PRISCILA NEDER MORATO

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EFEITO DO CONSUMO DAS PROTEÍNAS DO SORO DO LEITE, COMPONENTES PEPTÍDICOS E AMINOACÍDICOS NOS TRANSPORTADORES DE GLICOSE EM MÚSCULO DE RATOS

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Orientador: Prof. Dr. Jaime Amaya Farfán

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Alimentos e Nutrição da Faculdade de Engenharia de Alimentos da Universidade Estadual de Campinas, para obtenção do Título de Doutora em Alimentos e Nutrição – Área de Concentração: Nutrição Experimental e Aplicada a Tecnologia de Alimentos.

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RESUMO

As proteínas do soro do leite apresentam propriedades nutricionais e funcionais que influenciam a modulação de funções bioquímicas e fisiológicas. Estudos têm demonstrado que as proteínas do soro do leite (PSL), principalmente na forma hidrolisada (PSLH) possuem a capacidade de aumentar os níveis de glicogênio muscular. Considerando que a captação de glicose pela célula do músculo esquelético relaciona-se diretamente à atividade de proteínas transportadoras de glicose, este estudo se propôs realizar dois experimentos para conhecer os efeitos da PSL e da PSLH e de alguns dos seus produtos de hidrólise nos transportadores de glicose em músculos de ratos. No experimento 1, o objetivo foi verificar se o consumo de PSL e PSLH modulam a concentração de transportadores de glicose GLUT-1 e GLUT-4 na membrana plasmática (MP) de células musculares de animais sedentários e exercitados. Foram utilizados 48 ratos Wistar machos divididos em dois grupos: sedentários e exercitados, e cada um desses subdivididos em outros três, de acordo com a dieta, totalizando 6 grupos (n=8 por grupo). Os animais foram mantidos por 9 dias recebendo as dietas experimentais baseadas na AIN-93G, com as seguintes fontes protéicas: caseína (CAS), utilizada como controle, proteína do soro do leite (PSL), proteína do soro do leite hidrolisada (PSLH), e o animais exercitados foram submetidos a uma única sessão de exercício a 15m/min durante 60min um dia anterior ao sacrifício. Após o período experimental os animais foram sacrificados, os transportadores de glicose no músculo, GLUT-1 e GLUT-4, foram analisados por western blot. Adicionalmente, glicogênio, aminoácidos livres plasmáticos, insulina

e indicadores bioquímicos de saúde foram determinados por métodos padrões. O consumo de PSLH elevou significativamente as concentrações de GLUT-4 na MP e de glicogênio, enquanto GLUT-1, insulina e os indicadores de saúde não apresentaram alterações. Baseado nas evidências do experimento 1, de que o consumo de PSLH eleva os estoques de glicogênio muscular e que também aumenta a concentração do transportador de glicose GLUT-4 na membrana plasmática, o experimento 2 teve como objetivo identificar quais componentes da PSLH poderiam modular a translocação do transportador de glicose GLUT-4 para a MP em músculo esquelético. Foram utilizados 49 ratos Wistar machos divididos em 7 grupos (n=7), que receberam soluções orais de glicose 30% mais 0,55 g/kg de peso corpóreo os seguintes componentes da PSLH: a) glicose (controle); b) PSLH; c) L-isoleucina; d) L-leucina; e) L-leucina mais L-isoleucina; f) peptídeo L-isoleucil-L-leucina; g) peptídeo L-leucil-L-isoleucina. Após receberem as soluções, os animais foram sacrificados, o transportador de glicose GLUT-4 no músculo foi analisado por western blot. Também foram analisados glicogênio, glicemia, insulina, aminoácidos livres plasmáticos e musculares, e indicadores bioquímicos de saúde por métodos clássicos. Entre os componentes testados da PSLH, o peptídeo leucil-isoleucina e o aminoácido isoleucina se mostraram mais eficientes em translocar GLUT-4 para a MP, favorecendo a captação de glicose pelo músculo esquelético. Os resultados obtidos nos experimentos indicam que o consumo da PSLH e de seus componentes ao aumentarem a translocação de GLUT-4 para a membrana plasmática, poderiam auxiliar no tratamento ou prevenção do diabetes do tipo II.

Palavras-chave: proteínas do soro do leite, GLUT-4, glicogênio, peptídeos bioativos, aminoácidos de cadeia ramificada.

ABSTRACT

The milk whey proteins (WP) exhibit nutritional and functional properties which result in the modulation of the biochemical and physiological functions. Studies have shown that the WP, especially those in the hydrolyzed form (WPH), has the capacity to increase muscle glycogen levels. Considering that glucose uptake by the skeletal muscle cell is directly related to the activity of the glucose transporter proteins, the present study proposed to carry out two experiments to determine the effects of WP and WPH and of some of their hydrolysis products on the glucose transporters in rat muscles. The objective of experiment 1 was to verify if the consumption of WP and WPH are able to modulate the concentration of the glucose transporters GLUT-1 and GLUT-4 in the plasma membrane (PM) of muscle cells in sedentary and exercised animals. Forty-eight male Wistar rats were used, divided into sedentary and exercised groups, each of which was sub-divided into three sub-groups according to the diet, giving a total of 6 groups (n=8 per group). The animals were maintained for 9 days on experimental diets based on AIN-93G with the following protein sources: casein (CAS) used as the control, whey protein (WP) and whey protein hydrolysate (WPH). The exercised animals were submitted to a single exercise session for 60 min at 15m/min one day prior to euthanasia. After the experimental period, the animals were euthanized, and the muscle glucose transporters GLUT-1 and GLUT-4 analyzed by western blot. In addition, glycogen, free plasma amino acids, insulin and the biochemical health indicators were analyzed by standard techniques. The consumption of WPH significantly increased the concentrations of GLUT-4 in the PM and of glycogen,

whereas GLUT-1, insulin and the health indicators remained unaltered. Based on evidence from experiment 1 that the consumption of WPH raised the muscle glycogen reserves and also the concentration of the glucose transporter GLUT-4 in the plasma membrane, the second experiment was designed to identify which WPH components could modulate translocation of the glucose transporter GLUT-4 to the PM in the skeletal muscle of the animals. Forty-nine male Wistar rats were used, divided into 7 groups (n=7), who were orally fed 30% glucose solutions plus 0.55 g/kg of body weight of the following WPH components: a) glucose (control); b) WPH; c) L-isoleucine; d) L-leucine; e) L-leucine plus L-isoleucine (50:50 mixture of both amino acids); f) L-isoleucyl-L-leucine peptide or g) L-leucyl-L-isoleucine peptide. After receiving the solutions, the animals were euthanized and the GLUT-4 determined by western blot. Glycogen, glycemia, insulin, free plasma and muscle amino acids, and the biochemical health indicators were also analyzed by classical methods. Of the WPH components tested, the peptide L-leucyl-L-isoleucine and the amino acid L-isoleucine were shown to be more efficient in translocating GLUT-4 to the PM, favoring the capture of glucose by the skeletal muscle. The results obtained from these experiments indicated that the consumption of WPH and its components increased GLUT-4 translocation to the plasma membrane, and could aid in the treatment and prevention of type ll diabetes.

Keywords: milk whey proteins, GLUT-4, glycogen, bioactive peptides, branched chain amino acids.

SUMÁRIO

LISTA DE ABREVIATURAS

AIN93-G	*American Institute of Nutrition*
Akt	Proteína quinase B
AMP	Adenosina monofosfato
AMPK	Proteína quinase ativada por 5´-AMP
ATP	Adenosina trifosfato
BCAAs	Abreviatura inglesa para aminoácidos de cadeia ramificada
CAS	Caseína
ECA	Enzima conversora da angiotensina I
FAO	Food and Agriculture Organization
GLUT	Transportador de glicose
GLUT-1	Transportador de glicose 1
GLUT-4	Transportador de glicose 4
LDL	Lipoproteína de baixa densidade
PI-3K	Fosfatidilinositol 3-quinase
MP	Membrana Plasmática
PKC	Proteína quinase C
PSL	Proteínas do soro do leite
PSLH	Proteína do soro do leite hidrolisada
TG	Triacilglicerol

Introdução Geral

INTRODUÇÃO GERAL

O conceito de alimentação saudável em vigor considera importante, além do consumo de nutrientes reconhecidos como necessários para o adequado funcionamento e desenvolvimento do organismo, a inclusão de compostos funcionais na dieta, que têm sido relatados como capazes de atenuar ou prevenir doenças crônicas não transmissíveis, tais como câncer, doenças cardiovasculares e diabetes. Entre as medidas preventivas com vistas à redução de riscos dessas patologias, deve ser considerada também a prática de atividade física.

O leite é uma importante fonte protéica alimentar. Além de seus atributos nutricionais, esse alimento e os seus componentes, incluindo o soro têm sido alvo de pesquisas que visam entender os benefícios, os mecanismos de ação e as quantidades de consumo necessárias para que os componentes naturalmente presentes nesse alimento possam gerar efeitos que contribuam para a manutenção da saúde ou prevenção de certas doenças.

As proteínas do soro do leite (PSL) são reconhecidas pela qualidade nutricional e digestibilidade, rápida absorção e por serem ricas em aminoácidos indispensáveis e de cadeia ramificada (BCAAs) (HULMI, LOCKWOOD, STOUT, 2010). Além disso, as proteínas do soro de leite são consideradas nutrientes de atividade funcional, capazes de modular algumas respostas fisiológicas do organismo (MARSHALL, 2004; PACHECO et al., 2005).

As PSL, principalmente em sua forma hidrolisada (PSLH), têm sido associadas à capacidade de recuperar e/ou preservar os níveis de glicogênio no músculo esquelético (TASSI, AMAYA-FARFAN, AZEVEDO, 1998; PIMENTA et al.,

2006; MORIFUJI et al., 2005; MORIFUJI et al., 2010, 2011). O glicogênio muscular é a principal fonte de energia durante o exercício prolongado de moderada a alta intesidade (JEUKENDRUP, 2011), assim o aumento nos estoques de glicogênio poderia melhorar a performance e evitar a fadiga, além de favorecer rápida recuperação da depleção causada pelo exercício.

Sabe-se que o consumo dessas proteínas eleva os estoques de glicogênio, e que a captação de glicose pelo músculo esquelético está relacionada à atividade de proteínas transportadoras de glicose. Dessa maneira, é razoável imaginar que as PSL poderiam influenciar a ativação e/ou concentração de transportadores de glicose. Entretanto, não foram encontrados dados na literatura sobre o possível efeito dessas proteínas nos transportadores de glicose.

Assim, no presente estudo, inicialmente procurou-se investigar se a PSL, e principalmente a PSLH apresentam efeitos nos transportadores de glicose no músculo, visando conhecer o mecanismo envolvido no estímulo a biossíntese de glicogênio, e posteriormente quais os componentes presentes na PSLH que poderiam influenciar na ação dessa proteína na captação de glicose pelo músculo e subsequente síntese de glicogênio.

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OBJETIVOS

OBJETIVO GERAL

Determinar se o consumo da PSL e da PSLH exerce influência sobre as proteínas transportadoras de glicose sódio-independentes (GLUTs).

Avaliar alguns dos componentes formados durante a produção de PSLH no tocante à capacidade de influenciar a ação dessa proteína na captação de glicose pelo músculo.

OBJETIVOS ESPECÍFICOS

Analisar os parâmetros envolvidos no influxo celular de glicose no músculo:

- translocação dos transportadores de glicose GLUT-4, GLUT-1 para a membrana plasmática.

- ativação de proteínas da via da insulina (Akt/p-Akt, p85) e AMPK

- níveis de glicogênio no músculo esquelético, coração e fígado

- insulina plasmática

- glicose sérica

- aminoácidos livres plasmáticos

- indicadores bioquímicos de saúde

Capítulo 1

CAPÍTULO 1 - REVISÃO BIBLIOGRÁFICA

1 Proteínas do soro do leite

As proteínas do soro do leite industrial, mais propriamente denominado de “soro de queijo”, correspondem aproxidamente a 20% do total da proteína do leite bovino e incluem as proteínas β-lactoglobulina, α-lactalbumina, albumina de soro bovino, lactoferrina, imunoglobulinas e o glicomacropeptídeo (MARSHALL, 2004).

A proteína do soro do leite (PSL) pode ser encontrada comercialmente na forma de concentrados protéicos, que apresentam geralmente de 29 a 89% de proteína, de isolados protéicos contendo de 90-95% de proteína e hidrolisados protéicos, obtidos pela hidrólise parcial dos concentrados de maior teor protéico e apresentando conteúdo médio de 80% de proteínas em sua composição. Os hidrolisados possuem em sua composição peptídeos, assim como aminoácidos livres formados durante o processamento (MARSHALL, 2004; HULMI, LOCKWOOD, STOUT, 2010).

O perfil de aminoácidos indispensáveis do conjunto das proteínas contidas na PSL supera as recomendações da *Food and Agriculture Organization* - FAO para a maioria deles, sendo considerada de elevado valor nutricional, comparada a outras fontes protéicas, incluindo a caseína, a proteína isolada de soja e a proteína do ovo (AMAYA-FARFAN et al., 2007; HULMI, LOCKWOOD, STOUT, 2010).

As proteínas do soro do leite são facilmente digeridas e rapidamente absorvidas pelo intestino, porque permanecem solúveis nas condições ácidas do

estomago, diferente da caseína que coagula, sendo considerada uma proteína de digestão lenta (BOIRIE et al., 1997). O metabolismo rápido se mostra atrativo para a recuperação em situações de estresse metabólico, como na fase após o exercício físico. O consumo das proteínas do soro do leite resulta em rápido aumento de aminoácidos no plasma, estimulando a síntese e manutenção protéica (TANG et al., 2009).

O consumo da PSL hidrolisada estimulou a síntese protéica em músculo esquelético de indivíduos jovens, em repouso e após exercício de resistência, superando a caseína e a proteína de soja. Os resultados foram associados à rápida digestão e absorção de PSLH, e ao maior conteúdo de leucina fornecido por essa proteína (TANG et al., 2009).

Em estudo realizado por Pennings et al. (2011) foi investigado a cinética de absorção e síntese protéica muscular depois da ingestão de PSL, caseína e caseína hidrolisada em indivíduos idosos saudáveis. Os resultados mostraram que o consumo de PSL, em comparação a caseína intacta e a hidrolisada, eleva mais rapidamente e em maior concentração os aminoácidos indispensáveis. A PSL também foi mais efetiva que a caseína, na forma intacta e hidrolisada, na promoção da síntese protéica muscular.

Evidências demonstram que a PSL apresenta potencial na redução do risco de doenças cardiovasculares, apresentando efeitos benéficos em diferentes processos associados a desordens metabólicas. O consumo de PSL por indivíduos com sobrepeso ou obesos, por exemplo, reduziu os níveis de colesterol total, lipoproteínas de baixa densidade (LDL) e triacilgliceróis (TG) comparado ao grupo que recebeu caseína ou ao grupo controle que recebeu glicose. O resultado

foi obtido após 12 semanas de suplementação com o consumo diário de 54 g de proteína (PAL, ELLIS, DHALIWAL, 2010). O consumo de uma única dose de PSL reduziu os níveis pós-prandiais de triacilglicerol (TG) em mulheres no estágio pós-menopausa com sobrepeso ou obesas (PAL, ELLIS, HO, 2010).

