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"CARACTERIZAÇÃO MOLECULAR E FUNCIONAL DA ENZIMA GLUTAMATO DESIDROGENASE (GDH) EM ILHOTAS DE RATOS SUBMETIDOS À RESTRIÇÃO PROTÉICA E SUPLEMENTADOS COM LEUCINA"

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Orientador: Prof. Dr. Everardo Magalhães Carneiro

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Aos meus Pais,

Irmãos

e Avós.

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À Deus, que jamais nos abandona e aos caminhos que Ele nos dá para que atravessemos.

Aos meus pais, que me colocaram no mundo, me deram o meu caráter e jamais me abandonaram.

Aos meus irmãos, que contribuíram para a formação desse caráter e que me acompanharam de perto ou de longe no trajeto da minha vida, sempre soube do amor deles por mim.

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"Porque a sabedoria deste mundo é loucura diante de Deus."

I Coríntios 3:19a

"Dizem que sou louco por eu ser assim, mais louco é quem me diz e não é feliz!"

Mutantes (Rita Lee, Arnaldo Batista e Sérgio Batista)

"Que me conste, ainda ninguém relatou o seu próprio delírio; faço-o eu, e a ciência mo agradecerá. Se o leitor não é dado à contemplação destes fenômenos mentais, pode saltar o capítulo; vá direto à narração. Mas por menos curioso que seja, sempre lhe digo que é interessante saber o que se passou na minha cabeça durante uns vinte a trinta minutos."

Brás Cubas em Memórias Póstumas de Brás Cubas, Capítulo VII "O Delírio", por *Machado de Assis*.

"(...)Deus tem autoridade sobre a morte, perguntou um dos optimistas, São as duas caras da mesma moeda, de um lado o rei, do outro a coroa, Sendo assim, talvez tenha sido por ordem de deus que a morte se retirou, A seu tempo conheceremos o motivo desta provação, entretanto, vamos por os rosários a trabalhar, Nós faremos o mesmo, refiro-me às orações, claro está, não aos rosários, sorriu o protestante (...) procissões nunca fizeram parte das manias que cultivamos, tornou a sorrir o protestante. E nós, perguntou um dos filósofos optimistas em um tom que parecia anunciar o seu próximo ingresso nas fileiras contrárias, que vamos fazer (...), Para começar, levantar a sessão, respondeu o mais velho, E depois, Continuar a filosofar, já que nascemos para isso, e ainda que seja sobre o vazio, Para quê, Para quê, não sei, Então porquê, Porque a filosofia precisa tanto da morte como as religiões, se filosofamos é por saber que morreremos, monsieur de montaigne já tinha dito que filosofar é aprender a morrer."

José Saramago, escritor português em Intermitências da Morte

"(...)

É verdade que se podia criticar muita coisa no meu pai, mas nunca achei chato conversar com ele. Ele é o tipo de pessoa que nunca iria se contentar com uma vida de mecânico. Se dependesse de mim, ele teria direito a um salário do governo como filósofo. Certa vez ele mesmo disse algo nesse sentido: "Temos ministérios para tudo, mas não para a filosofia. E até os países grandes acham que podem dar conta de suas tarefas sem ela".

Com o peso daquela herança que eu tinha sobre os ombros, tentava participar das conversas filosóficas que meu pai sempre começava quando não estava falando de mamãe. Naquele momento, eu disse:

- O fato de o universo ser tão vasto não significa necessariamente que a nossa Terra seja um grão de ervilha.

Meu pai sacudiu os ombros e acendeu outro cigarro. No fundo não estava particularmente interessado na opinião dos outros quando falava sobre a vida e sobre os astros. Nesse ponto era um homem seguro demais quanto à sua própria opinião. Em vez de comentar minha afirmação, disse:

- Com mil diabos, Hans-Thomas, de onde vêm as pessoas como nós? Você já pensou a respeito disso?

É claro que já tinha pensado muitas vezes; mas como sabia que minha resposta não ia adiantar muita coisa, deixei que ele continuasse falando. Nós nos conhecíamos fazia tanto tempo, meu pai e eu, que eu sabia que aquilo era o melhor.

