levels of saturated fatty acids from high-fat and caloric diet [3, 4].

C57BL/6 mice develop obesity, insulin resistance (IR), *diabetes mellitus*, advanced fatty liver, and fatty pancreatic diseases when submitted to a high-fat diet (HFD), mainly enriched with saturated fatty acids [5]. The composition of fatty acids on diet can be an important modulator of lipid metabolism and inflammation. Some studies have showed that an increase in circulating levels of mono- or polyunsaturated fatty acids improves insulin sensitivity and ameliorates hepatic steatosis [6, 7]. Sunflower oil is rich in

mono- (MUFA) and polyunsaturated fatty acids (PUFA), mainly linoleic acid, a member of the n-6 family.

Diet enriched with sunflower oil decreases plasma triacylglycerol and has beneficial effects on plasma lipid profile [8]. Moreover, acute administration of sunflower oil in rats potentially prevents the gastric damages generated by indomethacin administration through increased anti-inflammatory response [9]. However, mice fed with sunflower oil-enriched diet present increased IL-6 (a pro-inflammatory cytokine) levels in white adipose tissue [8]. Thus, further studies are required to adequately determine the pro- or anti-inflammatory effects of n-6 fatty acids.

Adipose tissue, skeletal muscle, liver, and immune cells have been identified as critical targets for the disruption in metabolic and inflammatory signaling in obesity. The imbalance between metabolic alterations and inflammatory signaling pathways leads to insulin resistance, hepatic steatosis, and related diseases [4, 10].

In this study, we evaluated the effect of sunflower oil supplementation on inflammation and insulin sensitivity in mice fed a balanced diet and a HFD (rich in saturated fatty acids). The protecting or potentiating effect of sunflower oil supplementation on insulin resistance and inflammation was investigated. Whole body (Insulin Tolerance Test—ITT and Glucose Tolerance Test—GTT) and skeletal muscle (glucose uptake and metabolism) insulin sensitivity was examined. Inflammatory markers were investigated in liver, adipose tissue, skeletal muscle, and peritoneal macrophages. The strategy used was to investigate the effect of sunflower oil supplementation in mice fed a balanced diet and a HFD. Under this last condition, a clear inflammatory state is established and so a possible anti-inflammatory effect could be pronounced.

2. Materials and Methods

2.1. Animals. All animal studies were performed according to protocols approved by the Animal Care and Use Committee from the Institute of Biomedical Sciences, University of São Paulo. C57BL/6 male mice (8 weeks-old) were used for the study. Animals were housed in a room with light-dark cycle of 12-12 h and temperature of $23 \pm 2^\circ\text{C}$. Animals were divided in four groups: (a) control diet (CD), (b) high-fat diet (HFD), (c) control diet supplemented with n-6 (CD + n-6), and (d) high-fat diet supplemented with n-6 (HFD + n-6). During the first 4 weeks preceding the induction of obesity by HFD, all the four experimental groups were fed *ad libitum* with a control diet (76% carbohydrates, 9% fat, 15% proteins). CD + n-6 and HFD + n-6 were supplemented with sunflower oil (n-6 PUFA source) by oral gavage at 2 g per Kg of body weight, three times per week, during 12 weeks. This dosage of oil was chosen based on previous studies using different oils from our group and others [11–13]. CD and HFD received water at the same dose.

2.2. HFD-Induced Obesity and Insulin Resistance. After the first 4 weeks, animals from the HFD and HFD + n-6 groups received high-fat diet (26% carbohydrates, 59% fat, 15%

proteins) during the next 8 weeks. CD and CD + n-6 groups remained on the control diet. Supplementation with n-6 (sunflower oil, 2 g/Kg b.w.) was kept until the end of the 12 weeks.

2.3. Glucose and Insulin Tolerance Tests. Tolerances to glucose (GTT) and to insulin (ITT) were evaluated after 6 h fasting. For GTT, mice were intraperitoneally injected (i.p.) with glucose (2 g/Kg body weight). Blood glucose measurements were performed at 0, 15, 30, 45, 60, and 90 min after glucose injection. Glucose concentration versus time was plotted and the area under the curve (AUC) was calculated for each animal. For ITT, animals were i.p. injected with insulin (Humulin R, Lilly, 0.75 UI/kg b.w.) and glucose measurements were performed at 0, 10, 20, 30, 40, 50, and 60 min after injection. Glucose concentration versus time was plotted and the glucose lowering rate was calculated.

In both tests, blood samples were collected from the tail vein. For GTT, serum glucose was measured by colorimetric assay commercially available (PAP Liquiform Glucose, Labtest) and for ITT, glucose was measured by using glucometer (One Touch Ultra, Johnson & Johnson).

2.4. Serum Parameters Analysis. After 6 hours fasting, animals were anesthetized and blood was collected by puncturing the orbital plexus. Serum glucose, triacylglycerol, total cholesterol, LDL-cholesterol, and HDL-cholesterol were determined by colorimetric assays (Labtest Diagnostics, Lagoa Santa, MG, Brazil).

2.5. Responsiveness to Insulin in Isolated Soleus Muscle. Animals were euthanized by cervical dislocation and soleus muscles rapidly and carefully isolated, weighed (8–10 mg), attached to stainless steel clips to maintain resting tension, and preincubated for 30 min, at 37°C , in Krebs-Ringer bicarbonate buffer (KRBB) containing 5.6 mM glucose and 1% bovine serum albumin (BSA), pH 7.4, pregassed for 30 min with 95% O_2 /5% CO_2 , with agitation at 100 oscillations per min. After this period, muscles were transferred to other vials containing the same buffer, but added of $0.3 \mu\text{Ci/mL}$ D-[^{14}C]-glucose and $0.2 \mu\text{Ci/mL}$ 2-deoxy-D-[2,6- ^3H]-glucose. Phenylethylamine (0.2 mL), diluted 1:1 v/v in methanol, was added into a separate compartment for $^{14}\text{CO}_2$ adsorption. Incubation was then performed for 1 h under similar conditions, in the absence or presence of 7 nM insulin. Gasification with 95% O_2 /5% CO_2 was maintained during the preincubation and the first 15 min of the incubation period and then stopped.

After the incubation period, muscles were briefly washed in cold KRBB at 4°C , dried on filter paper and frozen in liquid N_2 . Samples were processed for measurements of uptake of 2-deoxy-D-[2,6- ^3H]-glucose, D-[^{14}C]-glucose incorporation, [^{14}C]-glycogen synthesis, and decarboxylation of D-[^{14}C]-glucose, according to methods described by Challiss et al. [14], Espinal et al. [15] and Leighton et al. [16], respectively, and routinely have been used by our group [11, 17–22].

TABLE 1: Obesity characteristics from mice fed with control diet (CD) or high-fat diet (HFD), supplemented or not with sunflower oil (rich in n-6 fatty acids).

	CD	HFD	CD + n6	HFD + n6
Body weight gain (g)	2.90 ± 0.42	11.30 ± 0.91*	3.99 ± 0.65	10.33 ± 0.50 [#]
Food ingestion (g/day/animal)	3.61 ± 0.091	2.44 ± 0.164*	4.53 ± 0.158*	2.45 ± 0.033 [#]
Food efficiency (body weight gain (g)/food ingestion (g))	0.014 ± 0.06	0.083 ± 0.022*	0.016 ± 0.008	0.075 ± 0.011 [#]
Epididymal adipose tissue (mg)	737.9 ± 89.4	1777.0 ± 234.3*	555.1 ± 45.5	1450.0 ± 176.8 [#]

Oral supplementation with sunflower oil rich in n-6 (2 g/Kg body weight, three times a week, oral gavage) or water, started four weeks before feeding or not with HFD, maintained until the end of experimental protocol. Animals were fed with HFD or CD for additional eight weeks. Mean ± SEM ($n = 8-10$). Data were analyzed by two-way ANOVA and Bonferroni post-test. * $P < 0.05$ versus CD; [#] $P < 0.05$ versus CD + n6.

2.6. Analysis of Inflammatory Parameters

2.6.1. Tissue Cytokine and Adipokine Content Measurements.

Mice were euthanized on CO₂ chamber and visceral adipose tissue (epididymal, retroperitoneal, and mesenteric), liver, gastrocnemius muscle, and peritoneal macrophages rapidly collected. About 100 mg of liver, gastrocnemius muscle and retroperitoneal adipose tissue were used for the determination of TNF- α , IL-6, and IL-10 content. Tissues were homogenized in RIPA buffer (0.625% Nonidet P-40, 0.625% sodium deoxycholate, 6.25 mM sodium phosphate, and 1 mM ethylenediamine tetra acetic acid at pH 7.4), containing 10 g/mL of a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Homogenates were centrifuged at 12,000 g for 10 min at 4°C, supernatant was collected, and protein concentration determined using Bradford assay (Bio-Rad, Hercules, CA, USA). Bovine serum albumin was used as a protein reference. Likewise, about 150 mg of epididymal adipose tissue were cultured in DMEM sterile medium (Gibco), containing 10% FBS, 2 mM glutamine and antibiotics for 24 h, at 37°C, 5% CO₂ humidified air environment. Thereafter, medium culture was collected and used for the determination of leptin, adiponectin, and resistin contents. For all determinations, we used colorimetric immunoassays ELISA (DuoSet kits, R&D System).

2.6.2. Peritoneal Macrophage Isolation and Culture. Cytokine and nitric oxide (NO) production was evaluated in macrophages obtained by washing the peritoneal cavity with 6 mL RPMI culture medium (Gibco), containing 10% FBS and 4 mM glutamine. Macrophage-rich cultures (more than 90% of the cells were F4/80⁺) were obtained by incubating peritoneal cells in 24-well polystyrene culture plates for 2 h at 37°C in a 5% CO₂, humidified air environment. Nonadherent cells were removed by washing with RPMI. Adherent cells were then incubated with 2.5 μ g/mL of LPS (*E. coli*, serotype 0111:B4, Sigma Chemical Company, USA) for 24 h [23]. Medium was collected for determination of IL-6, IL-10, and TNF- α by ELISA, and nitrite content by Griess method [24].

2.7. Statistical Analysis. Data are presented as mean ± SEM. All groups were compared by two-way ANOVA following Bonferroni posttests. $P < 0.05$ was considered to be significant.