Em camundongos, o consumo de dieta hiperlípidica (60% do valor calórico total) contendo proteína do soro leite (18%) e elevados valores de cálcio (1,8% carbonato de cálcio) resultou em menor ganho de peso e gordura corporal comparada a uma dieta similar, mas com a substituição da fonte protéica pela caseína (PILVI et al., 2007).

O consumo de PSL tem também auxiliado na redução da hipertensão em humanos. A ingestão de bebida com PSL e PSLH durante 6 semanas, reduziu a pressão sanguínea de indivíduos jovens pré-hipertensos ou que apresentavam hipertensão de estágio I, e não alterou a pressão sanguínea em indivíduos normotensos. Observou-se também redução nos níveis de colesterol total e lipoproteínas de baixa densidade (LDL) (FLUEGEL et al., 2010).

A PSL tem mostrado capacidade imunomoduladora, provavelmente por fornecer elevada concentração de aminoácidos sulfurados, principalmente cisteína, que é precursor de glutationa, um importante antioxidante intracelular (MARSHALL, 2004).

A suplementação com PSL e a prática de exercício físico de resistência resultou em alterações de parâmetros antioxidantes e redução de risco de fatores cardiovasculares em indivíduos com sobrepeso. O tratamento foi realizado durante 6 semanas e resultou em melhor capacidade antioxidante total, aumento nos níveis de glutationa e vitamina C, redução de lipoproteína de baixa densidade

(LDL) e triacilgliceróis (TG). A combinação do exercício de resistência com o consumo da PSL foi mais efetiva, comparada ao consumo da PSL sem exercício, para melhorar o sistema de defesa antioxidante e na redução de fatores de riscos associados a doenças cardiovasculares (VATANI, GOLZAR, 2012).

Os efeitos fisiológicos que resultam do consumo de PSLH têm sido estudados em ratos exercitados. Moura et al. (2013) mostraram que o consumo de PSLH resultou na maior concentração de HSP70, uma proteína produzida pelo organismo para compensar os efeitos do estresse, a qual é considerada um antioxidante complementar e parte do sistema de defesa celular. Assim, as evidências apontam que a PSLH poderia conferir um efeito protetor ao organismo de ratos submetidos ao exercício.

Nery-Diez et al. (2010) mostraram que o consumo das PSL apresenta efeito anti-estresse em ratos, representado pela diminuição da atividade da glutaminase intestinal. Foi observado também, que o consumo das PSL favorece redução significativa de enzimas indicadoras de lesão muscular (LDH, CK) em atletas futebolistas em período de competição (LOLLO, AMAYA-FARFAN, SILVA, 2011).

A suplementação com PSL melhorou o estado nutricional de pacientes com esclerose lateral amiotrófica, doença neurodegenerativa progressiva (SILVA et al., 2010).

1.1. Características específicas das proteínas do soro do leite

Entre outras características das PSL, destacamos na sequência aquelas que são interessantes para a implementação deste estudo:

1.1.2. Estímulo à secreção de insulina

As proteínas do soro do leite possuem capacidade insulinotrópica, sendo amplamente eficientes em elevar a secreção de insulina após sua ingestão quando comparada à proteína de soja (MORIFUJI, SAKAI, SUGIURA, 2005) ou mesmo quando comparada à caseína (POWER et al., 2009). Dois fatores apontados para esse efeito são: a) a alta velocidade de digestão e consequente elevação das concentrações de aminoácidos plasmáticos; b) perfil aminoacídico, rico em aminoácidos indispensáveis, principalmente em leucina, reconhecidamente estimuladora da secreção de insulina.

A suplementação aguda com proteína do soro hidrolisada, em contraste com o isolado protéico (proteína não-hidrolisada), promoveu o aumento transitório da leucina com subsequente aumento da insulina plasmática em ratos. Os autores sugerem que maior capacidade insulinotrópica da PSLH também pode estar associada a presença de peptídeos bioativos, formados durante o processo de hidrólise da proteína, e que poderiam estimular a secreção pancreática de insulina (TOEDEBUSCH et al., 2012).

Os efeitos do consumo da PSL podem ser diferentes no curto e longo prazo. A PSL demonstrou efeito insulinotrópico em curto prazo quando consumida por indivíduos obesos. No entanto, após 12 semanas, o consumo da proteína reduziu os níveis de insulina plasmática e melhorou a sensibilidade a insulina (PAL, ELLIS, DHALIWAL, 2010). Os autores relataram que embora o mecanismo ainda não estivesse elucidado, a elevada concentração de aminoácidos insulinogênicos, principalmente os de cadeia ramificada, assim como de peptídeos

bioativos, presentes na proteína ou liberados durante a digestão, possam ter sido responsáveis pelos efeitos observados.

Ainda, com relação ao metabolismo de glicose, Bernard et al. (2011) verificaram em ratos, que a ingestão de carboidrato adicionado de uma mistura de aminoácidos composta por cisteína, metionina, valina, leucina e principalmente isoleucina, não resultou no aumento dos níveis de insulina quando comparado ao consumo de carboidrato sozinho. No entanto, houve melhora da tolerância a glicose, que segundo os autores, parece estar relacionada à maior entrada de glicose no músculo. Esta resposta não foi associada com os níveis séricos de insulina, e sim com a ativação de proteínas envolvidas na regulação da entrada de glicose no músculo.

Petersen et al. (2009) observaram em indivíduos saudáveis redução da glicemia pós-prandial (37,5%) após o consumo de uma única dose contendo 50g de carboidrato mais PSL (20 g), tendo como controle o consumo do carboidrato sem a proteína. Foram testadas as doses de 5, 10 e 20g de PSL, sendo a maior dose de proteína recebida a que resultou menores níveis glicêmicos. Os autores sugerem que a ingestão da PSL, uma proteína rica em leucina, aminoácido considerado insulinogênico, promove a maior secreção de insulina e a conseqüente redução dos níveis de glicose sanguínea. No entanto, a insulina plasmática não foi analisada nesse estudo.

1.2.2 Capacidade de recuperar e preservar o glicogênio

A capacidade de realização de um exercício físico prolongado de média a alta intensidade está relacionada à quantidade de glicogênio muscular disponível (JEUKENDRUP, 2011). O exercício promove a depleção do glicogênio que está associada à queda do rendimento e a fadiga. A restauração dos estoques de glicogênio no músculo seguido do exercício é um dos fatores mais importantes e determinantes no tempo de recuperação.

A síntese de glicogênio no músculo, após a depleção causada pelo exercício, pode ser dividida em duas fases. Inicialmente, há uma rápida reposição de glicogênio muscular independente de insulina. Em seguida, inicia-se uma fase mais lenta e prolongada de síntese de glicogênio, a qual é insulinodependente. A recuperação do glicogênio pode levar até 24 horas, dependendo da extensão da depleção e da dieta consumida (JENTJENS, JEUKENDRUP, 2003).

Dentre os fatores limitantes da síntese de glicogênio no músculo, destaca-se a atividade da enzima glicogênio sintase, que catalisa a síntese de glicogênio. Outro importante fator é o transporte de glicose para dentro da célula, dada a necessidade de que este substrato esteja disponível para síntese de glicogênio (REN et al., 1994; KUO, BROWNING, IVY, 1999; KUO et al., 2004).

Diversos estudos, com ratos, mostram que o consumo de PSL, na forma isolada ou hidrolisada é capaz de aumentar a concentração muscular e hepática de glicogênio (TASSI, AMAYA-FARFAN, AZEVEDO, 1998; PIMENTA et al., 2006; MORIFUJI et al., 2005b; MORIFUJI et al., 2010b, 2011). Quando comparadas à proteína da dieta controle, as PSL oferecem vantagem, no entanto, as PSLH

mostram vantagem adicional, elevando a concentração de glicogênio significativamente acima dos níveis ocasionados pelas PSL, em situação de pré- ou pós-exercício (MORIFUJI et al., 2010b, 2011).

O consumo de suplemento contendo carboidrato e PSLH aumentou a recuperação de glicogênio muscular em ciclistas, mensurado 6 horas após o exercício, comparado ao consumo de suplemento isoenergético contendo apenas carboidrato. Os autores afirmam que a adição de proteínas a carboidratos, pode favorecer a síntese de glicogênio quando ingeridos no início da recuperação e quando a depleção causada pelo exercício é moderada (BERARDI et al., 2006).

Faria et al. (2012) verificaram aumento no glicogênio cardíaco em ratos sedentários alimentados com PSLH quando submetidos à exaustão. O estudo mostrou que a PSLH poderia conferir efeito protetor ao coração em situações de estresse quando comparado à proteína do soro do leite isolada ou a caseína. Por ser um órgão vital, o coração pode responder de maneira diferente a condições catabólicas, como no jejum e no exercício. A concentração dos níveis de glicogênio é importante para a manutenção dos processos vitais e sobrevivência do músculo cardíaco (TAEGTMEYER, 2004).

1.2.3 Elevação da síntese de ácidos graxos musculares

Buscando compreender como as PSL melhoram o desempenho físico, Morifuji et al. (2005a) observaram que o consumo de PSL aumentou significativamente a atividade e a expressão da enzima ácido graxo sintetase nos músculos, sugerindo que as PSL podem estimular o acúmulo e uso de

triacilgliceróis musculares, que seriam utilizados como fonte energética durante o exercício, poupando o glicogênio muscular (que quando exaurido pode limitar o desempenho no exercício) (VAN LOON, 2004).

1.2.4 Aumento no tempo de exaustão

Resultados com ratos treinados consumindo PSLH têm sugerido que essas proteínas conferem vantagens para o desempenho físico por meio do aumento no tempo de exaustão (PIMENTA et al., 2006). O consumo das PSLH ampliou o tempo de exaustão dos animais, sem, no entanto, reduzir proteínas séricas e musculares e nem aumentar concentrações de lactato, mostrando que o consumo das PSLH pode ser um recurso ergogênico nutricional efetivo durante o treinamento físico (TASSI, AMAYA-FARFAN, AZEVEDO, 1998; PIMENTA et al., 2006).

1.2.5 Presença de peptídeos bioativos

As PSLH são as PSL que passaram por um processo de quebra da cadeia peptídica (hidrólise), formando peptídeos de diversos tamanhos. As proteínas do leite são consideradas precursoras de diferentes peptídeos biologicamente ativos. Esses peptídeos são inativos enquanto inseridos na sequência primária da proteína, e podem ser liberados por meio da digestão gastrointestinal do leite ou durante o processamento do alimento (MEISEL, 2005; KORHONEN, 2009).

Sabe-se que as proteínas dietéticas, durante a digestão, podem ser absorvidas como aminoácidos, di e tripeptídeos. Morifuji et al. (2010a) detectaram

dipeptídeos contendo BCAAs em plasma de humanos, após a ingestão de PSLH. Os níveis plasmáticos de Val-Leu e Ile-Leu aumentaram significativamente após a ingestão da proteína hidrolisada comparada a proteína não hidrolisada. Os autores sugerem que os peptídeos presentes nas proteínas hidrolisadas, produzidos durante o processamento por enzimas industriais que clivam ligações peptídicas específicas, podem ser mais biodisponíveis comparados aos peptídeos produzidos durante o processo digestão por enzimas gastrointestinais.

Os peptídeos bioativos têm sido definidos como fragmentos específicos de proteínas que causam impacto positivo nas funções ou nas condições do organismo, e que podem influenciar a saúde (KORHONEN, 2009).

Os peptídeos derivados das proteínas do leite exercem atividades nos sistemas digestivos, cardiovascular, imune e nervoso. Estudos têm identificado grande número de peptídeos com bioatividades específicas. Os efeitos benéficos na saúde atribuídos aos peptídeos derivados do leite estão relacionados às atividades antimicrobiana, antioxidativa, antitrombótica, anti-hipertensiva e imunomodulatória (KORHONEN, PIHLANTO 2006; MILLS et al., 2011).

Peptídeos com atividade anti-hipertensiva demonstraram inibir a enzima conversora da angiotensina I (ECA), impedindo a síntese da angiotensina II, um potente vaso constritor. Os peptídeos Val-Pro-Pro e Ile-Pro-Pro reduziram a pressão sanguínea em ratos espontaneamente hipertensos. Após 12 semanas, a pressão sistólica nos grupos que receberam os peptídeos e leite fermentado contendo esses peptídeos foi menor comparada ao grupo controle que recebeu água. Os autores relataram que o efeito anti-hipertensivo dos peptídeos do leite pode estar relacionado à atividade inibitória da ECA (SIPOLA et al., 2001).

Os peptídeos Val-Pro-Pro e Ile-Pro-Pro, obtidos da fermentação do leite com *Lactobacillus helveticus*, têm sido identificados como um dos mais importantes inibidores de ECA (KORHONEN, PIHLANTO, 2006).

Pins e Keenan (2006) verificaram em humanos pré-hipertensos ou em hipertensão de estágio I, que a dose diária de 20 g de PSLH, reduziu a pressão sanguínea após uma semana de tratamento. Esse efeito se manteve ao longo das 6 semanas de estudo. Observou-se também redução nos níveis de lipoproteína de baixa densidade (LDL). Os autores concluíram que o consumo da PSLH, por conter os peptídeos bioativos, pode auxiliar no tratamento do risco de doenças cardiovasculares.

Os peptídeos derivados da lactoferrina apresentaram atividade antimicrobiana para bactérias como *Escherichia, Listeria, Salmonella*. O efeito antimicrobiano parece estar relacionado à capacidade da lactoferrina em quelar ferro, privando os microrganismos desse nutriente. Os peptídeos parecem afetar a permeabilidade da membrana celular das bactérias (SÉVERIN, WENSHUI, 2005).

No sistema imune, certos peptídeos parecem modular a proliferação de linfócitos, regular a produção de algumas citocinas, e estimular a atividade de macrófagos (GAUTHIER, POULIOT, SAINT-SAUVEUR, 2006).

Além desses efeitos, estudos apontam que os peptídeos formados na hidrólise da PSL são capazes de modular o metabolismo energético. Morifuji et al. (2009) estudando as PSLH, isolaram dipeptídeos que continham aminoácidos de cadeia ramificada (Leu, Ile ou Val) e incubaram músculos de ratos Wistar em solução tampão, a cada vez, com 1 mM de Ile-Val, Leu-Val, Val-Leu, Ile-Ile, Leu-Ile, Ile-Leu ou Leu-Leu. Os autores concluíram que os músculos incubados com os

dipeptídeos selecionados tiveram aumento no conteúdo de glicogênio e que as PSLH possuem peptídeos bioativos, com capacidade de elevar os estoques de glicogênio muscular.

1.2.6 Presença de aminoácidos de cadeia ramificada

As proteínas do soro do leite apresentam elevada concentração de aminoácidos de cadeia ramificada (BCAAs – leucina, isoleucina, valina). Esses aminoácidos são metabolizados principalmente no músculo esquelético, onde são utilizados como substrato energético e também como precursores da síntese de outros aminoácidos e proteínas (PLATELL et al., 2000).

Os BCAAs podem agir na regulação da síntese protéica, secreção de insulina, redução da fadiga periférica e central induzida pelo exercício físico. Além disso, acredita-se que os BCAAs contribuam para o metabolismo energético como fonte de energia e de substratos para síntese de intermediários do ciclo do ácido cítrico e para a gliconeogênese (BLOMSTRAND 2006a; YOSHIZAWA, 2012).