- Você sabe o que sua vó me disse um dia? Ela disse ter lido na Bíblia que Deus está lá no céu e ri das pessoas que não acreditam nele.

- E por quê? - perguntei. Perguntar era sempre mais fácil do responder.

- Muito bem... - começou meu pai. - Se há um Deus, que nos criou, então de uma certa forma somos "artificiais" aos seus olhos. Falamos besteira, discutimos e brigamos entre nós. Depois nos separamos e morremos, compreende? Somos superinteligentes: sabemos construir bombas atômicas e foguetes para ir à Lua. Mas nenhum de nós se pergunta de onde veio. A gente simplesmente se contenta em estar por aqui, dividindo com os outros este espaço.

- E é nessa hora que Deus ri de nós?

-Exatamente. Se *nós* fôssemos capazes de criar um ser artificial, Hans-Thomas, nós também iríamos rachar o bico de rir se esse ser artificial saísse por aí falando um monte de bobagens sobre os índices da bolsa de valores ou sobre corridas de cavalos, por exemplo, sem se perguntar a coisa mais simples e mais importante de todas: "de onde é que eu vim?".

E foi exatamente o que ele fez antes de prosseguirmos viagem:

- Devíamos ler mais a Bíblia, meu caro. Depois que Deus criou Adão e Eva, ele ficou andando pelo jardim do Éden, observando os dois. Verdade... Ele ficava atrás dos arbustos e árvores observando direitinho tudo o que os dois faziam, entende? Deus não conseguia tirar os olhos deles , de tão fascinado que estava com a sua criação e não o critico por isso. Não, não... posso entendê-lo muito bem!

Meu pai apagou o cigarro e com isso a pausa chegou ao fim. Fiquei pensando que apesar de tudo eu era uma garoto de sorte: afinal, durante aquela viagem à Grécia ia poder usufruir de umas trinta ou quarenta paradas como aquela.

Dentro do carro, tirei novamente a lupa do estojo que anão misterioso tinha me dado decidi usá-la para examinar a natureza mais de perto . Se me deitasse no chão e ficasse olhando um bom tempo uma formiga ou uma flor, talvez conseguisse descobrir alguns segredos. E então eu poderia dar um pouco de sossego de natal ao meu pai."

Hans-Thomas e seu pai, a caminho de Atenas, Grécia,
em O Dia do Curinga, Parte *Espadas*,
Capítulo "Ás de Espadas, ...Deus está lá no céu e ri das pessoas que não acreditam nele...".
Por *Jostein Gaarder*.

Todo bem procede de Deus

Cântico de Romagem. De Salomão

127 *Se o senhor não edificar a casa, em vão trabalham os que a edificam; se o*
SENHOR não guardar a cidade, em vão viagia a sentinela.
[2]*Inútil vos será levantar de madrugada, repousar tarde, comer o pão que*
penosamente granjeaste; aos seus amados ele o dá enquanto dormem.
(...)

BÍBLIA SAGRADA, *Salmos.*

Thank You

Lookin at the devil
Grinning at his gun
Fingers start shaking
I begin to run

Bullets start chasing
I begin to stop
We begin to wrestle
I was on the top

(I wanna)
Thank you
Falletinme
Be Mice Elf
Agin

(…)

Stiff all in the collar
Fluffy in the face
Chit Chat Chatter tryin
I begin to run

Thank you for the party
I could never stay
Many things is on my mind
Words in the way

(I wanna)
Thank you
Falletinme
Be Mice Elf
Agin

Thank you
Falletinme
Be Mice Elf
Agin

Dance to the music
All night long
Everyday people
Sing a simple song

Mama's so happy
Mama starts to cry
Papa's still singin:
"We can make it if we try"