3. Results

3.1. Exposure to HFD Induces Obesity Associated with Glucose and Insulin Intolerance. Animals fed with HFD for eight weeks showed increased (by 3.8 fold) body weight gain when compared to those fed with CD. Despite reduced food ingestion, the food efficiency of HFD was 6 fold higher than CD. Moreover, epididymal adipose tissues were increased by HFD. Sunflower oil did not change body weight gain, food efficiency, or adipose tissue increased (Table 1).

HFD increased fasted glucose in 18% (Table 2). An increase of 63% in area under the curve in glucose tolerance test (GTT) was also observed in animals treated with HFD, demonstrating glucose intolerance, which was associated with insulin intolerance, as observed by the reduction in glucose clearance during insulin tolerance test (ITT). Supplementation with sunflower oil maintained fasted glucose levels similar to CD. However, the supplementation did not prevent the glucose and insulin intolerance induced by HFD. Furthermore, sunflower oil supplementation induced insulin intolerance itself (CD + n-6 group), reducing the clearance of glucose similar to HFD without supplementation. Total cholesterol and LDL cholesterol levels were increased by HFD and sunflower oil supplementation in association with HFD showed protective effect in the prevention of the increased LDL cholesterol and cholesterol total levels found in HFD.

3.2. HFD Impaired Glucose Uptake and Metabolism in Soleus Muscle. Soleus muscles from animals fed with HFD did not respond to insulin stimulus in relation to glucose uptake and metabolism (Figure 1), characterizing muscle insulin resistance state. This effect was not prevented or reduced by sunflower oil supplementation. Interestingly, the supplementation itself (CD + n-6 group) increased basal glucose uptake and glucose oxidation, but it failed to respond to the insulin stimulus when compared to the control group (CD group).

3.3. HFD and Sunflower Oil Supplementation Induced Inflammation. Peritoneal macrophages stimulated with LPS showed increased production of nitrite, TNF α , IL6, and IL10 when compared with unstimulated cells (Figure 2). Macrophages from animals with obesity induced by HFD for 8 weeks showed a inflammatory profile, with increased production of nitrite, TNF- α , and IL-6, associated with decreased content of IL-10 when stimulated with LPS.

TABLE 2: Metabolic parameters from mice fed with control diet (CD) or high-fat diet (HFD), supplemented, or not with sunflower oil (rich in n-6 fatty acids).

	CD	HFD	CD + n6	HFD + n6
Fasted glucose (mg/dL)	186.1 ± 5.66	221.0 ± 8.38*	173.8 ± 4.75	196.8 ± 10.66
GTT test (AUC)	7987 ± 779.3	13090 ± 643.9*	7568 ± 1359	14790 ± 1731 [#]
ITT test (% glucose/min)	2.97 ± 0.19	2.13 ± 0.21*	1.94 ± 0.30*	1.98 ± 0.22
Total cholesterol (mg/dL)	148.2 ± 6.72	192.7 ± 11.96*	164.3 ± 14.00	166.3 ± 12.33
LDL cholesterol (mg/dL)	127.4 ± 5.15	165.1 ± 14.49*	128.2 ± 13.77	136.7 ± 13.22
HDL cholesterol (mg/dL)	14.73 ± 0.83	15.52 ± 1.86	19.33 ± 2.21	15.00 ± 1.15
Triglycerides (mg/dL)	68.61 ± 5.56	63.00 ± 4.40	84.70 ± 8.53	72.78 ± 8.71

Oral supplementation with n-6 (2 g/Kg body weight, three times a week, gavage) or water have started four weeks before HFD and continued until the end of experimental period. Animals were fed HFD or CD for eight weeks. GTT: glucose tolerance test; ITT: insulin tolerance test. Mean ± SEM ($n = 6-15$). ANOVA two-way with Bonferroni post tests. * $P < 0.05$ when compared with CD; [#] $P < 0.05$ when compared with CD + n6.

TABLE 3: Adipokine production in adipose tissue from mice fed with control diet (CD) or high-fat diet (HFD), supplemented, or not with sunflower oil (rich in n-6 fatty acids).

	CD	HFD	CD + n6	HFD + n6
Epididymal adipose tissue ^a				
Leptin	42.51 ± 8.75	81.38 ± 9.90*	62.42 ± 8.80	88.03 ± 10.96
Adiponectin	659.9 ± 39.94	697.6 ± 43.85	730.3 ± 76.68	649.8 ± 45.38
Resistin	625.1 ± 100.8	528.6 ± 71.65	913.0 ± 181.0	327.0 ± 42.15 [#]

Oral supplementation with n-6 (2 g/Kg body weight, three times a week, gavage) or water started four weeks before HFD and continued until the end of experiment. Animals were fed with HFD or CD for eight weeks. Approximately 150 mg of tissue were incubated in DMEM medium for 24 h and the supernatant were used for measures. Mean ± SEM ($n = 7-11$). ANOVA two-way with Bonferroni post tests. * $P < 0.05$ when compared with CD; [#] $P < 0.05$ when compared with CD + n6. ^aValues are expressed as pg/mL/mg tissue.

Interestingly, supplementation with sunflower oil induced similar inflammatory response in macrophages. For TNF- α only, the association of HFD and n-6 supplementation (HFD + n-6 group) showed beneficial response, decreasing this cytokine production.

In general, HFD induced and increase in the pro-inflammatory cytokine IL6 and decrease in the anti-inflammatory cytokine IL10 contents in the insulin target tissues (liver, skeletal muscle, and adipose tissue; Figure 3). Interesting, sunflower oil supplementation itself (CD + n-6 group) for 12 weeks increased the content of the proinflammatory cytokine IL6 in all tissues. However, when the sunflower oil was associated with HFD (HFD + n-6 group), cytokine concentrations were similar to that found in the HFD group, in liver and adipose tissue.

Adipokines production by epididymal adipose tissue was determined after 24 h culture. Adipose tissues from HFD animals showed increased production of leptin (Table 3). Resistin was reduced only when HFD was associated to sunflower oil supplementation (HFD + n-6 group) in comparison to CD + n6 group.

4. Discussion

This is the first study to investigate the effects of sunflower oil supplementation on inflammation and insulin sensitivity in mice fed a balanced diet and a HFD. We showed that HFD induces decreased glucose tolerance (as demonstrated

by GTT) and insulin sensitivity (as observed in ITT) in the whole body. Surprisingly, this last effect was also verified in the CD + n6 group. Moreover, proinflammatory response was increased in the HFD group without prevention or attenuation by sunflower oil supplementation. This effect was also observed in CD + n6 group. Lipid profile was impaired by HFD. Animals from HFD group showed an increase in total and LDL cholesterol plasma levels. These alterations were ameliorated by sunflower oil supplementation (HFD + n6 group).

HFD has been associated with insulin resistance and inflammatory condition, contributing to the development of several related diseases, such as obesity, diabetes mellitus type 2, metabolic syndrome, cancer, and cardiovascular diseases [25–27]. Various strategies have been investigated in order to decreasing insulin resistance and inflammation, including natural and modified compounds, cyclooxygenase inhibitors, and physical exercise [28–35]. Since skeletal muscle tissue represents 50–60% of the body weight and it is the main insulin-responsive tissue in association to liver and adipose tissue, it has been proposed that skeletal muscle dysfunctions (reduced oxidative capacity and impaired insulin sensitivity) play a central role in the abnormalities of chronic diseases [21, 36–40]. In this work, we investigated the effect of sunflower oil supplementation on insulin sensitivity (in whole animal *in vivo* and in soleus muscle *in vivo*) and inflammatory condition (liver, skeletal muscle, adipose tissue) in mice fed a balanced diet and a HFD. In recent study, Bjerme et al. [41] showed that isocaloric diet with n-6

FIGURE 1: Effect of sunflower oil supplementation (rich in n-6 fatty acids) on glucose uptake and metabolism in isolated soleus muscle from C57BL/6 mice. The muscles were incubated in Krebs Ringer buffer, containing 5.6 mM glucose, 0.3 $\mu\text{Ci/mL}$ of $[\text{U-}^{14}\text{C}]$ -D-glucose and 0.2 $\mu\text{Ci/mL}$ of 2-deoxy-[2,6- ^3H]-D-glucose, in the absence (white bars) or presence (black bars) of 7 nM insulin, for one hour. At the end of the incubation period, muscles were proceeded to analyze glucose uptake and metabolism as described in Materials and Methods. Results are presented as mean $\pm$ SEM ($n = 10$ –16). Two-way ANOVA and Bonferroni posttest were used for statistical analysis. § $P < 0.05$ versus the insulin stimulated (+insulin) of the same group; * $P < 0.05$ versus CD with insulin; # $P < 0.05$ versus CD + n6 with insulin.

PUFA for 10 weeks reduce liver fat and modestly improves metabolic conditions, without weight loss in humans. In addition, it has been proposed that n-6 PUFA reduce risks of cardiovascular diseases, by improving lipid profile [42]. This improvement in lipid profile was found in our study, whereas HFD + n6 group showed decreased plasma LDL and total cholesterol levels.

Some studies have shown that n-6 PUFA intake positively correlate with obesity and insulin resistance [43, 44]. Treatment with subcutaneous injection of sunflower oil by 7 days in *Wistar* rats impaired insulin sensitivity by decreasing glucose clearance during ITT and GLUT-4 expression and translocation in white adipose tissue (WAT), with no effect in skeletal muscle [7]. In the CD + n6 group, KiTT was

reduced, but glucose uptake and metabolism in isolated soleus muscles were not altered after insulin stimulus. These results suggest that sunflower oil supplementation *per se* is not able to impair insulin response in skeletal muscle, but it possibly reduces total insulin sensitivity by decreasing the response to the hormone in other tissues, such as liver and adipose tissue. This proposition has to be investigated in further studies. In addition, both groups fed with HFD (HFD and HFD + n6 groups) showed skeletal muscle resistance to insulin, showing no beneficial effects of n-6 PUFA on peripheral insulin sensitivity.

Obesity is accompanied by a chronic low grade subclinical inflammation promoted by an increase in fuel availability, leading to infiltration of macrophages into adipose tissue.