Estudos com BCAAs sugerem que a leucina e a isoleucina podem estar envolvidas na regulação do metabolismo de glicose (DOI et al., 2007; NISHITANI et al., 2005). A isoleucina foi relatada como um aminoácido com capacidade hipoglicemiante, que estimula a entrada da glicose no músculo esquelético sem elevar os níveis de insulina plasmáticos (DOI et al., 2003; WANG et al., 2012). No fígado, a isoleucina inibiu a gliconeogênese, diminuindo a atividade de enzimas glicogênicas e a produção de glicose. O mecanismo foi associado ao efeito hipoglicemiante da isoleucina (DOI et al., 2007; YOSHIZAWA, 2012).

A suplementação aguda ou crônica, durante 6 semanas, com isoleucina, melhorou o metabolismo de glicose em camundongos intolerantes à glicose e diabéticos (tipo II). Os resultados mostram que a isoleucina reduziu os níveis de glicose sanguínea e melhorou a sensibilidade à insulina dos animais (IKEHARA et al., 2008).

Bernard et al. (2012) verificaram que uma mistura de aminoácidos, composta principalmente por isoleucina, na presença de insulina, favoreceu a entrada de glicose em músculo de ratos. Nesse estudo, a translocação de GLUT-4 foi quantificada e associada ao aumento na fosforilação da proteína AS160, revelando ser independente da ativação das proteínas PI-3 quinase e AMPK.

Diversos estudos verificaram que a isoleucina favorece a captação de glicose no músculo, mas não promove a síntese de glicogênio (DOI et al., 2003; DOI et al., 2005; DOI et al., 2007; NISHITANI et al., 2005). Embora o mecanismo ainda não tenha sido esclarecido, Doi et al. (2007) sugerem que a isoleucina promova a entrada de glicose no músculo, que é oxidada imediatamente após a sua captação e consequentemente, diminua os níveis de AMP, melhorando o estado de energia do tecido.

A leucina exerce importante papel no metabolismo protéico, promovendo a síntese protéica por meio de mecanismos envolvendo mTOR/p70S6k (BLOMSTRAND et al., 2006b). Lollo et al. (2012) investigaram o efeito da suplementação com PSL acrescida com diferentes doses de leucina (0, 3, 4.5 e 6%) nas proteínas mTOR e p70S6K em diafragma de ratos sedentários e exercitados. Os autores verificaram uma relação dose-dependente, a dose mais elevada de leucina resultou na maior ativação das proteínas estudadas.

A leucina também estimula a secreção de insulina, favorecendo o anabolismo muscular. No metabolismo de glicose, a leucina não foi associada à maior entrada de glicose no músculo, apesar de favorecer a liberação de insulina (DOI et al., 2007). Doi et al. (2005) verificaram que a administração oral de leucina (1,35 g/kg de peso corpóreo) não aumentou, após uma hora da administração, o transporte de glicose em músculo de ratos.

Baum et al. (2005) verificaram, em ratos, que uma única dose oral de leucina não causou efeito na ativação da proteína PI3-quinase, uma importante proteína da via da insulina, envolvida na entrada de glicose no músculo. Além disso, os dados indicam que a leucina apresentou efeitos mínimos nos níveis de insulina plasmática e não alterou a glicemia e a captação de glicose nos músculo dos animais.

Por meio de análise de músculo esquelético de ratos, Iwanaka et al. (2010) mostraram que a leucina inibiu o transporte de glicose estimulado pela insulina, no entanto, favoreceu o transporte de glicose via contração muscular. Os resultados obtidos com a leucina foram associados principalmente a ativação das proteínas mTOR/p70S6K.

Evidências indicam que os aminoácidos leucina e isoleucina podem agir de maneira distinta no metabolismo de glicose. A leucina estimula a liberação de insulina no pâncreas, mas não promove a entrada de glicose dependente de insulina. A isoleucina estimula a entrada de glicose no músculo e não contribui para a síntese de glicogênio, no entanto, poderia favorecer a produção de ATP ou agir no metabolismo de lipídeos (DOI et al., 2003).

2. Transportadores de glicose

A glicose fornecida pela dieta deve ser transportada através da circulação para as células alvo. A entrada da glicose na célula é realizada por proteínas na membrana plasmática, que podem ser classificadas, de acordo com a funcionalidade, em 2 tipos: a) co-transportadores glicose/sódio, que compreende uma grande variedade de transportadores de glicose dependentes de sódio; b) transportadores de glicose sódio-independente (GLUTs) (WRIGHT et al., 2001; MUECKLER, 1994; JOOST, THORENS, 2001). Os GLUTs permitem o influxo de glicose através da membrana plasmática e diferem entre si em relação à especificidade pelo substrato, propriedades cinéticas e expressão tecidual. Os transportadores de glicose são uma família composta por 14 membros, as primeiras variantes parecem ser as principais, e têm sido foco de estudos que buscam caracterizar os fluxos de glicose, tanto em situações fisiológicas como fisiopatológicas (THORENS, MUECKLER, 2010).

Na sequência, na Tabela 1 são listados alguns dos principais transportadores de glicose e suas características: expressão tecidual, constante de Michaelis (K_m) que indica a afinidade do transportador pelo substrato, e as principais funções dos transportadores de glicose.

Tabela 1. Características dos transportadores de glicose sódio-independentes (GLUTs)

Transportador	Presença principal	K_m	Função(ões) principal(is)	Referência
GLUT1	Todos os tecidos	baixo	Influxo celular de glicose em glicemia fisiológica	(GOROVITS, CHARRON, 2003)
GLUT2	Fígado e pâncreas	alto	Influxo celular de glicose em glicemia elevada / secreção de insulina	(GOROVITS, CHARRON, 2003)
GLUT3	Cérebro e tecido muscular	alto	Influxo celular de glicose em glicemia elevada	(STUART, WEN, JIANG, 1999)
GLUT4	Músculo esquelético, cardíaco e tecido adiposo	alto	Influxo celular de glicose em glicemia elevada induzida por insulina	(DOHM, 2002)
GLUT5	Intestino delgado, testículos e rins	alto	Alta afinidade por frutose	(CHEESEMAN, 2008)
GLUT6	Cérebro, leucócitos e baço	baixo	Influxo celular de glicose em glicemia fisiológica	(DOEGE et al., 2000)
GLUT7	Intestino delgado e grosso	alto	Transporte de glicose e frutose	(CHEESEMAN, 2008)

GLUT-4 e GLUT-1 são os principais transportadores de glicose presentes no músculo esquelético. Em menor quantidade, está presente o GLUT-1 que exerce papel importante na entrada basal de glicose, sendo GLUT-4 o principal transportador de glicose presente no músculo esquelético (JENTJENS, JEUKENDRUP, 2003).

2.1. Transportador de Glicose 4 (GLUT-4)

A entrada de glicose no músculo é um processo complexo. Para ser metabolizada, a glicose precisa migrar do sangue para o interstício e deste, para o espaço intracelular, e assim ser fosforilada em glicose-6-fosfato. A migração da glicose do sangue para o interstício é determinada, entre outros fatores, pelo fluxo sanguíneo no músculo e pela permeabilidade endotelial da glicose. O número de transportadores de glicose e a atividade destes determinam a capacidade do transporte de glicose do interstício para o espaço intracelular (WASSERMAN, AYALA, 2005).

A capacidade do exercício físico em melhorar a ação da insulina e desse modo influenciar o aumento da entrada de glicose no músculo esquelético tem sido relatada em vários estudos (KUO et al., 2004; CHRIST-ROBERTS, MANDARINO, 2004; DOHM, 2002; REN et al., 1994). No entanto, os mecanismos bioquímicos e moleculares envolvidos no efeito do exercício físico agudo e do treinamento físico no metabolismo de glicose ainda estão sendo determinados.

No músculo esquelético, o transporte de glicose para dentro da célula é realizado principalmente pelo transportador GLUT-4. Sob condições basais, a maior parte das moléculas de GLUT-4 encontra-se em vesículas no espaço intracelular. Em resposta à insulina ou à contração do músculo, GLUT-4 é translocado para a membrana plasmática, onde é inserido para realizar o transporte de glicose para dentro da célula (DOHM, 2002).

O exercício promove o aumento do transportador de glicose GLUT-4 na membrana plasmática, o qual torna possível uma rápida entrada de glicose e maior estoque de glicogênio no músculo (HOLLOSZY, 2008).

Christ-Roberts e Mandarino (2004) relataram que o exercício agudo, isto é, aquele realizado em uma única sessão, resultou na maior translocação de GLUT-4 para a membrana plasmática, e também aumento na atividade da enzima glicogênio sintase. Com relação ao treinamento físico, os autores verificaram aumento na expressão de proteínas envolvidas no metabolismo de glicose, incluindo o transportador GLUT-4, Akt e glicogênio sintase.

Ren et al. (1994) verificaram, em ratos, aumento de 50% da expressão da proteína GLUT-4 após 16 horas de uma única e prolongada sessão de exercício, e aumento de 100% após 16 horas de uma segunda sessão de exercício no dia seguinte. O aumento de GLUT-4 foi acompanhado por maior transporte de glicose estimulado por insulina. Em estudo realizado por Host et al. (1998), os resultados encontrados indicam que a expressão de GLUT-4 tem meia-vida curta e muda rapidamente. Os autores verificaram que após 40 horas do término do exercício físico, os níveis de GLUT-4 foram semelhantes aos níveis encontrados no grupo controle, sem exercício.

2.2. Sinalização de GLUT-4 por insulina

A translocação do GLUT-4 para a membrana plasmática é estimulada por alguns fatores, entre eles, a insulina e o exercício físico. A insulina desencadeia seus efeitos biológicos mediante a interação com seu receptor e fosforilação de

proteínas. O receptor de insulina (IR) é uma proteína com atividade tirosina-quinase intrínseca. A ligação da insulina em seu receptor provoca alteração conformacional que resulta na autofosforilação dessa proteína (CARVALHEIRA, ZECCHIN, SAAD, 2002) e que em seguida, fosforila uma série de proteínas. Isso permite a modulação da subunidade regulatória p85 da fosfatidilinositol-3 quinase (PI3-K) (WATSON, PESSIN, 2001) que é uma proteína de importância fundamental na regulação das ações da insulina. Esta proteína é formada por uma subunidade regulatória (p85) e uma subunidade catalítica (p110), que catalisa a fosforilação dos fosfoinositídeos e recruta proteínas como a Akt (KROOK, WALLBERG-HENRIKSSON, ZIERATH, 2004). A ativação da Akt eleva a captação de glicose através do aumento da translocação do GLUT-4 (ŽDYCHOVA, KOMERS, 2005), da mesma forma, PKCδ e PKCλ aumentam a captação de glicose através da ativação e translocação do GLUT-4 (KHAN, PESSIN, 2002; LIU et al., 2010).

2.3. Sinalização de GLUT-4 por exercício

Além da insulina, o exercício físico também aumenta a translocação de GLUT-4 para a membrana plasmática. Muitos estudos demonstram que a prática regular de atividade física aumenta a sensibilidade à insulina (SHAW et al., 2010) e a proteína AMPK (proteína quinase ativada por 5´-AMP) pode ter papel relevante nesse processo.

A AMPK é uma proteína formada por uma subunidade catalítica (α), que apresenta duas isoformas (α 1 e α 2) e duas subunidades regulatórias (β e γ)

(HARDIE, CARLING, 1997) ativadas por fosforilação (treonina 172) quando há elevação na razão AMP:ATP (HARDIE et al., 2003). Sua ativação estimula vias geradoras de ATP e inibe vias anabólicas que consomem o ATP, tais como a síntese de proteínas. O aumento do fornecimento de substrato energético para a célula mediado pela ativação da AMPK acontece também durante o exercício (altas concentrações de AMP) através da translocação de GLUT-4 para a membrana plasmática aumentando o transporte de glicose para o músculo, assim como a insulina, no entanto por via de sinalização diferente (MCGEE et al., 2003).

A AMPK funciona como um sensor de energia da célula muscular. Durante períodos de grande demanda de energia no músculo, como durante o exercício físico, a ativação da AMPK aumenta o transporte de glicose e diminui a atividade da enzima glicogênio sintase (GS), favorecendo a oxidação da glicose. Em períodos de repouso, a ativação de AMPK no músculo parece favorecer o armazenamento de glicose (FRIEDRICHSEN et al., 2012).

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Capítulo 2

Preparado para Molecular Nutrition and Food Research

CAPÍTULO 2 - WHEY PROTEIN HYDROLYSATE INCREASES GLUT-4 IN PLASMATIC MEMBRANE

Whey protein hydrolysate increases the translocation of GLUT-4 to the plasmatic membrane independent of the insulin response in sedentary and exercised Wistar rats

Short title: Whey protein hydrolysate increases GLUT-4 in plasmatic membrane

Abbreviations

Akt – Protein kinase B

ALT – Alanine aminotransferase

AST – Aspartate amino transferase

CAS – Casein

CK – Creatine kinase

GLUT-1 – Glucose transporter 1

GLUT-4 – Glucose transporter 4

LDH – Lactate dehydrogenase

p85 – PI 3 Kinase p85 alpha

PM – Plasmatic membrane

UC – Uric acid

WP – Whey protein

WPH – Whey protein hydrolysate

Abstract

Whey protein (WP) and whey protein hydrolysate (WPH) have the recognised capacity to increase glycogen stores. The objective of this study was to verify if consuming WP and WPH could modulate the concentration of the glucose transporters GLUT-1 and GLUT-4 in the plasmatic membrane (PM) of the muscle cells of sedentary and exercised animals. Forty-eight animals, divided into 6 groups (n=8 per group), were treated and fed with experimental diets for 9 days as follows: a) control–casein (CAS); b) WP; c) WPH; d) CAS exercised; e) WP exercised; and f) WPH exercised. After the experimental period, the animals were sacrificed; GLUT-1 and GLUT-4 were analysed by western blotting; and the glycogen, aminogram, insulin levels and biochemical health indicators were analysed using standard methods. Consumption of WPH significantly increased the concentrations of GLUT-4 in the PM and glycogen, whereas the GLUT-1 and insulin levels and the health indicators showed no alterations.

Keywords: glucose transporters, glycogen, dietary protein, whey protein, insulin resistance

1. Introduction

Different food proteins may affect muscle metabolism in different fashions, as shown when the effects of whey proteins (WP) are compared to those of casein (CAS) [1-3]. One difference between WP and CAS is that WP stimulates an increase of fatty acid synthesis in the muscle accompanied by a concomitant decrease in fatty acid synthesis in the liver, considered to be a positive effect on lipid metabolism [4]. Hydrolysis of the protein alone could alter the biological function of the protein, thus affecting the metabolism [5]. For example, it has been suggested that a slight change in the physicochemical form of the protein when presented to the animal can be enough to influence the general metabolism, apparently as a result of the various peptides that are generated during partial enzymatic hydrolysis of whey proteins (WPH) [3,6-8]. The bioactive peptides present in WPH might be capable of reducing the levels of creatine kinase in both exercising rats [1] and soccer players [9].

Alterations in glycogen metabolism have most likely been the most frequently reported positive feature resulting from substituting WP with WPH [7, 8, 10]. Morifuji et al. [6] showed that from seven possible dipeptides containing the branched-chain amino acids found in milk-whey proteins, the peptide Ile-Leu was capable of increasing the uptake of glucose by isolated rat muscle. These reports suggest that WP promotes physiological responses different from those of CAS, such as greater endurance exercise performance [11], better post-exhaustion glycemic levels and higher glycogen levels [7, 8, 10]. Muscle glycogen is the

primary fuel source during prolonged moderate-to-high intensity exercise [12], so an increase in glycogen could increase performance.