(I wanna)
Thank you
Falletinme
Be Mice Elf
Agin

(…)
Flaming eyes
Of people fear
Burning into you

Many men
Are missing much
Hating what they do

Youth and truth
Are making love
Dig it for a starter

Dying young
Is hard to take
Selling out is harder

(I wanna)
Thank you
Falletinme
Be Mice Elf
Agin

SLY AND THE FAMILY STONE THANK YOU FALETTINME BE MICE ELF AGIN
(1969, Epic Records)
by **Dave Mathews Band**

I can see clearly now the rain is gone!!!
It´s gonna be a bright, bright, bright...
bright, sunshine day!!!
Jimmy Cliff

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Lista de Abreviaturas

$[Ca^{2+}]_i$- Concentração de cálcio intracelular

ADP- Bifosfato de adenosina

AGL- Ácidos graxos livres

Anova- Análise de variância

AOA- Ácido aminooxiacético

ATC- Ácido tricarboxílico

ATP- Trifosfato de adenosina

BCATm- Transaminase mitocondrial de aminoácidos de cadeia ramificada

BCH- Ácido 2-aminobiciclo[2,2,1]heptano-2-carboxílico

EGCG- Epigalocatequina galato

GDH- Glutamato desidrogenase

GLN- Glutamina

GLU- Glutamato

GLUD1- Gene da glutamato desidrogenase 1

GLUD2- Gene da glutamato desidrogenase 2

GLUT-2- Transportador de glicose - 2

GTP- Trifosfato de guanosina

H^+- Íons hidrogênio

HI/HA: Hiperinsulinemia/hiperamonemia

INS-1- Células β de insulinoma de rato-1

IR- Receptor de insulina

IRS-1- Substrato do receptor de insulina -1

K^+- Íon potássio

KIC- Ácido α-cetoisocapróico

LEU- Leucina

LP- Hipoprotéica

LPL- Hipoprotéica suplementada com LEU

M3- Receptor muscarínico 3

mTOR- Proteína alvo da rapamicina em mamíferos

NP- Normoproteíca

NPL- Normoproteíca suplementado com LEU

S6K-1- Proteína S6 quinase-1

p85- Subunidade regulatória da PI3K de 85 kDa

PDG- Glutaminase dependente de fosfato

PDX-1- Fator homeobox pancreático-duodenal

PI3K- Fosfatidil inositol 3-quinase

PKA- Proteína quinase A

PKC- Proteína quinase C

RNAm- Ácido ribonucléico mensageiro

RPS29- Proteína ribossomal S29

RESUMOS

RESUMO

A glutamato desidrogenase (GDH) é uma enzima mitochondrial que cataliza a reação reversível de glutamato a α-cetoglutarato. Nas ilhotas pancreáticas, está associada com a secreção de insulina por aumentar a concentração de ATP. Ratos alimentados com dieta hipoprotéica apresentam secreção de insulina diminuída. A suplementação com leucina (LEU) aumenta a secreção de insulina em resposta a agentes insulinotrópicos. O presente estudo investigou a influência da suplementação com LEU na expressão da GDH e seu envolvimento com a secreção de insulina em ratos desnutridos e suplementados com LEU. Ratos machos foram alimentados com dietas normo- (17%, **NP**) ou hipoprotéicas (6%, **LP**) por oito semanas. Após, foram divididos e suplementados com LEU (1,5%) na água de beber (**NPL** e **LPL**) pelas quatro semanas seguintes. O conteúdo protéico de GDH no cérebro, fígado, rim e músculo esquelético não diferiu entre os grupos. Nas ilhotas LP, a expressão da GDH estava diminuída e a suplementação com LEU aumentou a expressão de RNAm restaurando o conteúdo protéico a valores similares a NP. A secreção de insulina estimulada por agentes insulinotrópicos ou inibidores, combinados ou não, estava diminuída em ilhotas LP comparada com NP. A suplementação com LEU aumentou a secreção de insulina a valores similares a NP, exceto quando as ilhotas LPL foram incubadas com EGCG. Ilhotas LP tiveram diminuição na $[Ca^{2+}]_i$ quando expostas a GLN+BCH. A suplementação com LEU restaurou esses parâmetros aos valores de NP. Frente a esses resultados, podemos concluir que a diminuição na expressão da GDH induzida pela dieta LP foi central ao pâncreas endócrino e está associada à redução da secreção de insulina observada nas ilhotas LP. A suplementação com LEU foi capaz de restaurar a expressão da GDH, contribuindo para o aumento da secreção de insulina observado nas ilhotas

LPL. Além disso, a GDH pode, ainda, estar associada com a secreção de insulina pelo acoplamento estímulo/secreção via regulação da $[Ca^{2+}]_i$.