FIGURE 2: Nitric oxide and cytokine production in peritoneal macrophages collected from mice fed with control diet (CD) or high-fat diet (HFD), supplemented or not with sunflower oil (rich in n-6 fatty acids, 2 g/Kg b.w., three times a week, by oral gavage). Animals were fed with HFD or CD for eight weeks. Peritoneal macrophages were collected and cultured for 24 h in the absence (white bars) or presence (black bars) of 2.5 μ g/mL LPS. Nitric oxide (a), TNF- α (b), IL-6 (c), and IL-10 (d) were measured as described in Material and Methods. Total number of cells in peritoneal exudates was not different among the groups (data not shown). Data are presented as mean $\pm$ SEM ($n = 5-10$). The CD + n6 group had just 5 samples up to the detection limit in the TNF- α assay. Two-way ANOVA and Bonferroni post-test were used for statistical analysis. All LPS stimulated conditions were different from unstimulated; * $P < 0.05$ versus CD with LPS; # $P < 0.05$ versus CD + n6 with LPS; ° $P < 0.05$ versus HFD with LPS.

This condition results in enhanced inflammatory response, which stimulates the production of cytokines by adipose tissue [45]. Moreover, in obesity, increased lipotoxicity caused by high nonesterified fatty acids (NEFA) available, and triacylglycerol content leads to an increase in inflammatory markers and cytokine production in multiple organs [46, 47]. NEFA increases inflammatory response by activating TLR-4 [48]. HFD increased inflammatory response in macrophages, showing high content of IL-6, TNF- α , and NO, and low content of IL-10 (anti-inflammatory cytokine)

when compared to the control group. In peripheral tissues, IL-6 content was increased in the liver and gastrocnemius muscle whereas IL-10 content was decreased in liver and retroperitoneal adipose tissue. Adipose tissue showed increased production of leptin in the HFD and HFD + n6 groups, compared with mice fed with control diet, and resistin was reduced in the HFD + n6 group compared with CD + n6, in epididymal adipose tissue. Whereas in retroperitoneal adipose tissue, IL-6 was elevated in the HFD and CD + n6 groups compared with CD, IL-10 was reduced

FIGURE 3: Tissue cytokine concentrations of TNF α (a), IL6 (b), and IL10 (c) in adipose tissue (retroperitoneal), liver, and gastrocnemius skeletal muscle from mice treated with control diet (CD) or high-fat diet (HFD), supplemented or not with sunflower oil (rich in n-6 fatty acids). Oral supplementation with n-6 (2 g/Kg body weight, three times a week, gavage) or water started four weeks before HFD and continued until the end of experiment. Animals were feed with HFD or CD for eight weeks. Cytokines were measured in tissue homogenate. Values are expressed as pg/mg protein. Mean $\pm$ SEM ($n = 4-6$). The statistical analysis was performed using two-way ANOVA with Bonferroni post tests. * $P < 0.05$ when compared with CD; # $P < 0.05$ when compared with CD + n6.

in the HFD and CD + n6, and adiponectin was reduced only in CD + n6. These results showed the inflammatory characteristics the sunflower oil supplementation and the high fatty diet fed.

Our results showed that sunflower oil supplementation causes a clear pro-inflammatory response in mice fed on control diet and it does not have additive or prophylactic effects in mice fed on HFD. Fatty acid effects on immune system have been studied since 1970's decade [49]. Linoleic acid is an essential fatty acid that leads to inflammatory response by increasing arachidonic acid production, a fatty acid related to elevated pro-inflammatory mediator generation [50]. High intake of linoleic acid has been associated to the development of chronic diseases in several studies [51, 52]. However, few studies have been carried out on the direct effect of this fatty acid on inflammatory markers [53].

In conclusion, our results showed that sunflower oil supplementation has pro-inflammatory effects in macrophages and insulin-sensitive peripheral tissues and induced insulin resistance *in vivo* and *in vitro*. These effects were demonstrated in mice fed a balanced diet and pronounced in mice fed a HFD. Interestingly, sunflower oil supplementation partially prevented the dislipidemia found in mice submitted to HFD, but it did not improve or even worsened inflammatory state and insulin resistance induced by this diet.

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APÊNDICE D

Fish oil supplementation for two generations increases insulin sensitivity in rats

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Abstract

We investigated the effect of fish oil supplementation for two consecutive generations on insulin sensitivity in rats. After the nursing period (21 days), female rats from the same prole were divided into two groups: (a) control group and (b) fish oil group. Female rats were supplemented with water (control) or fish oil at 1 g/kg body weight as a single bolus for 3 months. After this period, female rats were mated with male Wistar rats fed on a balanced chow diet (not supplemented). Female rats continued to receive supplementation throughout gestation and lactation periods. The same treatment was performed for the next two generations (G1 and G2). At 75 days of age, male offspring from G1 and G2 generations from both groups were used in the experiments. G1 rats did not present any difference with control rats. However, G2 rats presented reduction in glycemia and lipidemia and improvement in *in vivo* insulin sensitivity (model assessment of insulin resistance, insulin tolerance test) as well as *in vitro* insulin sensitivity in soleus muscle (glucose uptake and metabolism). This effect was associated with increased insulin-stimulated p38 MAP kinase phosphorylation and lower n-6/n-3 fatty acid ratio, but not with activation of proteins from insulin signaling (IR, IRS-1 and Akt). Global DNA methylation was decreased in liver but not in soleus muscle. These results suggest that long-term fish oil supplementation improves insulin sensitivity in association with increased insulin-stimulated p38 activation and decreased n-6:n-3 ratio in skeletal muscle and decreased global DNA methylation in liver.

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Keywords: Omega-3; Dietary intervention; Glucose metabolism; Insulin resistance; Skeletal muscle

1. Introduction

Evidence suggests that omega-3 (n-3) polyunsaturated fatty acids (PUFAs), mainly eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, have positive impact on health in humans and animals models. These fatty acids have been suggested to provide beneficial effects on several disorders such as dyslipidemias, cardiovascular diseases, hypertension, insulin resistance, cancer, type 2 diabetes mellitus (DM2) and metabolic syndrome [1–5]. Lack of n-3 PUFA in the diet has been related to increased risk for DM2 and cardiovascular diseases [6,7], while dietary enrichment with n-3 PUFA has been associated with improved insulin sensitivity and reduced risk factors for several diseases [8,9].

Diet enrichment with fish oil prevents insulin resistance and obesity induced by a high-fat diet [10–16], a high-sucrose diet [17–23], both [24–26] and tumor necrosis factor (TNF) infusion [27]. In addition, dietary enrichment with n-3 PUFA prevents or reduces insulin

resistance and/or cardiovascular risks in animal models of obesity [28,29], metabolic syndrome [30,31] and diabetes [32], as well as in humans with overweight [33–35], obesity [36], diabetes [37,38] and aging [39,40]. However, in some studies, n-3 PUFA had no effect or even impaired insulin sensitivity and/or cardiovascular risk factors in obese Zucker rats [41], diet-induced obese humans [42] and diabetic patients [37,43,44]. Differences are probably due to variations in the experimental model or design, treatment time and n-3 PUFA dose, for example.

Supplementation with n-3 PUFA has several effects on diet-induced obese animals and obese humans, including the following: (a) reduction in body weight, adiposity, total body fat and lipolysis [16,23,30]; (b) reduction in glucose plasma concentration, insulin, lipids (triacylglycerol, total cholesterol and nonesterified fatty acids) and in some inflammatory markers (tumor necrosis factor- α [TNF- α], interleukin-6 [IL-6], C-reactive protein [CRP], and plasminogen activator inhibitor-1 [PAI-1]) [26,34,37,45,46]; (c) improvement in the glucose tolerance test, insulin sensitivity [insulin tolerance test (ITT), homeostasis model assessment of insulin resistance (HOMA-IR)] and insulin signaling (insulin binding, Akt, ERK) [25,29,47,48]; (d) decrease in hepatic steatosis and lipid accumulation and their derivatives in liver, skeletal muscle and pancreas [20,28,49,50]; (e) improvement in plasmatic levels of adipokines (leptin, adiponectin

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and visfatin) [2,51,52]; (f) increase in the n-3 fatty acid derivatives in the phospholipids (hepatocytes, adipocytes and myocytes) [4,11,13,24,36,47,53,54]; (g) modulation of gene expression towards glucose metabolism (glucose transporter-4 [GLUT-4]), lipid oxidation (carnitine palmitoyltransferase-1 [CPT-1], acyl-CoA oxidase [ACO], and uncoupling protein-3 [UCP-3]) and hepatic glucose production (glucose-6-phosphatase [G6Pase], malic enzyme, glucose-6-phosphate dehydrogenase [G6PDH]), and phosphoenolpyruvate carboxykinase [PECK]), rather lipid accumulation (decreased acetyl-CoA carboxylase [ACC], fatty acid synthase [FAS], sterol regulatory element-binding protein-1 [SREBP-1], and nuclear factor Y [NFY]) [5,14,22,31,32,55–57]; (h) reduction in other cardiovascular risks (reduced blood pressure and platelet aggregation, improved endothelial function) [9,33,39] and (i) increase in resolvins, protectins and maresins and decrease in proinflammatory eicosanoids [58–61].

Studies in healthy animals or humans, however, have failed to show beneficial effects of fish oil supplementation on insulin sensitivity [1,42,62]. In addition, the mechanisms involved are not completely known yet. Thus, the aim of this work was to evaluate the long-term effect of fish oil supplementation for two generations on insulin sensitivity in rats by measuring the following parameters: (a) glucose uptake and metabolism in incubated soleus muscle; (b) plasma lipid [free fatty acids, triacylglycerol, total cholesterol, low-density lipoprotein (LDL) cholesterol and high-density lipoprotein (HDL) cholesterol] levels; (c) ITT; (d) insulin signaling pathways (insulin receptor, IRS-1, Akt and p38 MAP kinase); (e) translocation of GLUT-4 from cytoplasm to plasmatic membrane and (f) fatty acid profile in skeletal muscle. We hypothesized that n-3 PUFA supplementation has effect on healthy animals only after a long period of supplementation.