Two isoforms from the facilitative glucose transporter family, GLUT-4 and GLUT-1, are expressed in skeletal muscle [13]. GLUT-1 is present in very low amounts in skeletal muscle and has been suggested to influence basal glucose uptake by the muscle [14]. The abundance of GLUT-4 protein is a primary factor in determining the maximal rate of glucose transport into skeletal muscle. Under normal resting conditions, most of the GLUT-4 molecules reside in membrane vesicles inside the muscle cell. In response to insulin or muscle contractions, GLUT-4 translocates to the cell membrane, where it is inserted to increase glucose transport [15, 16].

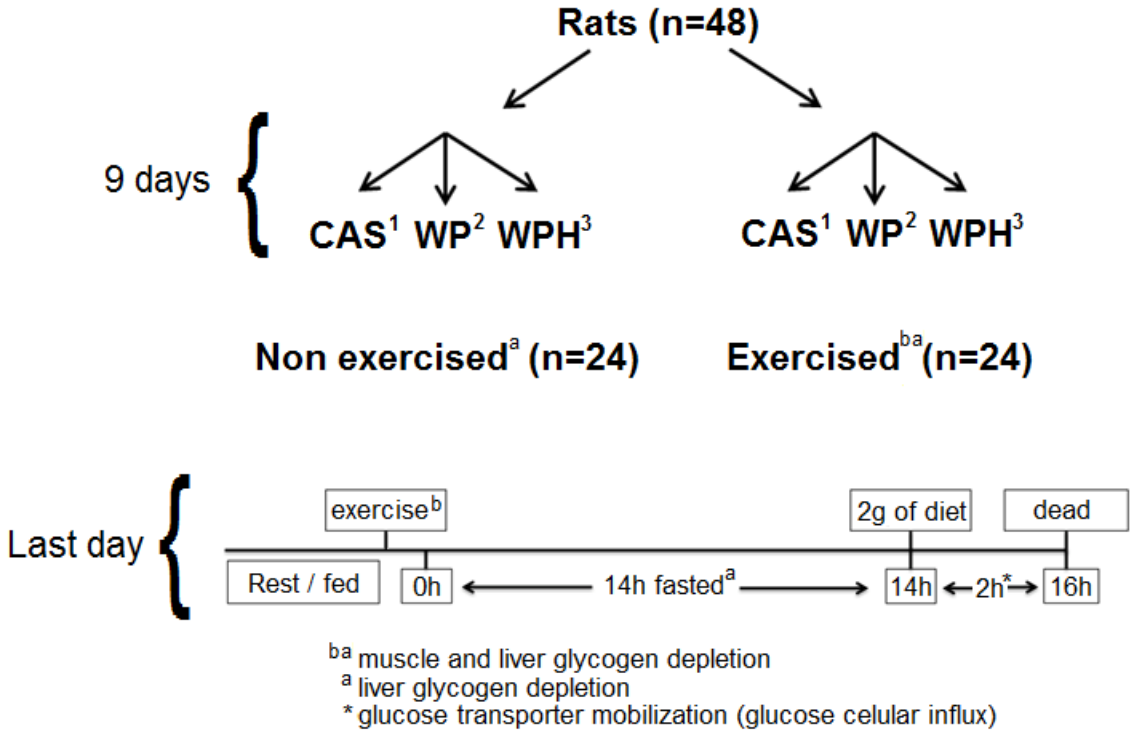
The present study was designed to investigate the possible effects produced by the consumption of WP and WPH, compared to those of CAS, with respect to several parameters of metabolism, which included the following: the levels of GLUT-4, GLUT-1, p85, Akt, phosphorylated-Akt, glycogen, serum insulin, and serum amino acids of Wistar rats. These parameters were assessed for the three types of protein under two states of physical activity, sedentary and exercised.

2. Methods

Forty-eight male Wistar rats (~100 g; n=8 per group) were divided into sedentary and exercised groups, and each group was fed casein (CAS, control), whey protein (WP) or whey protein hydrolysate (WPH) as their dietary protein source for a total of 9 days. On the last day, the rats reached ~ 150 g of body

mass and all of the animals had fasted overnight to arrive at similar glycogen reserves, but two hours before sacrifice, they each received 2 g of the appropriate experimental diet (Figure 1). The experimental protocol was based on Morifuji et al. [17]. Food consumption was determined every other day, and body mass evolution was monitored weekly. The research methodology was approved by the Ethics Committee on Animal Experimentation (CEEA-UNICAMP, protocol 2376-1/2011).

Figure 1. Experimental design



2.1. Training Protocol

Exercised rats underwent training on a treadmill, running for 60 minutes at 15 m/min 16 hours before sacrifice. A single exercise session enhances GLUT-4 translocation to the plasma membrane [15, 16, 18]. This procedure allowed one to carry out the analyses with the animals homogeneously depleted of glycogen and

at a moment of great influx of glucose into the cells when mobilization of the glucose transporters was maximum [18,19].

2.2. Experimental diets

The diets (Table 1) were isonitrogenous (approximately 12% protein, dry weight basis), isolipidic and isocaloric (approximately 360 kcal/100 g), and each was formulated following the recommendations of the American Institute of Nutrition, AIN93-G diet [20]. The three diets differed from each other with respect to the nature of the protein source; the control diet was casein (~ 88%, mesh 30, Conaprole, Uruguay) and the two experimental diets were whey protein diets, one of which was pre-hydrolysed (Hilmar product 8350, ~ 80% whey protein hydrolysate) and the other was not (Hilmar product 8000, ~ 80% whey protein concentrate). The complete amino acid profile of each protein was determined (Table 2). The degree of hydrolysis in the pre-hydrolysed whey protein was approximately 12.5%, and both whey proteins were donated by Hilmar Ingredients (Dalhart, TX, USA). The standard reference protein was casein. The molecular weight distribution of the WPH peptides showed 40·5% < 1 kDa, 26·7% between 1 and 5 kDa, and 15·6% between 5 and 20 kDa.

Table 1. Diets composition (g/kg of diet)

Ingredient	CAS[1] (g)	WP[2] (g)	WPH[3] (g)
Corn Starch	437.92	427.31	425.00
Dextrinised starch	145.42	141.90	141.13
Sucarose	110.16	107.50	106.92
WPH[3]	-------	-------	156.41
WP[2]	-------	152.77	-------
CAS[1]	135.96	-------	-------
Vegetable oil	70.00	70.00	70.00
Fiber (cellulose)	50.00	50.00	50.00
Mineral mixture	35.00	35.00	35.00
Vitamin mixture	10.00	10.00	10.00
L-Cystine	3.00	3.00	3.00
Choline bitartrate	2.50	2.50	2.50
Tert-butylhydroquinone	0.014	0.014	0.014

1 – CAS: casein; 2 – WP: whey protein; 3 – WPH: whey protein hydrolysate.

Table 2. Amino acid profile of the protein sources (g/100g of protein)

Amino Acid	CAS	WP	WPH
Asparagine	5.96	11.52	11.16
Glutamate	19.00	18.82	17.99
Serine	4.68	5.31	5.04
Glycine	1.39	1.74	1.75
Histidine	2.12	1.31	1.27
Arginine	3.03	2.66	2.31
Threonine	3.56	7.64	7.40
Alanine	2.30	5.11	4.89
Proline	8.85	5.89	5.68
Tyrosine	4.57	2.88	2.78
Methionine	**2.32**	**2.51**	**2.52**
Cystine	**0.16**	**1.48**	**1.60**
Isoleucine	4.51	6.88	6.97
Leucine	7.62	10.14	10.15
Valine	5.36	5.68	5.81
Phenylalanine	3.89	2.86	2.78
Lysine	6.62	9.20	9.48
MET+CYS[1]	**2.48**	**3,99**	**4,12**

[1]Sum of methionine and cysteine.

2.3. Biochemical parameters

Blood samples were collected in Vacutainers, maintained at 4℃ and then centrifuged at 3000g (4℃, 12 min) to obtain the se rum. The assessment of the serum included the following: uric acid, urea, aspartate amino transferase (AST), alanine aminotransferase ALT, creatine kinase (CK) and lactate dehydrogenase (LDH). The standard enzymatic spectrophotometric determinations were carried out employing Laborlab kits (São Paulo, Brazil). For the glycogen analysis, samples of the skeletal muscle, heart and liver were collected. Tissue glycogen was isolated and purified by precipitation with ethanol after basic digestion, and then it was quantified by the phenol-sulfuric acid method [21]. The serum insulin levels were measured using a rat/mouse insulin ELISA (Millipore), and the glucose concentrations were measured using an Accu-Chek Active glucometer (Roche Diagnostics, Mannheim, Germany).

2.4. Protein extraction and immunoblotting

The total protein content of the skeletal muscle was determined by the Lowry method [22]. For immunoblotting, the tissue homogenates were subjected to SDS-PAGE and transferred to a nitrocellulose membrane using the Wide Biocom Western blot system (Bridge of Weir, UK). The blots were probed with the appropriate antibodies against GLUT-4 (Abcam, Cambridge; catalogue number ab654, diluted 1:5000), GLUT-1 (Abcam catalogue number ab40084, diluted 1:1000), Akt 1/2/3 (H-136) antibody SC8312 (Santa Cruz Biotechnology, CA, USA

diluted 1:1000), p-Akt 1,2,3 Ser 473, antibody SC7985-R (Santa Cruz Biotechnology, CA, USA diluted 1:1000), and Anti-PI 3-Kinase p85, N-SH2 domain (catalogue number #06-496, Upstate Biotechnology NY, USA diluted 1:1000). To assess the levels of these proteins in the muscle, the loading control was tubulin (Abcam, Cambridge, catalogue number ab44928, diluted 1:1000), and the appropriate secondary antibody conjugated to peroxidase and the BM chemiluminiscence blotting system were used for detection. The bands were visualised by chemiluminiscence (GE – ImageQuant LAS4000, Piscataway, NJ, USA), and the band intensities were quantified by scanning and processed using the ImageJ program (v. 1.44 for Windows).

2.5. Amino acid composition of the protein sources and plasma free amino acids

The protein sources were hydrolysed at 110 ℃ in 6 M HCl for 24 h. The hydrolysed samples (wet basis) were then diluted in deionised water; α-aminobutyric acid was added as the internal standard (Sigma–Aldrich Corp., St Louis, MO), and the amino acids were derivatised with phenylisothiocyanate [20]. The plasma free amino acids were extracted using methanol and derivatised with phenylisothiocyanate [23]. The PTH-derivatives were chromatographed using a Luna C-18, 100 Å, 5 µm, 250 x 4.6 mm (Phenomenex, Torrance, CA), at 50℃. The mobile phase composed of eluents A (sodium acetate 0,56 M with triethylamine 0,05% and acetonitrile 0,06%, pH 6.4) and B (acetonitrile + water 60:40). The flow rate was 1 mL/min and detection was at 254 nm. Quantification was

performed by comparison with a standard mixture, and the internal standard was DL-2-aminobutyric acid (Sigma-Aldrich Corp, St Louis, MO, USA). The mixture was sonicated for 10 minutes and further homogenised for 1 hour, followed by centrifugation at 8,500g for 15 minutes. The supernatant was filtered through a 0.22 mm membrane, a 40 μL aliquot was derivatised as described above, and 20 μL was injected into the liquid chromatograph.

2.6. Statistical analysis

The results were subjected to statistical analysis using the software SPSS (Statistical Package for the Social Sciences, Chicago, United States), version 17.0. The data were tested for normality (Kolmogorov-Smirnov test) and homogeneity using the tools available therein. For the parametric data, the multivariate analysis of variance (ANOVA) was used, and the means were compared (Duncan test), adopting the value of $p < 0.05$ as the criterion for statistical significance.

3. Results

The consumption of whey protein and whey protein hydrolysate for 9 days resulted in a significant increase ($p<0.05$) in GLUT-4 translocation to the plasmatic membrane (Figure 2A). Phosphorylation of Akt at serine 473 (Figure 2E) was increased ($p<0.05$) by the consumption of whey protein hydrolysate in both groups, sedentary and exercised, whereas there was no difference in the serum insulin concentrations between the control group (casein as dietary protein) and the WPH

group. However, the consumption of WPH also increased glycogen concentrations in the heart, skeletal muscle and liver (Figure 2G-I).

Figure 2

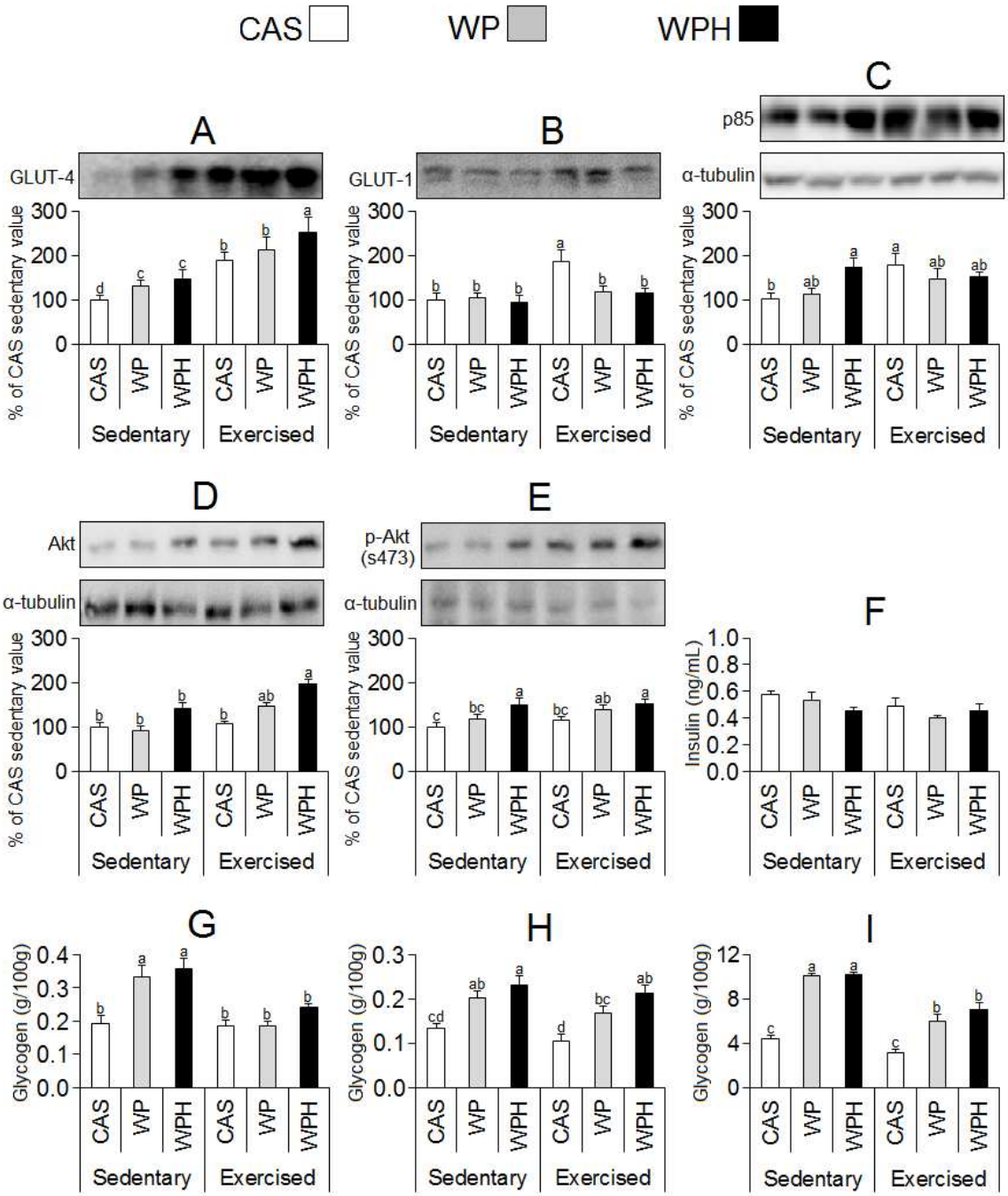


Figure 2. Means and standard error for the concentrations of: **A)** GLUT-4; **B)** GLUT-1; **C)** p85 / tubulin; **D)** Akt / tubulin; **E)** Akt phosphorylate (Ser 473) / tubulin in the skeletal muscle; (**F**) insulin in the serum; and of glycogen in the: **G)** heart; **H)** skeletal muscle and **I)** liver. The effect of 9 days of dietary whey protein (WP □, n=8) and whey protein hydrolysate (WPH ■, n=8) on sedentary and exercised (in treadmill) Wistar rats, with the control group receiving the standard diet with casein (CAS □, n=8). The AIN93-G WP and WPH diets were prepared by substituting casein of the AIN93 (standard) diet with whey protein or whey protein hydrolysate. Different superscript lowercase letters indicate significant differences between the groups. ANOVA was used, and the means were compared (Duncan test), adopting the value of $p< 0.05$ as the criterion for statistical significance.