ABSTRACT

Glutamate dehydrogenase (GDH) is a mitochondrial matrix enzyme that catalyzes the reversible reaction of glutamate to α-ketoglutarate. In the pancreatic islets, this enzyme is associated with insulin secretion by augmenting ATP levels. Protein malnourished rats displayed reduced insulin secretion. Leucine (LEU) supplementation augments the insulin secretion response to insulinotropic agents. The present study investigated the influence of LEU supplementation on GDH expression and its involvement with insulin secretion in malnourished rats supplemented with LEU. Male rats were fed normal- (17%, **NP**) or low-protein diet (6%, **LP**) for eight weeks. Half of rats of each group were supplemented with LEU (1.5%) in drinking water for the following four weeks (**NPL** and **LPL** groups). GDH protein content in brain, liver, kidney and skeletal muscles was not different in any group. GDH RNAm and protein content was reduced in LP islets and LEU supplementation augmented RNAm expression restoring protein content similar to NP. Insulin secretion was reduced in LP islets compared with NP when stimulated by insulinotropics agents or inhybitors, combinaned or not. LEU supplementation augmented insulin secretion to similar values as NP, an effect that was blunted when LPL islets were incubated with EGCG. LP islets showed lower $[Ca^{2+}]_i$ when exposed to GLN+BCH. LEU supplementation augmented these patterns similar to NP. Taken together, we may conclude that diminution in GDH expression induced by LP diet was central to endocrine pancreas and was associated with reduced insulin secretion observed in LP rats. LEU supplementation was able to restore GDH expression and it was capable to restore insulin secretion via GDH restoration. Yet, GDH may contribute to insulin secretion via Ca^{2+} regulation stimulus/secretion coupling.

INTRODUÇÃO

Glutamato Desidrogenase e Secreção de Insulina

A glutamato desidrogenase (GDH) é uma enzima homohexâmera, com pelo menos duas isoformas codificadas por genes distintos, *GLUD1* e *GLUD2*, e está localizada na matriz mitocondrial (**Shashidharan *et al.*, 1994; Mastorodemos *et al.*, 2005; Maechler *et al.*, 2006**). Ela catalisa a reação de deaminação do glutamato em α-cetoglutarato utilizando NAD e/ou NADP como co-fatores (**Shashidharan *et al.*, 1994; Kelly & Stanley, 2001; Plaitakis *et al.*, 2003; Frigerio *et al.*, 2008; Li *et al.*, 2010**). Essa enzima é expressa em altas concentraçãoes no fígado, cérebro, pâncreas e rins. No cérebro, onde é encontrado pelo menos duas isoformas da GDH (**Cho, *et al.*, 1995; Plaitakis & Zaganas, 2001; Mastorodemos *et al.*, 2005; Frigerio *et al.*, 2008**), ela pode transformar glutamato (GLU) em glutamina (GLN) ou, ainda, realizar a deaminação do glutamato (**Yu *et al.*, 1982; Anderson *et al.*, 2000**). No fígado, onde a GDH é proveniente do *GLUD1* (**Plaitakis *et al.*, 2003**) e no rim, possui um importante papel no metabolismo modulando a concentração de amônia (**Nissim *et al.*, 1999; Kelly & Stanley, 2001; Frigerio *et al.*, 2008; Li *et al.* 2010**). No pâncreas, a GDH é uma enzima chave no processo de secreção de insulina (**Sener & Malaisse, 1980; Bryla *et al.*, 1994; Kelly *et al.*, 2002**), estando envolvida no desenvolvimento da resposta secretória (**Gylfe, 1974; Sener & Malaisse, 1980; Carobbio *et al.*, 2004; Li *et al.*, 2006b; Maechler *et al.*, 2006; Carobbio *et al.*, 2009**). O controle do mecanismo de ação enzimática é efetuado via alostérica. Leucina (LEU) e ADP são uns dos principais efetores positivos e GTP, um modulador negativo (**Sener & Malaisse, 1980; Bryla *et al.*, 1994; Kelly, 2002; Fang *et al.*, 2002; Li *et al.*, 2010**).