2. Materials and methods

2.1. Materials

Enzymes and reagents for all assays were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Regular insulin was obtained from Eli Lilly and Company (Indianapolis, IN, USA). D-[U-¹⁴C]-glucose, 2-deoxy-D-[2,6-³H]-glucose and ECL Western Blotting System Kit were obtained from Amersham International Plc. (Bucks, UK). L-[1-¹⁴C]-glucose was obtained from NEN Life Sciences Products, Inc. (Boston, MA, USA). Anti-phosphoserine (473)-Akt, anti-phosphothreonine (308)-Akt and anti-phosphotyrosine (182)-p38 MAP kinase antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-phosphotyrosine, anti-insulin receptor- β and anti-IRS-1 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-GLUT-4 antibody was from Abcam (Cambridge, UK). Anti-IgG polyclonal antibody conjugated to horseradish peroxidase was obtained from ICN Biomedicals, Inc. (Irvine, CA, USA). Fish oil (obtained from cold sea fishes) was generously donated by Naturalis Nutrition and Pharma Ltd. (São Paulo, Brazil). Standard chow diet containing 4% fat was obtained from Nuvital Nutrients Ltd. (Curitiba, Brazil). Total fatty acid composition of the standard chow diet and fish oil was determined by high-performance liquid chromatography as previously described (Shimadzu model LC-10A, Shimadzu, Kyoto, Japan). Fish oil used in this study was composed by lauric (0.5%), myristic (11.4%), palmitic (14.2%), palmitoleic (0.9%), stearic (3.1%), oleic (8.7%), linoleic (0.5%), γ -linolenic (1.7%), arachidonic (12.1%), EPA (26.0%) and DHA (20.2%). Fatty acids from standard chow diet were composed by myristic (0.2%), palmitic (15.6%), stearic (4.2%), oleic (27.2%), linoleic (49.0%) and linolenic acid (2.9%).

2.2. Animals

Wistar rats were obtained from the Department of Physiology, Biological Sciences Building, Federal University of Paraná, Curitiba, Brazil. The animals were maintained at 12:12-h light–dark cycle, 23°C $\pm$ 1°C, and received a standard diet for rats containing 52% carbohydrates, 22% proteins and 4% lipids (CR-1, Nuvital-Nuvital Nutrients Ltd., Paraná, Brazil) and water *ad libitum*. The chow was removed 6 h before the beginning of the experiments, which was always carried out at 02:00 p.m. The Brazilian Animal Ethics Committee approved this study.

2.3. Study design

After the nursing period (21 days), female rats from the same prole were divided into two groups: (a) control group supplemented with water and (b) experimental group supplemented with fish oil. Both supplementations were daily and orally given at 1 g/kg body weight as a single bolus. Female rats were supplemented for 3 months [generation 0 (G0)]. After this period, female rats were mated with male Wistar rats fed

on a normal chow diet (not supplemented). The female rats continued to receive supplementation throughout gestation and lactation periods. The same treatment was performed for the next two generations (G1 and G2).

At 75 days of age, male offspring from G1 and G2 generations from both groups (control and fish oil) were used in the experiments. Body weight was determined every 2 days for adjustment of the fish oil supplementation and water administration. Daily food intake was measured in the last week of the experimental period.

2.4. Glucose uptake and metabolism in isolated soleus muscle

Soleus muscles were isolated and incubated as previously described [63,64]. The chow was removed 6 h before the beginning of the experiment, which was always carried out at 2:00 p.m. The rats were killed by cervical dislocation, and the muscles were rapidly and carefully isolated, split longitudinally in portions weighing 25–35 mg and preincubated, at 35°C, in Krebs–Ringer bicarbonate buffer containing 5.6 mM glucose, pH 7.4, pregassed for 30 min with 95% O₂/5% CO₂, with agitation at 100 oscillations/min for 30 min. Subsequently, the muscles were transferred to the same buffer with 0.3 μ Ci/ml D-[U-¹⁴C]glucose and 0.2 μ Ci/ml 2-deoxy-[2,6-³H]D-glucose and incubated under similar conditions for 1 h in the absence or presence of 0, 100, 1000 or 10,000 μ U/ml insulin. Phenylethylamine, diluted 1:1 v/v in methanol, was added into a separate compartment for ¹⁴CO₂ adsorption. After the incubation period, the muscles were briefly washed in saline at 4°C and frozen in liquid N₂. [¹⁴C]-glycogen synthesis (as estimated by D-[1-¹⁴C]-glucose incorporation into glycogen) was determined as described by Leighton and Cooper [65] and in our previous studies [66–68]. Lactate production was determined as described by Engel and Jones [69]. The decarboxylation of D-[1-¹⁴C]-glucose and the uptake of 2-deoxy-D-[2,6-³H]-glucose were measured as previously described [70–72]. For determination of the extracellular space, some muscles were incubated in the presence of 0.1 μ Ci/ml L-[1-¹⁴C]-glucose [72,73].

2.5. Determination of blood metabolites

Serum glucose concentration was measured by a glucose-oxidase-based assay kit as described by Trinder [74] at 505 nm. Serum cholesterol, high density lipoprotein (HDL) cholesterol and triacylglycerol (TAG) concentrations were determined as previously described [75,76] at 500 nm (cholesterol) and 540 nm (TAG). Serum lactate was assayed as described by Engle and Jones [69]. Insulin was measured by radioimmunoassay as previously described [77].

2.6. Insulin tolerance test

Insulin tolerance test was performed as previously described [78]. Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg body weight, intraperitoneal injection). Two international units of insulin per kilogram of body weight was intraperitoneally injected. Blood was collected from tail vein at 0 (basal), 5, 10, 15, 20, 25 and 30 min thereafter. Plasma was then separated, and serial glucose concentration of each animal was determined. Glucose disappearance rate (K_{ITT}) was calculated using the formula $0.693/t_{1/2}$. The glucose $t_{1/2}$ was calculated from the slope of the least square analysis of blood glucose concentration during the linear phase of decline [79].

2.7. Liver and muscle glycogen contents

Glycogen content of the liver, soleus and gastrocnemius muscles was measured by digesting 50–75 mg in 0.5 ml KOH solution (1 M) for 20 min at 70°C. After that, triethanolamine buffer and amyloglucosidase were added to the samples and then incubated for 2 h at 25°C. Samples were centrifuged at 800 g for 5 min, and the supernatant was added to glucose assay buffer [80]. Glycogen content was quantified by measuring the absorbance at 340 nm.

2.8. Fatty acid composition

Total lipid was extracted from gastrocnemius muscles according to Folch et al. [81] using chloroform–methanol (2:1), and fatty acid profile was determined by high-performance liquid chromatography [82]. The fatty acids were saponified using 2 ml of an alkaline methanol solution (1 mol/L NaOH in 90% methanol) at 37°C for 2 h in a shaking water bath. Afterwards, samples were acidified to pH 3 with HCl solution (1 mol/L). Fatty acids were then extracted 3 times with hexane and derivatized with 4-bromomethyl-7-coumarin, and the analysis was performed in a Shimadzu LC-10 high-performance liquid chromatograph using a C8 column (2.5 cm $\times$ 4.6 mm i.d.; particle size of 5 μ m) with a C8 pre-column (25 cm $\times$ 4.6 mm i.d.; particle size 5 μ m). Fatty acids were resolved using a mobile phase of acetonitrile–water (gradient from 77:23, vol/vol) at 1-ml/min flow rate and detected by fluorescence (325 nm, excitation; 395 nm, emission) [83]. The following fatty acids (Sigma-Aldrich, St. Louis, MO, USA) were used as standards: lauric (C12:0), myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1 n6), stearic (C18:0), oleic (C18:1 n9), linoleic (C18:2 n6), linolenic (C18:3 n6), arachidonic (C20:4), eicosapentaenoic (C20:5 n3), docosahexaenoic (C22:6 n3) and margaric (C17:0) acids. A calibration curve was established for each fatty acid standard in order to obtain correlation and regression coefficients. Limit for detection ranged from 1 to 10 ng of the fatty acids.

2.9. Insulin signaling in isolated soleus skeletal muscle

Muscles were incubated for 5–15 min in the absence or presence of 7 nM insulin. This period of insulin stimulation is sufficient to cause maximal phosphorylation of the proteins studied (data not shown). At the end of the incubation period, muscles were immediately homogenized in 0.6 ml extraction buffer (100 mM Trizma, pH 7.5; 10 mM EDTA; 10% SDS; 100 mM NaF; 10 mM sodium pyrophosphate; 10 mM sodium orthovanadate; at 100°C) for 30 s. Samples were boiled for 5 min and centrifuged at 13,000 g for 40 min at 4°C. Aliquots of supernatants were used for the measurement of total protein content, as described by Bradford [84]. Equal amounts of proteins of each sample were separated by using SDS gel polyacrylamide [85]. Western blotting was carried out following the method described by Towbin et al. [86]. The proteins of the gel were transferred to a nitrocellulose membrane at 120 V for 1 h. Nonspecific bounds were blocked by incubating the membranes with 5% defatted milk in basal solution (10 mM Trizma, pH 7.5; 150 mM NaCl; 0.05% Tween 20) at room temperature for 2 h. Membranes were washed in basal solution three times for 10 min each and then incubated with anti-phosphotyrosine, anti-IRS-1, anti-phosphoserine (473)-Akt, anti-phosphothreonine (308)-Akt or anti-phosphotyrosine (182)-p38 MAP kinase antibodies in basal solution containing 3% defatted milk at room temperature for 3 h. Membranes were washed again (three times for 10 min each) and incubated with anti-IgG antibody linked to horseradish peroxidase in basal solution containing 1% defatted milk at room temperature for 1 h. Following the washings, membranes were incubated with substrate for peroxidase and chemiluminescence enhancer (Amersham Pharmacia Biotech) for 1 min and immediately exposed to X-ray film for 1–20 min. Films were then revealed on the conventional manner.

In immunoprecipitation studies, muscle lysates were incubated with anti-insulin receptor- β or anti-IRS-1 (0.5 mg/ml) antibodies for 2 h and then incubated with protein A Sepharose for a further 2 h. Beads were then washed with Tris containing 1% Triton X-100, boiled into Laemmli buffer for 5 min and submitted to Western blotting analysis as described above using anti-phosphotyrosine antibody.