The following general health parameters were accessed: glucose, uric acid and urea. In addition, AST and ALT were accessed as hepatic health parameters, and CK and LDH were assessed as muscle damage indicators (Table 3). No differences ($p>0.05$) were observed.

Table 3 – Biochemical blood parameters in serum.

	Sedentary						Exercised					
	CAS		WP		WPH		CAS		WP		WPH	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Glucose[1]	137.13	3.70	142.25	4.30	140.38	4.13	141.88	5.70	135.13	3.50	140.00	3.05
LDH[A2]	778.92	67.89	954.02	34.43	947.48	25.82	855.64	58.57	940.01	30.45	949.75	36.58
CK[B2]	951.95	136.81	942.63	107.98	1262.58	132.24	1343.93	119.60	1104.57	105.31	1192.15	123.65
ALT[C2]	24.14	2.1	24.36	2.41	27.69	1.49	24.13	2.22	27.45	2.60	34.19	3.83
AST[D2]	114.64	11.19	128.12	3.21	145.18	7.43	126.32	7.50	134.23	9.59	120.79	17.06
HEMO[E1]	16.54^{bc}	1.06	20.67^{a}	0.81	18.30^{ab}	0.58	13.61^{d}	0.55	12.96^{d}	0.60	14.55^{cd}	0.67
TPROT[F1]	5.80^{ab}	0.36	5.83^{ab}	0.26	6.49^{a}	0.10	5.51^{ab}	0.22	4.92^{b}	0.28	5.39^{b}	0.18
Urea[1]	22.22^{b}	1.86	22.19^{b}	1.39	22.96^{b}	1.48	31.17^{a}	1.21	29.61^{a}	1.62	32.25^{a}	2.78
UA[1G]	0.58^{b}	0.08	0.97^{b}	0.21	0.94^{b}	0.07	1.77^{a}	0.33	2.18^{a}	0.45	0.66^{b}	0.04

1 – mg/DL; 2 – U/L. [A] LDH: lactate dehydrogenase; [B] CK: creatine kinase; [C] ALT: alanine aminotransferase; [D] AST: aspartate aminotransferase; [E] HEMO: hemoglobin; [F] TPROT: total proteins; [G] UA: uric acid. The effect of 9 days of dietary whey protein (WP) and whey protein hydrolysate (WPH) on the serum homeostasis parameters of sedentary and exercised (in treadmill) Wistar rats, with the control group receiving the standard diet with casein (CAS). The AIN93-G WP and WPH diets were prepared by substituting casein of the AIN93 (standard) diet with whey protein or whey protein

hydrolysate. Different superscript lowercase letters indicate significant differences between the groups. ANOVA was used, and the means were compared (Duncan test), adopting the value of $p< 0.05$ as the criterion for statistical significance.

The change in dietary protein had no effect ($p<0.05$) on growth, the weight of the organs, the organ weight / body weight relationship (data not shown) or the biochemical indicators of health and renal and liver function (Table 3). The complete amino acid profiles (Table 4) were obtained, and of the alterations detected, the most notable in the context of this study was the increase in the plasmatic levels of taurine in the exercised animals.

Table 4 – Mean (M) and standard error of the mean (SEM) of amino acids concentrations in plasma (µmol/L)

	Sedentary						Exercised					
	CAS		WP		WPH		CAS		WP		WPH	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
ASP	59,43^{b}	3,45	64,02^{ab}	2,56	66,39^{a}	2,19	65,54^{a}	3,21	67,05^{a}	3,69	65,39^{a}	2,42
GLU	68,70	2,13	57,20	2,46	27,08	0,84	29,45	1,00	20,36	1,14	15,68	0,63
HPRO	52,62	2,68	50,10	1,75	43,00	1,46	49,15	2,51	48,87	2,93	52,43	2,25
ASN	65,60	2,62	61,41	2,40	63,01	1,95	68,99	4,07	63,50	2,86	66,44	3,65
SER	158,30^{a}	5,54	125,27^{ab}	4,26	134,44^{ab}	5,51	168,11^{a}	6,72	151,27^{a}	6,96	104,40^{b}	5,74
GLN	661,50^{a}	27,12	651,40^{a}	38,43	703,86^{a}	28,15	554,14^{b}	26,60	569,75^{b}	20,51	557,25^{b}	20,06
GLY	174,35	9,24	165,37	5,46	287,97	15,26	218,08	9,16	164,57	8,23	284,61	10,25
HIS	13,86^{b}	0,69	24,79^{a}	0,92	25,90^{a}	0,85	16,31^{a}	0,77	19,67^{a}	0,75	15,50^{a}	0,56
ARG	21,43^{ab}	1,26	10,15^{b}	0,57	18,47^{b}	0,70	43,21^{a}	1,51	11,06^{b}	0,65	3,31^{b}	0,11
TAU	**168,83^{ab}**	**6,25**	**183,32^{a}**	**10,27**	**163,70^{ab}**	**8,35**	**111,47^{c}**	**3,34**	**155,39^{ab}**	**6,22**	**137,25^{b}**	**4,94**
THR	148,93^{c}	6,26	246,99^{ab}	12,60	249,29^{ab}	9,97	198,34^{bc}	8,13	284,09^{a}	9,66	198,86^{bc}	11,14
ALA	478,76	17,24	507,19	18,77	551,80	33,11	519,43	25,97	551,27	24,26	515,67	29,39

PRO	174,18^{a}	6,62	85,09^{b}	4,94	100,82^{b}	4,23	218,39^{a}	9,61	108,33^{b}	3,57	62,08^{b}	2,17
TYR	40,50^{d}	2,23	65,05^{bc}	3,12	60,70^{c}	3,16	59,48^{c}	2,26	84,16^{a}	3,20	76,02^{ab}	3,27
VAL	56,54^{a}	3,11	28,82^{b}	1,35	51,11^{a}	3,07	53,98^{a}	2,86	14,00^{b}	0,63	16,76^{b}	1,01
MET	**99,28^{b}**	**4,17**	**80,01^{b}**	**4,32**	**190,78^{a}**	**7,82**	**176,31^{a}**	**7,40**	**164,69^{a}**	**5,93**	**118,82^{b}**	**6,89**
CIS	**62,39**	**2,68**	**62,82**	**2,95**	**62,60**	**2,38**	**59,76**	**2,27**	**66,74**	**2,40**	**73,63**	**2,87**
ILE	30,31^{cd}	1,49	32,03^{bc}	1,86	21,15^{d}	0,72	30,78^{bc}	1,02	47,72^{a}	1,96	39,83^{ab}	1,55
LEU	9,47^{b}	3,20	18,71^{b}	1,07	43,94^{a}	1,85	13,66^{b}	0,57	7,18^{b}	0,35	13,37^{b}	0,67
PHE	20,87^{b}	0,94	32,65^{a}	1,47	15,87^{b}	0,94	13,26^{b}	0,76	32,24^{a}	1,13	20,83^{b}	0,79
TRP	55,66^{b}	2,28	104,04^{a}	5,41	79,69^{ab}	2,79	69,24^{ab}	3,39	60,48^{ab}	3,27	94,59^{ab}	5,20
LYS	466,60	20,06	410,89	18,49	459,01	17,44	440,04	22,00	511,41	27,62	421,97	13,92
Sum	3082,56	114,05	3067,32	159,50	3420,59	194,97	3177,11	168,39	3203,81	169,80	2954,67	177,28

ASP: aspartate, GLU: glutamate, HPRO: hydroxyproline ASN: asparagine, SER: serine, GLN: glutamine, GLY: glycine, HIS: histidine, ARG: arginine, TAU: taurine, THR: threonine, ALA: alanine, PRO: proline, TYR: tyrosin, VAL: valine, MET: methionine, CYS: cysteine, ILE: isoleucine, LEU: leucine, PHE: phenylalanine, TRP: tryptophan, LYS: lysine. Taurine and their precursors (MET and CYS) amino acids are bolded. The AIN93-G WP and WPH diets were prepared by substituting casein of the AIN93 (standard) diet with whey protein or whey protein hydrolysate. Different letters indicate significant differences between groups, ANOVA was used and means were compared (Duncan test), adopting the value of $p< 0.05$ as a criterion for statistical significance.

4. Discussion

Based on previous findings by the present [3,7,8] and other [10,24] authors clearly showing that the consumption of WP and WPH raised muscle and hepatic glycogen levels, the objective of the present study was to verify the effect that the consumption of WP and WPH had on the translocation of the glucose transporters GLUT-4 and GLUT-1 to the plasmatic membrane (PM), as compared to rats fed a standard diet (AIN93-G) with casein as the protein source. The results showed clearly that the consumption of WP and WPH increased the translocation of GLUT-4 (Figure 2A) when compared to casein-fed animals, whereas GLUT-1 (Figure 2B) was not responsive to the different proteins. This increase in GLUT-4 in the PM was consistent with increases in glycogen (Figure 2G-I) because with more glucose transporters in the cell plasmatic membrane, the availability of glucose and synthesis of glycogen could both increase. Physical exercise increased the potential for the translocation of GLUT-4 to the membrane [16, 25], and for all diets, the exercised animals demonstrated higher levels of GLUT-4 in the PM.

One of the primary methods to increase the concentration of GLUT-4 in the plasmatic membrane is through insulin-regulated trafficking [26]. However, in the present experiment, no increase was noted in the plasmatic insulin levels in the groups consuming WPH (Figure 2F). The results obtained for the proteins involved in the insulin signaling route suggested that the total (Figure 2D) and phosphorylated (Figure 2E) Akt residues in serine 473 increased significantly ($p<0.05$) in the groups consuming WP and WPH, with the greatest increase

occurring in the WPH group. Greater phosphorylation of Akt is capable of increasing the mobilization of GLUT-4 to the PM. In contrast, p85, which is also involved in insulin-regulated trafficking, was apparently not affected by the consumption of WP and WPH (Figure 2C). The translocation of GLUT-4 to the PM can also be stimulated in an insulin-independent way. Carneiro et al. [27] accomplished this through taurine activation of the insulin pathway, thus raising the GLUT-4 concentration in the plasmatic membrane independent of insulin. However, the molecular mechanism behind this effect has still not been elucidated [28]. In the exercised animals of the WP and WPH groups, the plasmatic concentrations of taurine (Table 4) were greater ($p<0.05$) than those in the control group consuming CAS, although there was no such significant difference amongst the sedentary animals ($p>0.05$). This could explain, at least in part, the greater translocation of GLUT-4 in the WP and WPH groups. After investigation of the amino acid composition of the WP and WPH, it was found they were rich in sulfurous amino acids (Table 2), and methionine and cysteine are endogenous precursors of taurine [27]. Thus, the consumption of WP or WPH provided a greater amount of substrate for the endogenous production of taurine than casein, and the presence of this amino acid facilitated activation of the insulin pathway and the cell capture of glucose, as indicated in the literature.

In addition, to explain the results for GLUT-4 (Figure 2A), it is notable that WPH contains peptides with the ability to increase glucose capture and glycogen synthesis in vitro [6]. Morifuji et al. [6] tested the capacity of cells to capture glucose when incubated with 7 different peptides present in WPH and observed that the peptide isoleucine-leucine significantly increased the capture of glucose

and also the synthesis of glycogen. Morifuji et al. [4] also reported that animals consuming WPH presented a greater capacity to synthesise intramuscular fatty acids, which could be the result of the influx of substrate into the cell for the synthesis of fatty acids. These are effects of WPH, which could be related to the peptides present in this protein, as it has already been reported that milk proteins are rich sources of these substances, which have an important, but not elucidated, metabolic role [5].

There was no difference between the groups (Table 3) with respect to the muscle damage markers CK and LDH [29], although soccer players supplemented for 8 weeks with WPH have shown a significant decrease in these markers [9], which normally tend to increase after exercise. In addition, chronic supplementation caused no significant alterations in the analysed liver (ALT and AST) or kidney (creatinine and uric acid) health parameters. The growth and anthropometric parameters did not suggest alterations in body and organ weight due to the difference in dietary proteins. Taken together, the biochemical and anthropometric parameters suggested that WP and WPH were generally safe for consumption by Wistar rats with respect to the health parameters analysed in this study.

In conclusion, the consumption of WP and WPH as dietary sources of protein for just 9 days was capable of increasing translocation of the glucose transporter GLUT-4 to the plasmatic membrane. The effect was significantly higher in the group consuming WPH, and even greater increases were observed when the animals performed aerobic exercise in addition to consuming WPH. This increase in the translocation of GLUT-4 was accompanied by increases in the

glycogen concentration but not by alterations in the insulin concentration. The biochemical and anthropometric parameters analysed also indicated that the exclusive consumption of WP or WPH as the dietary source of protein for 9 days was safe for the health and growth of the animals. These results should encourage new studies dealing with the potential of WP and especially WPH for the treatment or prevention of type II diabetes, a disease in which there is reduced translocation of GLUT-4 to the plasmatic membrane.

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Capítulo 3

Preparado para Food Chemistry

CAPÍTULO 3 - WHEY PROTEIN HYDROLYSATE COMPONENTS INCREASE TRANSLOCATION OF GLUT-4 TO THE PLASMA MEMBRANE

A dipeptide and an amino acid present in whey protein hydrolysate increase translocation of GLUT-4 to the plasma membrane in Wistar rats

Short title: Whey protein hydrolysate components increase translocation of GLUT-4 to the plasma membrane

Abbreviations

AMPK - 5´-AMP-activated protein kinase

Akt – Protein kinase B

ALT – Alanine aminotransferase

AST – Aspartate amino transferase

CK – Creatine kinase

GLUT-4 – Glucose transporter 4

LDH – Lactate dehydrogenase

p85 – PI 3 Kinase p85 alpha

PM – Plasmatic membrane

UC – Uric acid

WPH – Whey protein hydrolysate

Abstract

Whey protein hydrolysate (WPH) is capable of increasing muscle glycogen reserves and of concentrating the glucose transporter in the plasma membrane (PM). The objective of this study was to determine which WPH components could modulate translocation of the glucose transporter GLUT-4 to the PM of animal skeletal muscle. Forty-nine animals were divided into 7 groups (n=7) and received by oral gavage 30% glucose plus 0.55 g/kg body weight of the following WPH components: a) control; b) WPH; c) L-isoleucine; d) L-leucine; e) L-leucine plus L-isoleucine; f) L-isoleucyl-L-leucine dipeptide; g) L-leucyl-L-isoleucine dipeptide. After receiving these solutions, the animals were sacrificed and the GLUT-4 analyzed by western blot. Additionally, glycogen, glycemia, insulin and free amino acids were also determined by standard methods. Of the WPH components tested, the amino acid L-isoleucine and the peptide L-leucyl-L-isoleucine showed greater efficiency in translocating GLUT-4 to the PM and of increasing glucose capture by skeletal muscle.

Keywords: L-leucyl-L-isoleucine, L-isoleucine, glucose transporter, peptides, branched-chain amino acids

1. Introduction

Whey protein (WP) represents almost 20% of the total protein in bovine milk and has been recognized for its high nutritional quality, fast absorption and as a rich source of branched-chain amino acids (BCAAs) (Hulmi, Lockwood & Stout, 2010). Some properties associated with the whey proteins, especially in its hydrolyzed form, have been the subject of some investigations, properties such as the improved physical resistance of rats subjected to physical exhaustion (Pimenta, Abecia-Soria, Auler & Amaya-Farfan, 2006), a reduction in muscle injury enzyme indicators (LDH, CK) in soccer athletes during competition periods (Lollo, Amaya-Farfan & Carvalho-Silva, 2011), an increase in muscle fatty acids for use as an energy source during exercise (Morifuji, Sakai, Sanbongi & Sugiura, 2005a) and the capacity to recover the glycogen levels in the liver and skeletal muscle after exercise (Morifuji, Sakai, Sanbongi & Sugiura, 2005b; Pimenta et al., 2006; Morifuji, Kanda, Koga, Kawanaka & Higuchiet, 2010; Faria, Nery-Diez, Lollo, Amaya-Farfan & Ferreira, 2012).

Muscle glycogen synthesis is limited by the availability of glucose, and in skeletal muscle glucose transport occurs mainly via the glucose transport carrier proteins (GLUTs). GLUT-4 is the major glucose transporter isoform expressed in skeletal muscle, and thus the rate of muscle glucose transport is determined by the GLUT-4 concentration in the cell membrane, in response to insulin and/or muscle contraction (MacLean, Zheng & Dohm, 2000; Jentjens & Jeukendrup, 2003).

Previous studies showed that whey protein hydrolysate (WPH) and whey protein peptides containing BCAAs were capable of increasing the entrance of

glucose and glycogen synthesis in rat muscles (Pimenta et al., 2006; Morifuji, Koga, Kawanaka & Higuchi, 2009). However the glucose transporter expression was not analyzed in these studies and results obtained by the present authors were the first to show that WPH stimulated the translocation of GLUT-4 to the cell membrane (Morato, Lollo, Moura & Amaya-Farfan, 2012). Of the WPH components that could contribute to this effect, the following stand out: 1) BCAAs, the amino acids L-leucine and L-isoleucine improved glucose uptake in skeletal muscles, both *in vitro* and *in vivo* (Doi, Yamaoka, Nakayama, Mochizuki, Sugahara & Yoshizawa, 2005; Nishitani, Takehana, Fujitani & Sonaka, 2005). 2) Dipeptides composed of BCAAs, Morifuji et al. (2009) showed that the peptide L-isoleucyl-L-leucine, identified as the main BCAA-containing amino acid in WPH, stimulated glucose uptake and glycogen synthesis *in vitro*.

Based on the evidence that the consumption of whey protein hydrolysate increased the muscle glycogen reserves (Morifuji et al., 2005b; Pimenta et al., 2006; Morifuji et al., 2010; Faria et al., 2012) and also increased the glucose transporter concentration in the plasma membrane (Morato et al., 2012), the objective of the present study was to identify which whey protein hydrolysate components could have a relevant role in glucose capture. Thus the branched chain amino acids L-leucine and L-isoleucine and the peptides made up of these two amino acids, were tested *in vivo*, since it was already shown that both the BCAAs (Doi, Yamaoka, Fukunaga & Nakayama, 2003; Doi et al., 2005) and the peptides derived from them (Morifuji et al. 2009) could increase cellular glucose capture *in vitro*. This was the first study that analyzed the group of WPH components *in vivo*, considering their passage through gastrointestinal digestion.

2. Material and Methods

2.1. Animals

Forty-nine male Wistar rats (21 days old) reared in the Multidisciplinary Centre for Biological Research, University of Campinas, SP, Brazil, were housed (~22℃, 55% RH, inverted 12-hour light cycle) in individual growth cages, with access to commercial feed (Labina, Purina, Brazil) and water *ad libitum*. All experimental procedures were approved by the Ethics Committee on Animal Experimentation (CEEA-UNICAMP, protocol 2376-1/2011).

2.2. Experimental procedures

When the animals reached ~ 245 g of body mass, they were submitted to a glycogen depletion protocol consisting of the following 2 steps: 1) training on the treadmill, running for 60 minutes at 15 m/min (to deplete muscle glycogen); and 2) 16 hours fasting after exercising (to deplete hepatic glycogen). The experimental protocol was based on Morifuji et al. (2010). This procedure allowed one to carry out the analyses with the animals homogeneously depleted of glycogen and at a moment of great influx of glucose into the cells when mobilization of the glucose transporters was maximum (Ren, Semenkovich, Gulve, Gao & Holloszy, 1994; Kuo, Browning & Ivy, 1999). With the glycogen reserves depleted, the animals received by oral gavage the following solutions described (Figure 1A), and thirty minutes after oral ingestion of the solutions, were killed by decapitation and the blood and tissue samples immediately collected for analysis (Figure 1B).

Figure 1.

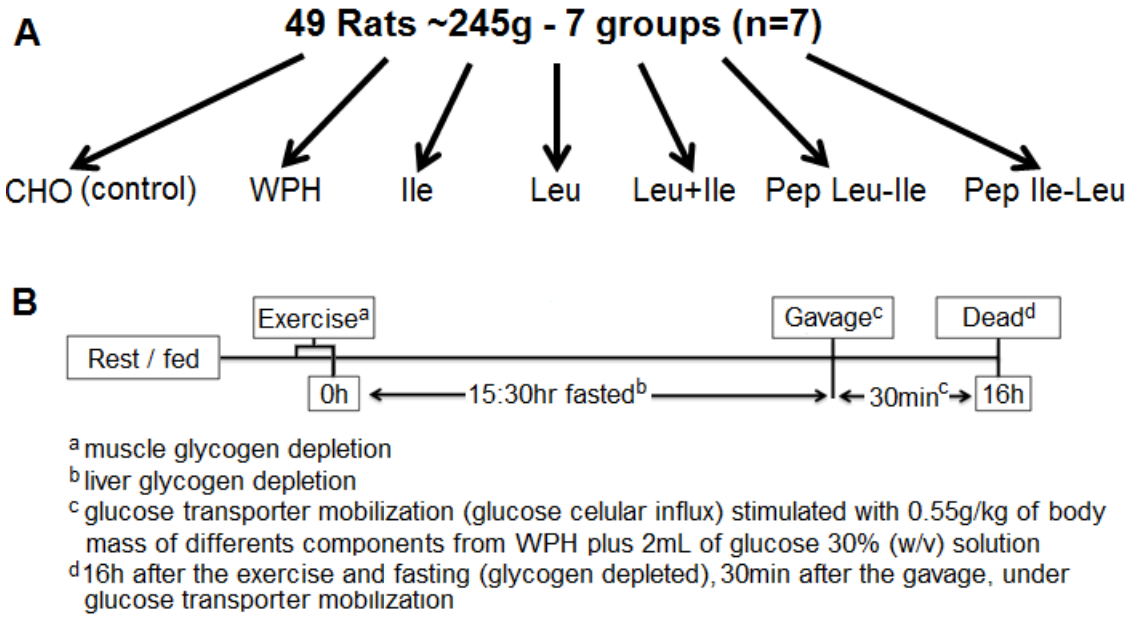

Figure 1A. Experimental design: Forty-nine animals were divided into 7 groups (n=7), each animal received by oral gavage 30% glucose plus 0.55 g/kg body weight of WPH components: CHO (glucose/control); WPH (whey protein hydrolysate); Ile: isoleucine; Leu: leucine; Leu+Ile: 50:50 mixture of the amino acids L-leucine plus L-isoleucine; Pep Ile-leu: L-isoleucyl-L-leucine peptide; Pep Leu-Ile: L-leucyl-L-isoleucine peptide. **1B. Experimental protocol**: The rats were sacrificed after the glycogen depletion state exercise-induced and fasting, but 30 min before the sacrifice the rats received a solution with different components from whey protein hydrolysate (WPH), so there be a glucose transporter mobilization at moment of dead.

2.3. Preparation of the oral solutions

Each animal received 0.55 g/kg body weight of the amino acid/peptide dissolved in a 30% glucose solution and the groups were as follows: a) CHO: 30% glucose (control group); b) WPH: whey protein hydrolysate, with the amount of liquid corrected according to the protein purity (78%) c) L-isoleucine d) L-leucine e)

a 50:50 mixture of the amino acids L-leucine plus L-isoleucine f) L-isoleucyl-L-leucine dipeptide g) L-leucyl-L-isoleucine dipeptide (Figure 1A).

The peptides (purity >98%) were acquired from BioBasic (Markham, Ontario, Canada), the amino acids L-leucine and L-isoleucine, at least 99.7% pure, donated by Ajinomoto (Sao Paulo, Brazil), and the WPH donated by Hilmar Ingredients (Hilmar, California, USA).

2.4. Biochemical parameters

Blood samples were collected in Vacutainers maintained at 4℃, and centrifuged at 3,000g (4℃, 12min) to obtain the se rum. To assess the serum, the following determinations were carried out: uric acid, urea, aspartate amino transferase (AST), alanine aminotransferase (ALT), creatine kinase (CK), lactate dehydrogenase (LDH), and glucose using spectrophotometer (Beckman-Coulter DU 640, Palo Alto, CA, USA) and Laborlab kits (São Paulo, Brazil). The serum insulin levels were measured using a rat/mouse insulin ELISA (Millipore, Billerica, MA, USA).

2.5. Determination of glycogen

The skeletal muscle and the myocardial and liver tissues were collected for the glycogen analysis. Tissue glycogen was isolated and purified by precipitation with ethanol from a basic digestion, and then quantified by the phenol-sulfuric acid

method (Lo, Russel & Taylor, 1970). The absorbance was read in a spectrophotometer (Beckman-Coulter DU 640, Palo Alto, CA, USA) at 490nm.

2.6. Western Blot analysis

The total protein content of the skeletal muscle was determined by the Lowry method (Lowry, Rosebrough, Farr & Randall, 1951). For immunoblotting, tissue homogenates were subjected to gel electrophoresis using SDS-PAGE and transferred to a nitrocellulose membrane. The blots were probed with the appropriate antibodies to assess the protein level of the GLUT-4 (Abcam, Cambridge; catalog number ab654 diluted 1:5000), Akt 1/2/3 (H-136) antibody SC8312 (Santa Cruz Biotechnology, CA, USA diluted 1:1000), p-AKT 1,2,3 Ser 473, antibody SC 7985-R (Santa Cruz Biotechnology, CA, USA diluted 1:1000), Anti-PI 3-Kinase p85, N-SH2 domain (catalog number #06-496, Upstate Biotechnology NY, USA diluted 1:1000), AMPK alpha 1 + AMPK alpha 2 phospho S485 + S491 (Abcam, Cambridge; catalog number ab39400 diluted 1:1000). To assess the levels of these proteins in the muscle, the loading control was tubulin (Abcam, Cambridge, catalogue number ab44928, diluted 1:1000).

The appropriate secondary antibody conjugated to peroxidase and the BM chemiluminescence blotting system, were used for detection. The bands were visualized using a GE, ImageQuant, model LAS 4000 instrument. Specific protein bands present in the blots were quantified using the digital program ImageJ (v. 1.44 for Windows).

2.7. Amino acid composition of the muscle and free amino acids of the plasma

The muscle and plasma free amino acids were extracted with methanol and derivatized with phenylisothiocyanate (White, Hart, Fry, 1986), and the PTH-derivatives chromatographed using a Luna C-18, 100 Å 5u, 250 x 4.6 mm (Phenomenex, Torrance, CA) column at 50°C. The mobile phase composed of eluents A (sodium acetate 0,56 M with triethylamine 0,05% and acetonitrile 0,06%, pH 6.4) and B (acetonitrile + water 60:40). The flow rate was 1 mL/min and detection was at 254 nm. Quantification was performed by comparison with a standard mixture, and the internal standard was DL-2-aminobutyric acid (Sigma-Aldrich Corp, St Louis, MO, USA). The mixture was sonicated for 10 minutes and further homogenised for 1 hour, followed by centrifugation at 8,500g for 15 minutes. The supernatant was filtered through a 0.22 mm membrane, a 40 µL aliquot was derivatised as described above, and 20 µL was injected into the liquid chromatograph.

2.8. Statistical Analysis

The data were analyzed using the software SPSS (Statistical Package for the Social Sciences), version 17.0. The results were tested for normality (Kolmogorov-Smirnov test) and homogeneity using the tools available therein. For parametric data, the multivariate analysis of variance (ANOVA) was used and the means compared (Duncan test). The level of significance was set at $p < 0.05$.

3. Results

Of the WPH components tested, given in equal amounts, the amino acid L-isoleucine increased translocation of GLUT-4 to the PM (Figure 2A) and reduced the blood glucose levels (Figure 2G). The phosphorylation of Akt at serine 473 (Figure 2D) was increased ($p<0.05$) by the peptide L-leucyl-isoleucine, which also presented higher levels of plasma insulin (Figure 2F). The glycogen concentration of the skeletal muscle showed no difference between the groups (Figure 2I).