2.10. GLUT-4 translocation

GLUT-4 translocation was performed as previously described [87]. Rats were anesthetized with sodium pentobarbital (50 mg/kg body weight, intraperitoneal

injection). Two international units of insulin per kilogram of body weight was intraperitoneally injected. Hindlimb muscles were collected at 0 (basal), 10, 20 and 30 min thereafter and immediately frozen in liquid N₂. About 1.5 g of each gastrocnemius muscle was homogenized in 15 ml Trizma-EDTA-sucrose buffer (10 mM Trizma, 1 mM EDTA, 250 mM sucrose). The samples were centrifuged at 760 g at 4°C for 10 min, and the supernatants were separated. The pellets were homogenized in 5 ml Trizma-EDTA-sucrose buffer and centrifuged as described above. The two supernatants were combined and centrifuged at 31,000 g at 4°C for 60 min. The resultant pellet (P1) was maintained on ice. The resultant supernatant was centrifuged at 190,000 g at 4°C for 60 min. The resultant pellet (P2) and P1 were then suspended in 0.3 ml Trizma-EDTA-sucrose buffer. Aliquots were used for total protein quantification as described by Bradford [84] using albumin as standard. Equal amounts of proteins of each sample were used for Western blotting analysis of GLUT-4 as described above. Translocation ratio was calculated by dividing the content of microsomal GLUT-4 by the plasma membrane GLUT-4.

2.11. Global DNA methylation

Total DNA methylation was investigated in soleus skeletal muscle and liver. Extraction of genomic DNA from liver and soleus skeletal muscle was performed as previously described [88]. Briefly, samples were homogenized in extraction buffer (0.1 mM NaCl, 20 mM Trizma, 25 mM EDTA, 0.5% SDS and 1 mg/ml proteinase K) and incubated at 55°C for 24 h, with mixing at 500 oscillations/min. After that, DNA was purified using phenol:chloroform:isoamyl alcohol (25:24:1, v/v) following precipitation with ethanol. Purified DNA pellet was resuspended in extraction buffer, and final DNA concentration and quality were determined at 260/280 nm.

Quantification of global DNA methylation from 100 ng of each sample was performed using the Imprint Methylated DNA Quantification Kit from Sigma-Aldrich (St. Louis, MO, USA) according to the instructions of the manufacturer's instructions.

2.12. Statistical analysis

The results are presented as mean $\pm$ S.E.M. and were analyzed by one-way analysis of variance followed by the Tukey posttest to evaluate the insulin response in soleus muscle (glucose uptake and metabolism) or by Student's *t* test for comparison between two groups. *P* < .05 was taken to indicate statistical significance.

Fig. 1. Effect of fish oil supplementation during two consecutive generations on insulin-stimulated glucose uptake (A) and metabolism (B–D) in skeletal muscle of Wistar rats. Values are presented as mean $\pm$ SEM. G1, generation 1; G2, generation 2. **P* < .05, ***P* < .01 and ****P* < .001 when compared to the respective control group.

Table 1

Body weight, food intake and glycogen content in the liver (LG), soleus (SG) and gastrocnemius (GG) muscles from control and fish oil supplemented rats for one (G1) or two generations (G2)

	Control	G1	G2
Body weight (g)	368.1±9.04	393.6±17.88	375.0±16.65
Food intake (g/day)	25.5±1.60	26.0±2.20	27.4±1.30
Plasma lactate (mM)	1.32±0.104	1.28±0.124	1.10±0.067
LG (μmol/g of tissue)	131.1±6.4	131.6±6.3	135.0±6.9
SG (μmol/g of tissue)	47.8±2.8	45.8±3.2	46.8±2.6
GG (μmol/g of tissue)	54.3±2.3	54.8±3.2	55.3±2.9

Values are presented as mean±S.E.M.

3. Results

3.1. Fish oil supplementation for two generations increased insulin-stimulated glucose uptake and metabolism in skeletal muscle

No difference was found in muscle glucose uptake and metabolism of control rats from G1 and G2 groups (data not shown). Supplementation with fish oil for one generation (G1) also did not alter these parameters (Fig. 1). However, when the supplementation was

prolonged for two generations (G2), there was an increase in insulin-stimulated glucose uptake and metabolism (Fig. 1). Since any significant effect was observed in G1 rats, further determinations were carried out in G2 rats only in order to find out the mechanisms involved.

3.2. Fish oil supplementation for two generations did not alter body weight, food intake and glycogen storage

Body weight and food intake were not altered by the supplementation with fish oil for two generations (Table 1). Glycogen content in the liver and muscles (soleus and gastrocnemius) was not also modified by the fish oil supplementation (Table 1).

3.3. Fish oil supplementation for two generations increased insulin sensitivity

Glycemia was reduced and insulinemia was not modified by fish oil supplementation for two generations. Insulin sensitivity, as indicated by R_{HOMA} (insulin resistance index), HOMA-IR (insulin

Fig. 2. Effect of fish oil supplementation during one (G1) or two generations (G2) on plasma lipid concentration in Wistar rats. Values are presented as mean±S.E.M.

sensitivity associated to insulin secretion) [89] and K_{ITT} (plasma glucose disappearance rate), was significantly increased by the fish oil supplementation (Fig. 2).

3.4. Blood lipid metabolite concentrations in G2 rats

Blood concentration of HDL cholesterol was not altered in the fish oil group. However, the treatment decreased plasma concentrations of free fatty acids, triacylglycerol, total cholesterol and LDL cholesterol (Fig. 3).

3.5. Fish oil supplementation for two generations had no effect on insulin receptor, IRS-1 and Akt phosphorylation, but increased p38 MAP kinase phosphorylation in soleus muscle

The effect of fish oil supplementation for two generations on content and maximal phosphorylation of insulin receptor, IRS-1, Akt and p38 MAP kinase is shown in Figs. 4 and 5. The content of all proteins was not modified by fish oil supplementation. Maximal phosphorylation of insulin receptor (phosphotyrosine), IRS-1 (phosphotyrosine) and Akt (phosphoserine 473 and phospho-

threonine 308) was not altered by fish oil (Figs. 4 and 5). However, insulin-stimulated p38 MAP kinase phosphorylation (tyrosine 182) was significantly increased by the supplementation (Fig. 5).

3.6. Fish oil supplementation increased insulin-stimulated GLUT-4 translocation in G2 rats

Translocation of GLUT-4 from microsomal vesicles to the plasma membrane was significantly increased by the fish oil supplementation for two generations as compared to the control group in all periods evaluated (10, 20 and 30 min) (Fig. 6).

3.7. Fish oil supplementation decreased n-6/n-3 polyunsaturated fatty acid ratio in skeletal muscles from G2 rats

Supplementation with fish oil for two generations increased n-3 PUFA content and decreased n-6 PUFA content in the skeletal muscle, resulting in a decreased n-6:n-3 ratio (Fig. 7).

Fig. 3. Effect of fish oil supplementation during two generations (G2) on plasma glucose (A) and insulin (B) levels, R_{HOMA} (C), HOMA-IR and K_{ITT} in Wistar rats. Values are presented as mean ± S.E.M.

Fig. 4. Effect of fish oil supplementation during two generations (G2) on total and tyrosine phosphorylated content (basal and insulin stimulated) of insulin receptor (IR) and insulin receptor-1 (IRS-1) in skeletal muscle of Wistar rats. Values are presented as mean±S.E.M.

Fig. 5. Effect of fish oil supplementation during two generations (G2) on total and phosphorylated content (basal and insulin stimulated) of Akt (phosphoserine 473 and phosphothreonine 308) and p38 in skeletal muscle of Wistar rats. Values are presented as mean±S.E.M.

Fig. 6. Effect of fish oil supplementation during two generations (G2) on the ratio of microsomal (M) and plasma membrana (PM) content of glucose transporter-4 (GLUT-4) in skeletal muscle of Wistar rats. Values are presented as mean $\pm$ S.E.M. * $P < .05$ and ** $P < .01$ when compared to the control group at the respective time.

3.8. Fish oil supplementation decreased global DNA methylation in liver but did not alter it in skeletal muscles from G2 rats

Global DNA methylation was significantly decreased in liver from G2 rats by 50% ($P < .05$). However, this parameter was not modified in soleus skeletal muscle (Fig. 8).

4. Discussion

The long-term effects of fish oil supplementation were evaluated in rats during two consecutive generations. We demonstrated that only fish oil supplementation for two generations increased systemic insulin sensitivity (R_{HOMA} , HOMA-IR and K_{ITT}) and insulin response in incubated soleus muscle (glucose uptake and metabolism, GLUT-4 translocation and p38 phosphorylation induced by insulin), and decreased n-6:n-3 PUFA ratio in skeletal muscle. These effects were

Fig. 8. Effect of fish oil supplementation during two consecutive generations on global DNA methylation in liver (A) and skeletal muscle (B) of Wistar rats. Values are presented as mean $\pm$ S.E.M. G1, generation 1; G2, generation 2.

associated with improved plasma lipid profile (reduced FFA, TAG, total and LDL cholesterol levels), decreased fasting glycemia and reduced global methylation in liver (but without effect in soleus muscle). Alterations in insulin-stimulated glucose uptake and metabolism in soleus muscle were not observed in G1 rats, suggesting

Fig. 7. Effect of fish oil supplementation during two generations (G2) on phospholipid composition in skeletal muscle of Wistar rats. Values are presented as mean $\pm$ S.E.M.

that the beneficial effects of fish oil on insulin sensitivity occur only when this supplementation is prolonged for two generations.

In previous studies, we reported several beneficial effects of long-term fish oil supplementation on Walker 256 tumor-bearing rats such as decreased plasma levels of lipids and inflammatory markers, as well as normalization of leukocyte function [90,91]. Thus, beneficial effects of long-term fish oil supplementation on Walker 256 tumor-bearing rats can be explained, at least in part, by the insulin sensitive effects on skeletal muscle, reducing cachexia development.