Figure 2

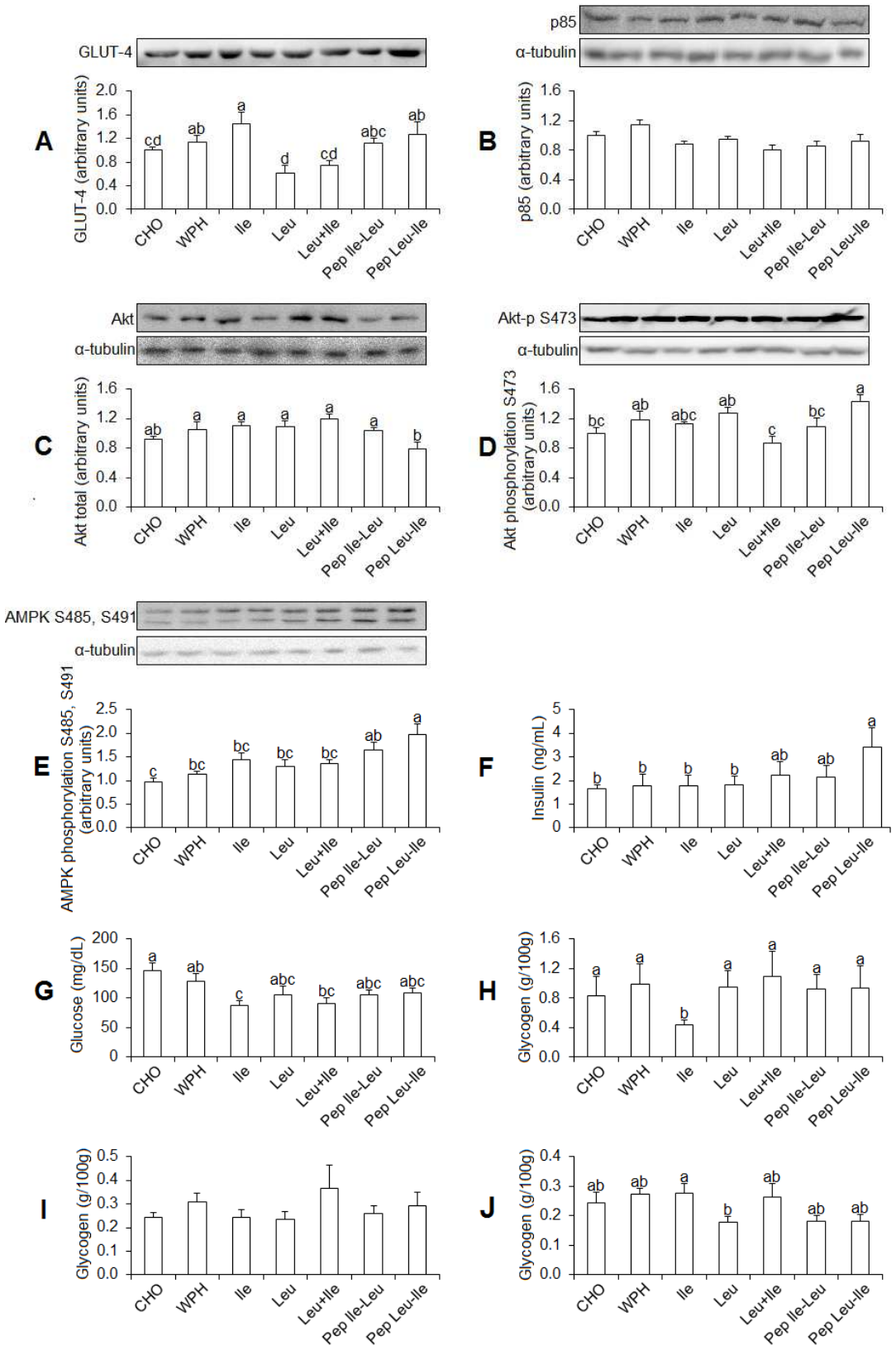
GLUT-4
A
GLUT-4 (arbitrary units)
p85
α-tubulin
B
p85 (arbitrary units)
Akt
α-tubulin
C
Akt total (arbitrary units)
Akt-p S473
α-tubulin
D
Akt phosphorylation S473 (arbitrary units)
AMPK S485, S491
α-tubulin
E
AMPK phosphorylation S485, S491 (arbitrary units)
F
Insulin (ng/mL)
G
Glucose (mg/dL)
H
Glycogen (g/100g)
I
Glycogen (g/100g)
J
Glycogen (g/100g)
CHO
WPH
Ile
Leu
Leu+Ile
Pep Ile-Leu
Pep Leu-Ile

Figure 2. The effect (means and standard errors) of administering oral solutions with 0.55g/kg of body weight dissolved in a 30% glucose after the glycogen depletion in Wistar rats: CHO (glucose/control); WPH (whey protein hydrolysate); Ile: isoleucine; Leu: leucine; Leu+Ile: 50:50 mixture of the amino acids L-leucine plus L-isoleucine; Pep Ile-leu: L-isoleucyl-L-leucine peptide; Pep Leu-Ile: L-leucyl-L-isoleucine peptide. The expression of the following proteins was determined by immunoblot: **A)** Glucose transporter 4 (GLUT-4); **B)** p85 / tubulin; **C)** Akt / tubulin **D)** Akt phosphorylate (Ser 473) / tubulin **E)** AMPK / tubulin in the skeletal muscle; and in addition for insulin (**F**) and glucose (**G**) in the serum; and for glycogen in the: **H)** liver; **I)** skeletal muscle and **J)** heart. Different superscript lowercase letters indicate significant differences between the groups. ANOVA was used and the means compared (Duncan test), adopting the value of $p< 0.05$ as the criterion for statistical significance.

The complete amino acid profiles of the plasma (Table 1) and muscle (Table 2) were determined. Higher concentrations of the amino acid L-isoleucine were detected in the plasma of the groups that received this free amino acid and WPH. In the muscle, the concentrations found for L-isoleucine and L-leucine were higher in the groups that received the free amino acids.

Table 1 – Plasma free amino acid profile (µmol/L) (mean values with their standard errors - SE)

	CHO		WPH		Ile		Leu		Leu+Ile		Ile-Leu		Leu-Ile	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
ASP	42.8^{a}	7.0	36.9^{abc}	0.6	27.9^{c}	0.2	44.2^{a}	2.0	28.7^{c}	0.6	38.5^{ab}	1.8	29.6^{bc}	0.2
GLU	15.0^{b}	0.4	18.5^{ab}	0.6	20.7^{a}	2.2	17.4^{ab}	0.4	15.4^{b}	0.7	15.4^{b}	0.6	20.9^{a}	1.7
HYPRO	41.6^{c}	1.1	53.4^{ab}	6.3	59.1^{a}	5.3	48.2^{abc}	1.5	43.4^{bc}	2.9	40.5^{c}	1.0	50.9^{abc}	2.0
ASN	83.5^{b}	3.2	117.7^{a}	13.1	122.4^{a}	6.1	80.8^{b}	0.0	77.0^{b}	2.6	86.7^{b}	1.3	84.4^{b}	1.4
SER	305.4^{b}	11.8	373.0^{a}	37.8	395.1^{a}	3.4	270.3^{b}	2.8	286.5^{b}	1.9	304.7^{b}	2.1	315.3^{b}	5.2
GLN	438.4^{b}	14.0	536.9^{a}	43.9	554.6^{a}	25.3	410.4^{b}	20.2	425.5^{b}	3.1	468.2^{b}	16.7	408.7^{b}	8.8
GLY	180.1^{b}	7.4	201.9^{a}	10.3	208.4^{a}	3.8	147.1^{a}	1.0	162.8^{a}	3.8	202.4^{a}	17.6	177.2^{a}	0.1
HIS	41.2^{ab}	1.4	44.5^{a}	2.9	49.0^{a}	4.7	30.4^{c}	2.0	35.4^{bc}	0.1	41.4^{ab}	3.1	34.9^{bc}	0.0
TAU	81.2^{b}	15.1	122.4^{a}	14.6	133.8^{a}	17.4	131.6^{a}	10.6	105.3^{ab}	2.8	145.4^{a}	12.7	112.0^{ab}	2.3
ARG	98.9^{bc}	2.9	112.1^{ab}	7.9	110.6^{ab}	11.0	116.2^{ab}	9.3	81.1^{c}	2.8	126.2^{a}	8.9	85.5^{c}	4.1
ALA	**258.9^{b}**	**2.0**	**331.4^{a}**	**18.4**	**302.6^{a}**	**15.7**	**217.1^{b}**	**0.1**	**252.6^{b}**	**13.1**	**218.9^{b}**	**1.9**	**242.6^{b}**	**19.7**
THR	466.6^{c}	18.2	561.4^{bc}	31.7	511.7^{bc}	8.2	738.5^{a}	31.5	436.6^{c}	1.7	516.3^{bc}	25.7	616.9^{ab}	105.2
PRO	191.4^{b}	9.2	245.7^{a}	10.9	247.5^{a}	29.4	215.7^{ab}	0.9	215.1^{ab}	1.4	218.4^{ab}	1.6	243.3^{a}	10.7
TYR	41.1^{a}	0.9	43.0^{a}	1.9	41.3^{a}	2.8	33.7^{b}	1.0	31.7^{b}	0.5	33.1^{b}	2.3	31.5^{b}	0.4

VAL	143.2^{b}	12.2	195.4^{a}	3.6	165.6^{b}	10.0	56.6^{c}	4.5	68.2^{c}	5.9	66.3^{c}	14.6	65.5^{c}	0.4
MET	36.9^{b}	6.3	35.0^{b}	2.2	60.5^{a}	0.1	17.2^{d}	1.5	28.8^{bc}	4.9	31.7^{bc}	2.3	22.9^{cd}	1.4
ILE	**92.7^{b}**	**2.9**	**189.5^{a}**	**5.3**	**191.6^{a}**	**20.7**	**29.8^{c}**	**0.8**	**92.8^{b}**	**9.5**	**67.4^{b}**	**2.0**	**71.6^{b}**	**1.0**
LEU	**49.4^{d}**	**5.1**	**197.4^{a}**	**6.0**	**129.2^{c}**	**1.9**	**156.3^{b}**	**3.0**	**49.7^{d}**	**3.8**	**38.4^{d}**	**0.9**	**40.0^{d}**	**0.2**
PHE	60.3^{ab}	0.8	64.8^{a}	3.1	53.5^{c}	3.5	34.9^{c}	1.1	36.5^{c}	0.3	39.6^{c}	3.9	34.0^{c}	1.5
TRP	142.8^{b}	6.1	175.0^{a}	15.7	127.8^{b}	8.6	93.3^{c}	7.7	96.1^{c}	0.6	102.5^{c}	4.0	90.1^{c}	0.1
ORN	86.4^{a}	4.0	74.4^{ab}	10.4	59.5^{bc}	9.2	41.4^{c}	7.7	38.7^{c}	4.3	47.6^{c}	2.2	43.7^{c}	3.6
LYS	406.4	36.8	571.7	65.5	453.9	39.0	533.3	66.0	517.1	90.4	543.6	77.3	526.9	93.0

ASP: aspartate, GLU: glutamate, HYPRO: hydroxyproline, ASN: asparagine, SER: serine, GLN: glutamine, GLY: glycine, HIS: histidine, TAU: taurine, ARG: arginine, ALA: alanine, THR: threonine, PRO: proline, TYR: tyrosine, VAL: valine, MET: methionine, ILE: isoleucine, LEU: leucine, PHE: phenylalanine, TRP: tryptophan, ORN: orinitine, LYS: lysine. Alanine, isoleucine and leucine are bolded. The effect of administering oral solutions with 0.55g/kg of body weight dissolved in a 30% glucose CHO: (glucose/control); WPH: whey protein hydrolysate; Ile: isoleucine; Leu: leucine; Leu+Ile: 50:50 mixture of the amino acids L-leucine plus L-isoleucine; Ile-Leu: L-isoleucyl-L-leucine peptide; Leu-Ile: L-leucyl-L-isoleucine peptide. Different letters indicate significant differences between groups, ANOVA was used and means were compared (Duncan test), adopting the value of $p < 0.05$ as a criterion for statistical significance.

Table 2 – Skeletal muscle free amino acid profile (µmol/Kg) (mean values with their standard errors - SE)

	CHO		WPH		Ile		Leu		Leu+Ile		Ile-Leu		Leu-Ile	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
ASP	27.2^{bc}	3.6	19.1^{c}	0.4	25.4^{bc}	5.3	34.0^{ab}	5.1	43.5^{a}	4.0	28.2^{bc}	0.0	27.9^{bc}	1.0
GLU	963.4^{b}	23.6	1041.2^{b}	116.2	1721.4^{a}	31.4	1467.0^{a}	224.2	1725.1^{a}	106.8	1078.7^{b}	30.7	1484.5^{a}	94.9
HYPRO	349.1^{de}	90.7	191.6^{e}	15.0	412.9^{cd}	111.6	614.8^{ab}	17.9	666.3^{a}	11.1	475.9^{bcd}	11.5	552.3^{abc}	30.5
ASN	273.1^{ab}	88.0	384.5^{a}	3.6	299.6^{ab}	89.3	133.9^{b}	26.5	139.6^{b}	15.0	255.1^{ab}	94.2	100.2^{b}	0.2
SER	171.5^{b}	30.1	147.6^{b}	0.0	248.9^{a}	0.0	253.5^{a}	37.2	265.1^{a}	21.0	162.0^{b}	12.4	208.1^{ab}	11.2
GLN	5517.0^{ab}	684.4	3749.3^{bc}	434.7	2799.1^{c}	113.9	3267.8^{c}	1298.6	848.2^{d}	58.0	5318.2^{ab}	267.3	6504.7^{a}	189.7
GLY	447.0^{ab}	93.2	277.7^{b}	27.5	402.8^{b}	81.5	628.4^{a}	75.8	619.0^{a}	17.0	392.5^{b}	39.1	456.7^{ab}	40.7
HIS	416.2^{ab}	91.0	259.0^{b}	12.7	411.1^{ab}	119.2	333.0^{b}	42.4	571.3^{a}	35.4	417.0^{ab}	48.7	404.0^{ab}	20.1
TAU	1804.2^{ab}	307.5	1115.7^{b}	43.9	2165.4^{a}	684.6	2525.4^{a}	268.1	1897.3^{ab}	18.5	1796.7^{ab}	40.9	1947.0^{ab}	85.9
ARG	1913.5^{ab}	276.0	1276.2^{b}	4.3	2150.6^{ab}	656.6	2611.6^{a}	241.9	2930.2^{a}	399.4	2155.0^{ab}	94.9	2033.6^{ab}	126.8
ALA	253.6^{bc}	52.2	155.9^{c}	9.8	248.5^{bc}	71.6	347.8^{ab}	53.6	394.7^{a}	39.5	255.3^{bc}	15.7	233.4^{bc}	7.4
THR	509.2^{b}	38.9	703.3^{a}	16.3	234.8^{cd}	72.1	273.5^{c}	87.8	108.8^{d}	7.9	89.0^{d}	2.2	701.4^{a}	6.2
PRO	782.6^{a}	34.6	386.5^{b}	126.0	590.0^{ab}	145.6	321.4^{b}	128.5	739.2^{a}	29.6	605.0^{ab}	26.2	533.6^{ab}	28.8
TYR	21.0^{abc}	3.2	10.1^{d}	2.1	19.1^{bcd}	2.5	27.6^{ab}	4.2	30.7^{a}	6.0	21.3^{abc}	1.3	14.4^{cd}	0.8

VAL	291.8^{ab}	39.2	151.8^{b}	45.3	328.7^{ab}	88.4	297.2^{ab}	64.5	400.8^{a}	89.5	271.5^{ab}	50.9	153.7^{b}	8.3
MET	14.5^{ab}	3.5	18.2^{ab}	2.8	12.6^{ab}	3.2	22.2^{a}	5.3	21.4^{ab}	5.4	14.2^{ab}	1.0	9.7^{b}	1.0
ILE	**137.8^{bc}**	**22.9**	**123.0^{bc}**	**13.8**	**255.8^{a}**	**22.9**	**161.2^{bc}**	**21.1**	**168.8^{b}**	**28.4**	**121.3^{bc}**	**16.0**	**100.3^{c}**	**8.3**
LEU	**203.8^{d}**	**19.0**	**163.3^{d}**	**5.6**	**270.6^{c}**	**22.9**	**505.0^{a}**	**31.2**	**387.6^{b}**	**0.0**	**513.3^{a}**	**0.0**	**470.9^{a}**	**4.1**
PHE	14.6	1.0	14.5	1.3	14.8	1.1	21.0	4.7	21.2	5.2	15.1	1.2	12.8	0.0
TRP	62.4^{ab}	4.4	66.7^{ab}	12.7	50.5^{ab}	11.0	85.0^{a}	2.6	68.1^{ab}	13.4	62.0^{ab}	13.2	43.8^{b}	3.9
ORN	51.9	11.6	90.3	23.7	53.2	17.1	42.4	25.7	37.5	5.9	46.9	7.0	45.0	0.3
LYS	1728.5^{ab}	504.6	2428.3^{a}	812.1	1341.7^{ab}	296.1	1837.4^{ab}	327.4	1892.4^{ab}	227.8	1099.3^{ab}	75.8	948.5^{b}	9.3

ASP: aspartate, GLU: glutamate, HYPRO: hydroxyproline, ASN: asparagine, SER: serine, GLN: glutamine, GLY: glycine, HIS: histidine, TAU: taurine, ARG: arginine, ALA: alanine, THR: threonine, PRO: proline, TYR: tyrosine, VAL: valine, MET: methionine, ILE: isoleucine, LEU: leucine, PHE: phenylalanine, TRP: tryptophan, ORN: orinitine, LYS: lysine. Isoleucine and leucine are bolded. The effect of administering oral solutions with 0.55g/kg of body weight dissolved in a 30% glucose CHO: (glucose/control); WPH: whey protein hydrolysate; Ile: isoleucine; Leu: leucine; Leu+ Ile: 50:50 mixture of the amino acids L-leucine plus L-isoleucine; Ile-Leu: L-isoleucyl-L-leucine peptide; Leu-Ile: L-leucyl-L-isoleucine peptide. Different letters indicate significant differences between groups, ANOVA was used and means were compared (Duncan test), adopting the value of $p< 0.05$ as a criterion for statistical significance.

The following general health parameters were assessed: uric acid and urea. AST and ALT were also assessed as hepatic health parameters, and CK and LDH as muscle damage indicators (Table 3).

Table 3. Mean and standard error of the mean (SE) of biochemical blood parameters

	Albumin[a]		TPROT[b]		AST[c]		ALT[d]		LDH[e]		CK[f]		Urea[g]		Uric Acid[h]	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
CHO[i]	3.55	0.20	4.92	0.38	40.14[b]	5.48	20.20[a]	2.22	837.61	95.38	1409.43	286.51	31.34	2.00	1.76[a]	0.30
WPH[j]	3.29	0.16	4.59	0.30	47.71[ab]	2.37	19.41[ab]	1.48	751.26	68.94	1287.55	210.63	44.36	5.80	1.94[a]	0.26
Ile[k]	3.29	0.09	4.89	0.21	48.37[ab]	4.38	16.26[ab]	1.48	686.50	39.66	1097.32	135.18	32.14	3.46	1.30[ab]	0.12
Leu[l]	3.32	0.09	5.08	0.38	48.45[ab]	3.83	17.14[ab]	1.17	711.01	76.58	1207.05	207.95	37.72	6.59	1.19[b]	0.06
Leu+Ile[m]	3.26	0.07	4.78	0.30	42.13[ab]	2.62	14.60[b]	0.95	644.68	74.82	1145.89	180.32	35.71	2.74	1.45[ab]	0.22
Ile-Leu[n]	3.06	0.32	4.98	0.63	52.41[ab]	6.37	15.58[ab]	1.58	689.20	61.82	1313.64	202.42	35.18	3.27	1.39[ab]	0.24
Leu-Ile[o]	3.37	0.12	5.44	0.39	54.53[ab]	2.55	16.03[ab]	0.69	672.33	53.29	1199.41	192.36	34.99	4.36	1.44[ab]	0.06

[a] g/dL; [b] TPROT: total proteins (mg/dL); [c] AST: aspartate aminotransferase (U/L); [d] ALT: alanine aminotransferase (U/L); [e] LDH: lactate dehydrogenase (U/L); [f] CK: creatine kinase (U/L); [g] mg/dL, [h] mg/dL. The effect of administering oral solutions with 0.55g/kg of body weight dissolved in a 30% glucose, 30 minutes before the dead [l] CHO: (glucose/control) ; [j] WPH: whey protein hydrolysate; [k] Ile: isoleucine; [l] Leu: leucine; [m] Leu+ Ile: 50:50 mixture of the amino acids L-leucine plus L-isoleucine; [n] Ile-Leu: L-isoleucyl-L-leucine peptide; [o] Leu-Ile: L-leucyl-L-isoleucine peptide. Different superscript lowercase letters indicate significant differences between the groups. ANOVA was used and the means compared (Duncan test), adopting the value of $p< 0.05$ as the criterion for statistical significance.

4. Discussion

Considering that WPH has the ability to mobilize translocation of GLUT-4 to the plasma membrane of the cell (Morato et al., 2012), the objective of this research was to identify the WPH components, given in equal amounts, that could have made a significant contribution to the uptake of glucose and synthesis of glycogen, promoted by the *in vivo* ingestion of this protein, considering the effect of the digestive process on the WPH components. Of the WPH components tested, it was shown that, on a mass basis, the amino acid L-isoleucine significantly ($p<0.05$) increased the translocation of GLUT-4 to the PM (Figure 2A), this increase being consistent with the low blood glucose levels found in this group ($p<0.05$) (Figure 2G), since a greater concentration of GLUT-4 in the PM favors glucose capture by the muscle, decreasing the serum levels. In earlier studies, L-isoleucine had presented a hypoglycemic effect (Doi, Yamaoka, Fukunaga & Nakayama, 2003; Doi, Yamaoka, Nakayama, Sugahara & Yoshizawa, 2007), and, amongst the BCAAs, had promoted the greatest translocation of GLUT-4 in the skeletal muscle of rats with liver cirrhosis (Nishitani et al., 2005). The standard diet used in experimental animal nutrition research uses casein as the protein source, considering this protein as the reference (Reeves, Nielsen & Fahey, 1993). The composition of WPH (Table 4) shows about 50% more L-isoleucine, suggesting that this amino acid could be contributing to the effects of this protein in the translocation of GLUT-4 (Morato et al., 2012).

Table 4. Amino acid profile of the protein sources (g/100g of protein)

Amino Acid	CAS[a]	WPH[b]
Asparagine	5.96	11.16
Glutamate	19.00	17.99
Serine	4.68	5.04
Glycine	1.39	1.75
Histidine	2.12	1.27
Arginine	3.03	2.31
Threonine	3.56	7.40
Alanine	2.30	4.89
Proline	8.85	5.68
Tyrosine	4.57	2.78
Methionine	2.32	2.52
Cystine	0.16	1.60
Isoleucine	**4.51**	**6.97**
Leucine	7.62	10.15
Valine	5.36	5.81
Phenylalanine	3.89	2.78
Lysine	6.62	9.48

[a] CAS: casein; [b] WPH: whey protein hydrolysate

The amount of GLUT-4 in the PM is a primary factor in determining the maximal rate of glucose transport into the skeletal muscle. Under normal resting conditions, most of the GLUT-4 molecules can be found in the membrane vesicles inside the muscle cell. Insulin signalization is amongst the factors that most influence translocation of GLUT-4 to the PM (Dohm, 2002), which, by way of proteins such as AKT, p85, unleashes a cascade of signalizing events, culminating in the translocation of GLUT-4. However, the insulin levels were low in the group that received L-isoleucine (Figure 2F), although the translocation of GLUT-4 was high, thus in this case it seems that the translocation was independent of insulin. The group showing the highest insulin concentrations was the group that received the peptide L-leucyl-isoleucine, which corresponds to 16% of the total amount of dipeptides formed from the BCAAs present in WPH, and comes from β-lactoglobulin, one of the WPH fractions (Morifuji et al., 2009).

The higher insulin levels in the L-leucyl-isoleucine group were coherent with a greater activation of Akt, phosphorylated on the serine 473 residue (Figure 2D – p-Akt), this protein being phosphorylated by insulin signalization (Wang et al., 1999). On the other hand the group that received L-isoleucine showed the lowest p-Akt levels (Ser 473), coherent with the low plasma insulin concentrations, and reinforces evidence that L-isoleucine is capable of translocating GLUT-4 without insulin signalization, as described by Nishitani et al. (2005). The present findings also agree with a previously reported improvement in glucose tolerance in rats obtained by administering an amino acid mixture consisting mostly of L-isoleucine, despite the lack of increase in Akt/PKB phosphorylation (Bernard, Liao, Hara, Ding, Chen, Nelson et al., 2011). AMPK has been indicated as a protein capable of

inducing muscle glucose intake (Musi & Goodyear, 2003). The present results show greater activation of AMPK for the group that received the L-leucyl-isoleucine peptide, which presented elevated translocation of GLUT-4, similar to that found for WPH (Figure 2E). On the other hand the protein PI3-kinase (p85), involved in insulin signalization, was apparently unaffected by any of the WPH components analyzed in this study (Figure 2B). Bernard et al. (2012) found that a mixture of amino acids, composed mainly of L-isoleucine, did not activate the PI3-kinase in rat muscle.

In the present study, there was no difference between all groups with respect to the glycogen content of the skeletal muscle. However, the experimental design of the study was focused on the moment of greater mobilization of the glucose transporters, which would be a short space of time for the depleted glycogen levels to recover according to the protocol used, only 30 minutes after ingestion of the different nutrients (Figure 2I), since glycogen recovery can take up to 24 hours, depending on the extent of depletion and the diet consumed (Jentjens & Jeukendrup, 2003). On the other hand, in the heart muscles, the glycogen levels were higher for the animals who received the L-isoleucine, and lower for those who received L-leucine (Figure 2J). The heart is a vital organ and can respond differently to catabolic conditions such as fasting and exercise, preserving energy substrates such as glycogen (Faria et al., 2012). In the liver, the lowest glycogen levels were found for the group that received L-isoleucine (Figure 2H). This fact is coherent with the indications of Doi et al. (2007) and Yoshizawa (2012) who indicated that in the liver L-isoleucine inhibited gluconeogenesis, decreasing the

activities of the glycogenic enzymes and the production of glucose, mechanisms associated with the hypoglycemic effect of L-isoleucine.

The amino acid profiles of the plasma and muscle were determined. Amongst the results obtained, the amino acid alanine, a glucogenic substrate, presented higher levels in the plasma of the groups that received L-isoleucine and WPH (Table 1). Doi et al. (2007) found elevated levels of alanine in the plasma of rats that received L-isoleucine. The authors suggested a competitive effect between L-isoleucine and L-alanine for hepatic transport via the neutral amino acid transporter, increasing the levels of L-alanine in the plasma and decreasing the synthesis of glucose in the liver. The concentrations of the amino acid L-isoleucine were higher in the plasma of the groups that received this free amino acid and WPH, which is coherent with other results found in this study which suggested that this amino acid present in large amounts in WPH, could contribute to the effects of the protein in capturing glucose. In the muscle, the concentrations found for L-isoleucine and L-leucine were higher in the groups that received the free amino acids (Table 2).

With respect to the blood parameters, the muscle damage markers CK and LDH (Brancaccio, Maffulli, Buonauro & Limongelli*,* 2008) did not vary between all groups, and in addition, no significant alterations were found in the liver (ALT and AST) and kidney (uric acid and urea) in the groups that received the peptides and amino acids (Table 3).

5. Conclusions

In conclusion, when considering their passage through gastrointestinal digestion, the data obtained in this study showed that of the whey protein hydrolysate components tested *in vivo*, given in equal amounts, it appears that the peptide L-leucyl-L-isoleucine and the amino acid L-isoleucine were the components contributing more to the increase in translocation of the major glucose transporter, GLUT-4, and the entrance of glucose into the skeletal muscle. The plasma insulin levels, as also the phosphorylation of Akt, suggested that the peptide L-leucyl-L-isoleucine was an important insulinotropic factor, which, added to the high concentrations of L-isoleucine present in the whey protein hydrolysate and its capacity to mobilize glucose transporters to the plasma membrane, could explain, at least in part, the high concentration of GLUT-4 found in the plasmatic membrane and the consequently greater influx of glucose into the cell and the concentrations of glycogen in the animals fed on whey protein hydrolysate. In addition, it must be highlighted that in insulin resistance and in diabetes type 2, the plasma glucose level is high due to a lack of glucose transporters in the plasmatic membrane, a problem which could be overcome by a greater presence of GLUT-4 in the plasmatic membrane. Thus new studies should be carried out considering the potential of whey protein hydrolysate and its components in the treatment of insulin resistance, so as to take greater advantage of the functional role of whey protein hydrolysate.

6. Acknowledgements

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Conclusão Geral

CONCLUSÃO GERAL

O consumo de PSL e PSLH como fonte de proteína dietética aumenta a translocação do transportador de glicose GLUT-4 para a membrana plasmática, sendo o efeito significativamente maior no grupo que consumiu PSLH e realizou exercício aeróbio, do que naquele que consumiu o concentrado de PSL. O aumento na translocação de GLUT-4 foi acompanhado por maiores concentrações de glicogênio, mas sem haver alterações nas concentrações de insulina.

Dentre os componentes testados da PSLH *in vivo*, aqueles que parecem ter maior contribuição para o aumento na translocação de GLUT-4 e entrada de glicose no músculo esquelético, parecem ser o peptídeo leucil-isoleucina e a isoleucina livre. O peptídeo leucil-isoleucina é um importante fator insulinotrópico que somado às altas concentrações de isoleucina presente na PSLH e sua capacidade de mobilizar o transportador de glicose para a membrana plasmática, poderia explicar ao menos parcialmente a elevada concentração de GLUT-4 na MP e consequentemente o maior influxo de glicose no músculo dos animais que se alimentam com PSLH.

Esse estudo visou elucidar o mecanismo pelo qual o consumo das PSL e PSLH aumentam os níveis de glicogênio muscular, e encontrou importante efeito das proteínas e de seus componentes nos transportadores de glicose sódio-independentes, revelando novas possibilidades para o uso da PSLH como alimento funcional. Os resultados aqui apresentados mostram como a ciência e a tecnologia podem extrair maior benefício de um alimento de consumo tradicional, aproximando mais as características de alimento e medicamento.

Estes resultados devem encorajar novos estudos abordando o potencial da PSLH e dos seus componentes no tratamento ou prevenção da resistência à insulina e do diabetes tipo II, doença em que há menor translocação de GLUT-4 para a membrana plasmática. Revela-se importante o desenvolvimento de estudo com animais que apresentem essas patologias para verificar se as proteínas do soro do leite teriam efeito similar ao encontrado nos ratos saudáveis. Além disso, estudar se os efeitos obtidos com animais poderiam ser também conseguidos em humanos.

ANEXO I. Aprovação da Comissão de Ética

UNICAMP

CEUA/Unicamp

Comissão de Ética no Uso de Animais
CEUA/Unicamp

C E R T I F I C A D O

Certificamos que o projeto **"Efeito do consumo de proteínas do soro de leite nos transportadores de glicose GLUT4"** (protocolo nº **2376-1**), sob a responsabilidade de **Prof. Dr. Jaime Amaya Farfan / Priscila Neder Morato**, está de acordo com os **Princípios Éticos na Experimentação Animal** adotados pela **Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL)** e com a legislação vigente, **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, e o **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**.

O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP - em 04 de abril de 2011.

Campinas, 04 de abril de 2011.

Profa. Dra. Ana Maria A. Guaraldo
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