The effects of fish oil supplementation on skeletal muscle from healthy individuals or rodents remain controversial. Several studies have failed to demonstrate positive effects of fish oil [37,41–44], whereas others have found marked changes of glucose metabolism and protein synthesis in skeletal muscle [92,93]. However, the effects are evidenced when fish oil is administered in insulin-resistant models, e.g., obesity induced by high-fat and/or high-sucrose diet, experimental type 2 *diabetes mellitus* and genetic models of metabolic syndrome, *diabetes mellitus* and obesity [1–5]. This is the first study to show a direct effect of a long-term fish oil supplementation on insulin response in skeletal muscle from healthy animals. Differently to the previous studies that usually evaluated the short-term effects of fish oil (up to 12 weeks), in the present study, we used animals for two generations. Effects of fish oil were not observed before this period (at the first generation), suggesting that fish oil has positive effects in healthy animals only after a prolonged period of time. These results can, at least in part, help to explain the findings from epidemiological studies that show low incidence of chronic diseases related to insulin resistance in populations with chronic n-3 PUFA consumption in their diet [1].

The mechanisms involved in the beneficial effects of n-3 PUFA are not completely known, but several propositions have been postulated, including the following: (a) activation of transcriptional factors (PPAR α , LXR2 and SREBP-1) in the liver, decreasing lipogenesis and raising fatty acid oxidation [14,22,56]; (b) activation of PPAR- γ and GPR120 signaling, and inhibition of TLR-4 and TNF- α pathways (adipocytes and macrophages), decreasing inflammation state [46,94,95]; (c) increase in AMPK phosphorylation and mitochondrial biogenesis (adipocytes), leading to an augmentation in fatty acid oxidation [2,96]; (d) reduction of lipid derivatives (DAG, TAG, ceramides, lysophosphatidylcholines, acyl-CoAs) production, reducing lipotoxicity and improving the activation of signaling pathways involved in insulin resistance development [21,50]; and (e) decrease of n-6/n-3 PUFA ratio in phospholipids, favoring insulin binding and signaling in target tissues [4,11,13,22,24,25,47,48].

Activation of p38 MAP kinase pathway has been demonstrated to be involved in the insulin-stimulated glucose uptake [97] and to be activated by several insulin-mimetic drugs when glucose uptake is improved [98–102]. So, this kinase might be involved in the effects of fish oil supplementation on the increased insulin sensitivity observed herein. p38 MAP kinase activation participates in glucose uptake mediated by insulin or insulin-mimetic compounds (e.g., homocysteine, curcumin, chromium picolinate, retinoic acid, berberine and vanadate) in different cells, including adipocytes, skeletal muscle cells and cardiomyocytes [98–102]; however, the mechanisms involved are not completely known yet. Direct effect of EPA has also been shown in human skeletal muscle cells, where the treatment with this fatty acid (for 24 h) induced an increased glucose uptake and oxidation in these cells [93]. Interestingly, insulin-stimulated glucose uptake is greatly reduced by pharmacological inhibitors (A291077, A304000, SB202190 or SB203580) of p38 MAP kinase [97], demonstrating the importance of this kinase in this process. Herein, skeletal muscles from long-term fish oil supplementation showed improved insulin-stimulated p38 MAP kinase activation associated with increased GLUT-4 translocation and glucose uptake and metabolism. Further studies using selective inhibitors are required to confirm the

participation of this pathway in the positive effects of fish oil on insulin sensitivity.

Decrease in the n-6:n-3 PUFA ratio in plasma membrane has been suggested to participate in the improved insulin sensitivity induced by n-3 PUFA. This decrease has been suggested to improve insulin binding and signaling in target tissues [4,11,13,22,24,25,47,48], but the mechanisms involved are not completely known yet. However, in the present study, the activation of several proteins involved in the insulin signaling pathway, including insulin receptor, IRS-1 and Akt, was not altered by long-term fish oil supplementation. This result suggests that the decreased n-6:n-3 PUFA ratio does not favor activation of early insulin signaling proteins, but it can be associated to improved activation of the downstream protein p38 MAP kinase, which can enhance GLUT-4 translocation from inner vesicles to plasma membrane.

Effects of fish oil supplementation on G2 rats but not in G1 rats suggest the involvement of some regulatory mechanisms related to epigenetic DNA alteration. According to this proposition, altered total DNA methylation has been found in different disordered conditions, including insulin resistance, glucose intolerance, metabolic syndrome and steatosis [103–105]. In order to investigate if fish oil modifies the profile of global DNA methylation, we evaluated this parameter in liver and soleus muscle. Fish oil supplementation for two generations decreased global DNA methylation in liver, but it had no effect on this parameter in soleus skeletal muscle. These results suggest that the increased *in vivo* insulin sensitivity in G2 rats is associated with decreased total DNA methylation in liver, but not in skeletal muscle. However, the absence of modified global DNA methylation in soleus muscle does not exclude the possibility of epigenetic modulation in specific genes by n-3 PUFA. This issue and the underlying mechanisms involved in the modulation of insulin sensitivity by DNA methylation remain to be investigated.

In summary, fish oil supplementation had positive effects on insulin sensitivity in healthy rats only when administered for a long-term period (two generations). These effects include improved *in vivo* and *in vitro* insulin sensitivity in association with increased insulin-stimulated p38 activation, decreased n-6:n-3 fatty acid ratio and improved GLUT-4 translocation in skeletal muscle, and reduced global DNA methylation in liver.

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APÊNDICE E

Research Article

Macadamia Oil Supplementation Attenuates Inflammation and Adipocyte Hypertrophy in Obese Mice

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Excess of saturated fatty acids in the diet has been associated with obesity, leading to systemic disruption of insulin signaling, glucose intolerance, and inflammation. Macadamia oil administration has been shown to improve lipid profile in humans. We evaluated the effect of macadamia oil supplementation on insulin sensitivity, inflammation, lipid profile, and adipocyte size in high-fat diet (HF) induced obesity in mice. C57BL/6 male mice (8 weeks) were divided into four groups: (a) control diet (CD), (b) HF, (c) CD supplemented with macadamia oil by gavage at 2 g/Kg of body weight, three times per week, for 12 weeks (CD + MO), and (d) HF diet supplemented with macadamia oil (HF + MO). CD and HF mice were supplemented with water. HF mice showed hypercholesterolemia and decreased insulin sensitivity as also previously shown. HF induced inflammation in adipose tissue and peritoneal macrophages, as well as adipocyte hypertrophy. Macadamia oil supplementation attenuated hypertrophy of adipocytes and inflammation in the adipose tissue and macrophages.

1. Introduction

The role of a diet with a higher content of unsaturated fatty acids, in place or concomitant to a diet with high content of lipids, has been appointed as an effective strategy to control metabolic disorders [1]. Monounsaturated fatty acids (MUFA) rich diet has been reported to decrease plasma total cholesterol and LDL-cholesterol and increase HDL-cholesterol levels [2–5]. Moreover, when saturated fatty acids

are replaced by MUFA in the diet of obese women, levels of inflammatory markers decrease, including IL-6 and visfatin in serum [6]. Macadamia nut oil is rich in monounsaturated fatty acids, containing approximately 65% of oleic acid (C18:1) and 18% palmitoleic acid (C16:1) of the total content of fatty acids [7]. Macadamia oil is the main source of palmitoleic acid in the human diet. Some studies have shown that diet rich in macadamia can improve the lipid profile [2, 8–10], but

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to date there is no studies on the effect of supplementation of macadamia oil on adipocyte hypertrophy and inflammation.

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In 2008, Cao and colleagues [11] showed that mice deficient in lipid chaperones $\alpha P2$ and mal1 present increased levels of palmitoleic acid in serum. Elevated levels of circulating palmitoleic acid restored sensitivity of insulin in liver and skeletal muscle, hepatosteatosis, and hyperglycemia, generated by high-fat diet. With this, the authors named this fatty acid as a lipokine, since palmitoleic acid has a hormonal-like effect [11].

The administration of high-fat diet in C57BL6 mice induces metabolic perturbations similar to those observed in humans. In fact, consumption of the high levels of saturated fatty acids is associated with overweight, visceral obesity, inflammation, dyslipidemia, and insulin resistance, in skeletal muscle, liver, and adipose tissue [12–17]. Saturated FFA promotes inflammation by interaction with toll-like receptor 4 (TLR4), activating NF κ B, JNK, and AP-1 pathways [18, 19].

A low grade inflammation is established with increase in plasma levels of IL-6, IL-1 β , prostaglandins, TNF- α , and leptin and decrease in the production and secretion of adiponectin, IL-10, and IL-4 [20, 21]. The increase in local inflammation is potentiated by the recruitment of macrophages to adipose tissue and polarization of M2 macrophages (macrophages type 2) to M1 macrophages (macrophages type 1) [16, 22, 23].

The aim of our study was to evaluate the effect of macadamia oil supplementation, rich in MUFA (palmitoleic and oleic acids), on adipose tissue and peritoneal macrophages inflammation in mice fed a balanced diet or high-fat diet rich in saturated fatty acids. We measured glucose uptake (2-6 deoxyglucose uptake) and mRNA content of proteins (GLUT-4; IRS-1) involved in insulin signaling in soleus muscle. The contents of IL-10, IL-6, TNF- α , and IL-1 β in peritoneal macrophages and adipose tissue were also determined. The adipocyte size was also evaluated.

2. Materials and Methods

2.1. Animals. All experiments were performed according to protocols approved by the Animal Care and Use Committee of the Institute of Biomedical Sciences, University of São Paulo. C57BL/6 male mice (8 weeks old) were used in this study. Animals were housed with light-dark cycle of 12-12 h and temperature of $23 \pm 2^\circ\text{C}$. Animals were divided into four groups: (a) control diet (CD), (b) high-fat diet (HFD), (c) control diet supplemented with macadamia nut oil (Vital Átman, Uchoa, SP, Brazil) (CD + MO), and (d) high-fat diet supplemented with macadamia oil (HF + MO). Control groups were run concomitantly. The oil composition is shown in Table 1 (Supplementary Material available online at <http://dx.doi.org/10.1155/2014/870634>). During the first 4 weeks preceding the induction of obesity by HFD, all groups were *ad libitum* fed a control diet (76% carbohydrates, 9% fat, and 15% proteins). Similar protocol has been used in our previous studies [24, 25]. CD + MO and HF + MO were supplemented by oral gavage at 2 g per Kg of body weight,

6

TABLE 1: Fatty acid composition of macadamia oil.

Fatty acid	%
C12:0 lauric acid	0.09
C14:0 myristic acid	0.82
C16:0 palmitic acid	8.45
C16:1n7 palmitoleic acid	19.11
C17:0 heptadecanoic acid	0.28
C16:2n4 9,12-hexadecadienoic acid	0.02
C16:3n4 6,9,12-hexadecatrienoic acid	0.06
C18:0 stearic acid	3.90
C18:1n9 oleic acid	56.35
C18:1n7 vaccenic acid	3.09
C18:2n6 linoleic acid (LA)	1.35
C18:3n3 linolenic acid (ALA)	0.12
C20:0 arachidic acid	2.79
C20:1n9 gondoic acid	2.18
C20:1n11 gadoleic acid	0.12
C22:0 behenic acid	0.75
C22:1n9 erucic acid	0.22
C22:5n3 eicosapentaenoic acid	0.30
SFA	16.08
MUFA	80.01
PUFA	1.83
PUFA n3	0.42
PUFA n6	1.35
n3/n6	0.31

SFA = saturated fatty acids, sum of C12:0, C14:0, C16:0, C17:0, C18:0, C20:0, and C22:0; MUFA = monounsaturated fatty acids, sum of C16:1, C18:1n7, C18:1n9, C20:1n9, C20:1n11, and C22:1n9; PUFA = polyunsaturated fatty acids, sum of C16:3n4, C18:2n6, C18:3n3, and C22:5n3; PUFA n3 = sum of C18:3n3 and C22:5n3; PUFA n6 = C18:2n6.

three times per week, during 12 weeks. This dosage of oil was chosen based on previous studies from our group using different oils with no signs of hepatic toxicity [24]. CD and HF diet received water at the same dose.

7

2.2. Serum Parameters Analysis. Serum triacylglycerol, total cholesterol, LDL-cholesterol, and HDL-cholesterol were determined by colorimetric assays (Labtest Diagnostics, Lagoa Santa, MG, Brazil). Serum glucose and insulin were measured using LABTEST colorimetric assay and radioimmunoassay (Millipore, Billerica, MA, USA), respectively, as described by Masi et al. (2012) [24]. The HOMA index was determined by calculating fasting serum insulin ($\mu\text{U/mL}$) $\times$ fasting plasma glucose (mmol L^{-1})/22.5. Leptin and adiponectin were measured using the protocol of the manufacturing R&D system.

8

2.3. GTT and ITT. Glucose tolerance test (GTT) and insulin tolerance test (ITT) were carried out in all groups after 6 h fasting at the end of the 10th and 11th weeks of treatment, respectively.

The methodologies used for GTT and ITT were similar to that described by Masi et al. (2012) [24].

TABLE 2: Primer sequences of the genes studies for real-time PCR.

Primer name	Forward	Reverse
RPL-19	5-AGC CTG TGA CTG CCA TTC-3	5-ACC CTT CCT CTT CCC TAT GC-3
GLUT-4	5-CAT TCC CTG GTT CAT TGT GG-3	5-GAA GAC GTA AGG ACC CAT AGC-3
IRS-1	5-CTC AGT CCC AAC CAT AAC CAG-3	5-TCC AAA GGG CAC CGT ATT G-3
CPT-1	5-CCT CCG AAA AGC ACC AAA AC-3	5-GCT CCA GGG TTC AGA AAG TAC-3
PGC1-a	5-CAC CAA ACC CAC AGA AAA CAG-3	5-GGG TCA GAG GAA GAG ATA AAG TTG-3
Perilipin 5	5-CAT GAC TGA GGC TGA GCT AG-3	5-GAG TGT TCA TAG GCG AGA TGG-3

TABLE 3: Effect of high fat diet, with or without supplementation of macadamia oil, on obesity characteristics.

	CD	CD + MO	HF	HF + MO
Initial body weight (g)	24.26 ± 5.02	24.77 ± 3.37	24.2 ± 3.42	24.4 ± 4.41
Final body weight (g)	26.2 ± 1.99	26.82 ± 3.32	34.49 ± 6.96 ^{##}	34.75 ± 4.96 ^{##}
Liver weight (g)	1.17 ± 0.14	1.10 ± 0.23	1.33 ± 0.52	1.24 ± 0.26
Mesenteric adipose tissue weight (g)	0.32 ± 0.14	0.28 ± 0.12	0.55 ± 0.28 ^{##}	0.52 ± 0.23 ^{##}
Epididymal adipose tissue weight (g)	0.63 ± 0.14	0.73 ± 0.34	1.45 ± 0.60 ^{##}	1.59 ± 0.70 ^{##}
Retroperitoneal adipose tissue weight (g)	0.21 ± 0.06	0.24 ± 0.12	0.53 ± 0.22 ^{##}	0.55 ± 0.17 ^{##}
Adiposity index (g)	1.16 ± 0.24	1.25 ± 0.53	2.53 ± 1.01 ^{##}	2.65 ± 1.01 ^{##}
Brown adipose tissue weight (g)	0.104 ± 0.06	0.116 ± 0.03	0.155 ± 0.06	0.128 ± 0.02

Values represent the means ± S.D. of the data obtained from analysis of 15 animals per group. * $P < 0.001$ versus CD; [#] $P < 0.001$ versus CD + MO.

9

2.4. Insulin Responsiveness in Incubated Soleus Muscle. Animals were euthanized on CO₂ chamber and soleus muscles rapidly and carefully isolated and weighed (8–10 mg). This protocol was described in [24, 25].

2.5. Haematoxylin and Eosin Staining. Adipose samples were fixed in formalin and paraffin embedded. Sections were prepared (5 μ M) using Leica EG1150H Machine. Haematoxylin and Eosin (H&E) staining was conducted using Leica Autostainer XL and Leica CV5030. Sections were mounted using DPX media (Fisher Scientific, Ireland) and analyzed using Nikon 80i transmission light microscope.

2.6. Extraction of Fatty Acids from Gastrocnemius Muscle and Gas Chromatographic Analysis. Gastrocnemius muscle fragments (100 mg) were subjected to lipid extraction. For this, 0.5 mL chloroform/methanol (2:1; v/v) was added to 100 mg of gastrocnemius sample, well-vortexed and incubated at room temperature for 5 min. Additional volumes of 1.25 mL chloroform and 1.25 mL deionized H₂O were then added, and finally, following vigorous homogenization for 3 min, samples were centrifuged at 1200 g for 5 min, at room temperature to obtain two phases: aqueous phase in the top and organic phase in the bottom containing. The organic phase was collected, dried, and suspended in isopropanol. Triglyceride content was then determined in the homogenate. After that, for fatty acid composition determination, gastrocnemius lipid extracts were dried using atmospheric N₂ for evaporation of the solvent without fatty acid oxidation. The fractions of neutral and polar lipids were separated from these extracts by using a column chromatography. The polar (phospholipids) and neutral (triglycerides) fractions were methylated (for formation of methyl esters), using acetyl

chloride and methanol. The methyl esters were analyzed in a gas chromatographer coupled to a flame ionizer detector (FID) (Varian GC 3900). Fatty acid composition was then determined by using standard mixtures of fatty acids with known retention times (Supelco, 37 Components).

For the analysis of fatty acids, a programmed chromatography was used with the characteristics described below. The reading was initiated at 170°C temperature for 1 minute and then a ramp of 2.5°C/min was employed to reach a final temperature of 220°C that was maintained for 5 min. The injector and detector were maintained at 250°C. We used the CP wax 52 CB column, with a 0.25 mm thickness, internal diameter of 0.25 mm, and 30 mm long, with hydrogen as the carrier gas.

2.7. Analysis of Inflammatory Parameters

2.7.1. Adipokines Content Measurements. Mice were euthanized on CO₂ chamber and retroperitoneal adipose tissue was rapidly collected. About 100 mg of retroperitoneal adipose tissue was used for the determination of TNF- α , IL-6, and IL-10 content. Adipose tissue was homogenized in RIPA buffer (0.625% Nonidet P-40, 0.625% sodium deoxycholate, 6.25 mM sodium phosphate, and 1 mM ethylenediaminetetraacetic acid at pH 7.4), containing 10 g/mL of a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Homogenates were centrifuged at 12,000 g for 10 min at 4°C, supernatant was collected, and protein concentration was determined using Bradford assay (Bio-Rad, Hercules, CA, USA). Bovine serum albumin was used as protein standard.

Ex Vivo Adipose Tissue Culture. Retroperitoneal adipose tissue explants (about 100 mg) were cultured in DMEM sterile medium (Gibco), containing 10% FBS, **2 mM glutamine**,

FIGURE 1: Effect of MO supplementation on adipose tissue histology. (a) Histological sections stained with H&E. (b) Area of adipocytes. CD = group of animals maintained on control diet; HF = group of animals fed high-fat diet; CD + MO = group of animals fed control diet supplemented with macadamia oil; HF + MO = group of animals fed high-fat diet supplemented with macadamia oil. The data are given as the means $\pm$ S.D. * $P < 0.05$; ** $P < 0.01$ ($n = 6$).

10

streptomycin, and penicillin for 24 h, at 37°C and 5% CO₂, humidified air environment. Thereafter, medium culture was collected and used for the determination of IL-1 β , adiponectin, and TNF- α , using ELISA assays (DuoSet kits, R&D System).

2.7.2. Peritoneal Macrophage Isolation and Culture. Cytokine and nitric oxide (NO) production were evaluated in macrophages obtained by washing the peritoneal cavity with 6 mL RPMI culture medium (Gibco), containing 10% FBS and 4 mM glutamine. Macrophage-rich cultures (more than 90% of the cells were F4/80⁺) were obtained by incubating peritoneal cells in 24-well polystyrene culture plates for 2 h at 37°C in a 5% CO₂, humidified air environment. Nonadherent cells were removed by washing with RPMI. Adherent cells were then incubated with 2.5 μ g/mL of LPS (*E. coli*, serotype 0111:B4, Sigma Chemical Company, USA) for 24 h [26]. Medium was collected for determination of IL-6, IL-10, and TNF- α by ELISA and nitrite content by Griess method [27].

2.8. Quantitative RT-PCR. Total RNA from the gastrocnemius muscle was extracted with Trizol reagent (Invitrogen Life Technologies, Grand Island, NY, USA), following the method described by Chomczynski and Sacchi [28]. Reverse transcription to cDNA was performed using the high-capacity cDNA kit (Applied Biosystems, Foster, CA, USA). Gene expression was evaluated by real-time PCR [29], using Rotor Gene (Qiagen) and SYBR Green (Invitrogen Life Technologies) as fluorescent dye. Primer sequences are shown in Table 2. Quantification of gene expression was carried out using the RPL-19 gene as internal control, as previously described [30].

2.9. Statistical Analysis. Results are presented as mean $\pm$ S.D. All groups were compared by using two-way ANOVA followed by Bonferroni posttest. Significance level was set at $P < 0.05$.

3. Result

3.1. Characterization of the Experimental Model

3.1.1. Body Composition. Mice fed high-fat diet showed increased body weight gain, hypercholesterolemia, and insulin resistance. These modifications were similar to those observed in our previous studies [24, 25]. Animals fed the high-fat diet (HF and HF + MO) for eight weeks showed increased (by 2-fold) body weight gain and visceral adiposity index as compared with CD and CD + MO (Table 1). The weights of the liver and the brown adipose tissue depot were not altered with diet or supplementation (Table 3). Although the visceral adiposity index of mice fed high-fat diet (HF and HF + MO) was greater than in animals that received control diet (CD and CD + MO), the HF group had an increase (by 1,62-fold) of adipocytes size compared to the control diet (Figures 1(a) and 1(b)), with statistical difference not evidenced in HF + MO group. No difference was evidenced by diet or supplementation in LDL-c, NEFA, and glycerol (data not shown). Moreover, the basal glycemia and K_{itt} were increased in both groups treated with high-fat diet (data not shown). Homa-IR index was increased in the HF group (by 3-fold) as compared to the other groups including the HF + MO (Figure 2(a)). This result suggests a beneficial effect of macadamia oil supplementation on insulin responsiveness in the HF group.

The peripheral insulin resistance was confirmed by glucose uptake in incubated *soleus* muscle (data not shown),

FIGURE 2: Insulin sensitivity and triacylglycerol content in skeletal muscle after 12 weeks. (a) HOMA-IR: homeostatic model assessment of insulin resistance; (b) GLUT-4 gene expression; (c) triacylglycerol content in gastrocnemius muscle. The data are given as the means $\pm$ S.D. In all experiments the animals were previously fasted for 6 hours. CD = group of animals maintained on control diet; HF = group of animals fed high-fat diet; CD + MO = group of animals fed control diet supplemented with macadamia oil; HF + MO = group of animals fed high-fat diet supplemented with macadamia oil. A.U. = arbitrary unit. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ ($n = 6$).

as also shown previously [24, 25]. In addition, both groups treated with high-fat diet showed decrease in GLUT-4 mRNA expression (Figure 2(b)). The HF group showed an increase in triacylglycerol content in gastrocnemius muscle, but this effect was blunted in HF + MO (Figure 2(c)). The fatty acid composition in neutral or polar lipid fractions remains unchanged regardless of the diet given and MO supplementation (Table 1 in Supplementary Material).

3.2. Macadamia Oil Supplementation Attenuates High-Fat Diet Induced Inflammation. The contents of the anti-inflammatory cytokine IL-10 were increased in the HF + MO group (approximately 4.09-fold), while IL-1 β concentration in the medium of adipose tissue explants was increased in the HF group (Figure 3(a)).

No difference was evidenced among other groups. When stimulated with LPS, macrophages from all groups showed increased IL-6 production (by 2.97-fold) (Figure 3D), whereas IL-10 and NO production were elevated in cells from the HF and CT + MO groups (2.39- and 4.08-fold compared to base line, resp.) (Figure 3A/E). No effect of LPS stimulation was observed on TNF- α production by macrophages from all groups (Figure 3C).

When stimulated with LPS, macrophages from the HF group showed an increase (by 2,41-fold) of IL-1 β production compared to unstimulated cells whereas the supplementation with MO abolished this elevation (Figure 3(b)). Similar results were found in NO production. MO attenuated nitrate production by LPS stimulation on macrophages from the HF group (Figure 3(a)). The production of IL-10 was decreased

FIGURE 3: Inflammatory parameters in adipose tissue homogenate and adipose tissue explant incubation medium. IL10 content in adipose tissue homogenate (a) and IL1- β in the adipose tissue explant incubation medium, after 24 hours measured by ELISA (b). The animals received water or macadamia oil orally, 2 g/kg b.w., with or without association with a high-fat diet. CD = group of animals maintained on control diet; HF= group of animals fed high-fat diet; CD + MO = group of animals fed control diet supplemented with macadamia oil; HF + MO = group of animals fed high-fat diet supplemented with macadamia oil. The data are given as the means $\pm$ S.D. * $P < 0.05$ ($n = 5-6$).

in the CT group compared to CT + MO and HF groups (by 1,88-fold) (Figure 3E). TNF- α and IL-6 production remained unchanged by diet or supplementation (Figure 3C, D). Levels of IL-1 β in culture medium of adipose tissue explants increased in HF group and were attenuated by macadamia oil supplementation (Figure 3B).

No significant difference was found in serum levels of adiponectin after 12 weeks of treatment (data not shown). As expected, leptin concentration was increased (by 5.69-fold) in the groups fed the high-fat diet (data not shown). The significant difference between the CD + MO and the CD + CT groups suggests that MO can enhance circulating leptin.

4. Discussion

We showed herein that twelve weeks of macadamia oil supplementation attenuate the increase in inflammation and adipocyte hypertrophy in mice fed a high-fat diet that exhibit signs of the metabolic syndrome.

High consumption of fat, sucrose, and industrialized foods in association with sedentary lifestyle is the main contributor to obesity and its related comorbidities, including dyslipidemias, insulin resistance, and cardiovascular diseases; evidence has been accumulated that low grade inflammation plays a key role in the obesity induced comorbidities [11, 31–37].

The increase in adipocytes size was attenuated by macadamia oil treatment. The increase in adipocyte diameter has been associated with disturbances in cellular homeostasis, such as insulin resistance, inflammation, and hypoxia [38]. The prevalence of large adipocytes increases leptin

production and secretion, as observed in our study [39]. The increase in leptin is associated with an elevation in low grade inflammation. Leptin is known to stimulate proinflammatory cytokines production in lymphocytes [40–42], monocytes [43], and macrophages [44].

Mice fed the HFD for 8 weeks exhibited increased IL-10 content in retroperitoneal adipose tissue. This result may be associated with the increase in peroxisome proliferator activated receptor- (PPAR-) gamma activity. This nuclear receptor increased the number of small adipocytes and raised the IL-10 [45, 46]. The increase of IL-10 content in adipose tissue leads to macrophage polarization (type 2) that is important for remodeling and tissue repair [47, 48]. Moreover, the increase in IL-10 content in adipocytes is associated with increased insulin sensitivity in adipose tissue [49, 50].

IL-1 β strongly induces the inflammatory response in innate immune cells [51], via JNK and NF κ B pathway [52]. IL-1 β is also a potent inducer of insulin resistance. This cytokine decreased insulin-stimulated glucose uptake via ERK activation [53]. Patients with high level of the circulating IL-1 β are associated with greater risk on development of type 2 diabetes [54]. Adipose tissue and peritoneal macrophages are two sources of IL-1 β , and macadamia oil supplementation was effective in decreasing the production of this cytokine in both. However, unexpectedly, the CDM showed an increased IL-1 β production after LPS stimulation in peritoneal macrophages.

NO production is increased in LPS-stimulated macrophages being more pronounced in mice fed high-fat diet [24]. We demonstrated herein that the same pattern and the supplementation with macadamia oil prevented the production of NO by peritoneal macrophages from HF mice.

FIGURE 4: Nitric oxide and cytokine production by peritoneal macrophages. Peritoneal macrophages were collected and cultured for 24 h in the absence (white bars) or presence (black bars) of 2.5 μ g/mL LPS. Nitric oxide (a), IL-1 β (b), TNF- α (c), IL-6 (d), and IL-10 (e) were measured. CD = control diet; HF = high-fat diet; CD + MO = control diet + macadamia oil; HF + MO = high-fat diet + macadamia oil. The data are given as the means $\pm$ S.D. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ ($n = 5-6$).

Other bioactive compounds, such as epigallocatechin gallate and resveratrol [55], decrease NO production by macrophage inhibition through of MAP kinase, JNK, and NFkB signaling [56].

In conclusion, macadamia oil supplementation attenuated inflammation and adipocyte hypertrophy in obese mice.

Conflict of Interests

The authors declare that they have no conflict of interests with the presented data.

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Manuscript #	BJN-2014-021945
Current Revision #	0
Submission Date	13th Aug 14
Current Stage	Under Review
Title	DNA methylation changes induced by a high-fat diet and fish oil supplementation
Running Title	Lipid diet modulates Pparg DNA methylation
Manuscript Type	Research Article
Special Section	N/A
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Manuscript Comment	There are two tables in the article file.
Corresponding Author	Catia Amaral (amaral_catia@yahoo.com.br) (University of Sao Paulo)
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Abstract	This study aimed to investigate the global changes in DNA methylation and methylation of the promoter region of the peroxisome proliferator-activated receptor gamma (Pparg) gene resulting from a high-fat diet and/or fish oil supplementation. Fish oil or water was orally administered to male mice for 12 weeks. After the first 4 weeks, the animals were fed a control diet or a high-fat diet until the end of the experimental protocol, when the epididymal fat, gastrocnemius muscle and liver were excised. High-fat diet-induced obesity led to a global increase in the level of DNA methylation in the muscle tissue and decreased methylation of the Pparg promoter. The administration of fish oil to mice receiving a high-fat diet resulted in the downregulation of muscle mRNA expression concomitant with the increased methylation of Pparg, suggesting an epigenetic mechanism. In addition, methylation of the Pparg promoter in the liver was correlated with body weight gain and liver weight. In conclusion, fish oil (rich in omega-3 polyunsaturated fatty acids - n-3 PUFAs) and a high-fat diet modified the global and Pparg promoter methylation levels. Fish oil supplementation exerted a protective effect against the altered methylation of the Pparg promoter induced by a high-fat diet in skeletal muscle.
Key Words	epigenetic, obesity, omega-3 fatty acids, Diet Induced Obesity (DIO)
Conflict of Interest	No , there is no conflict of interest that I should disclose, having read the above statement.
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