A importância fisiológica desta enzima tem-se demonstrado clinicamente através da síndrome de hiperinsulinemia-hiperamonemia (HI-HA), a qual se caracteriza por mutações no

sítio de ligação para GTP da GDH, perdendo o controle inibitório da enzima provocando hipoglicemia após o consumo de refeição rica em proteína (**Stanley *et al.*, 1998, Yorifuji *et al.* 1999**). Crianças que desenvolvem a síndrome congênita de HI-HA manifestam episódios recorrentes de hipoglicemia e elevação nos íons amônia no plasma (**Stanley *et al.*, 1998, Stanley *et al.*, 2004, Stanley *et al.*, 2009, Stanley *et al.*, 2010**). O aumento da atividade da enzima eleva a liberação de insulina em células β pancreáticas e, concomitantemente no fígado, há aumento de produção de amônia e redução de acetilglutamato, sendo este, um ativador alostérico dos primeiros passos da ureagênese (**Kelly & Stanley, 2001; Fang *et al.*, 2002; Li *et al.*, 2003; Stanley, 2004; Li *et al.*, 2004; Hoe *et al.*, 2006; Frigerio *et al.*, 2008**).

A atuação da GDH como enzima chave no controle do processo de secreção de insulina tem sido estudada desde a década de 70. Estudos utilizando um análogo não metabolizável da LEU, o ácido 2-aminobiciclo[2,2,1]heptano-2-carboxílico (BCH), e que, portanto, tem ação alostérica sobre a GDH, demonstraram a contribuição desta enzima na estimulação da secreção de insulina (**Gylfe, 1974; Sener & Malaisse, 1980**) e sua importância para manutenção da insulinemia no estado pós-absortivo (**Gao *et al.*, 1999**). Foi demonstrado em camundongos que a proteína sirtuina 4, uma ADP-ribosiltransferase mitocondrial, promove ADP-ribolização da GDH, reduzindo sua atividade enzimática em células β, limitando o metabolismo de GLN e GLU em gerar ATP (**Haigis *et al.*, 2006; Ahuja *et al.*, 2007**). Estudos recentes com inibidores da GDH, como o polifenol de chá verde, o epigalocatequina galato (EGCG) e o 5'-deoxipirodoxal, assim como o *knockout* desta enzima, reduzem a secreção de insulina (**Li *et al.* 2006a; Yang *et al.* 2003; Carobbio *et al.* 2009**). Já a superexpressão da GDH demonstrou aumentar a secreção de insulina, evidenciando seu papel chave na regulação metabólica deste processo (**Carobbio *et al.* 2004**). Em animais submetidos à restrição calórica, verificou-se que a atividade da GDH foi

amplificada causando aumento na secreção de insulina em resposta a GLN ou LEU (**Haigis *et al.*, 2006**).

Embora bem estabelecido o papel da GDH no controle da secreção de insulina, ainda faltam esclarecimentos sobre envolvimento na funcionalidade das células β (**Maechler *et al.* 2010**). Sugere-se que a GDH pode modular a secreção de insulina produzindo glutamato (**Maechler & Wollhein, 1999; Rubi *et al.* 2001; Casimir *et al.*, 2009**), apontado como segundo mensageiro necessário para secreção de insulina estimulada por glicose, ou como formadora de compostos anapleróticos que amplificam a resposta secretória de insulina (**Li *et al.* 2004; MacDonald *et al.*, 2008; Carobbio *et al.*, 2009; Odegaard *et al.*, 2010**).

De maneira geral, os aminoácidos de cadeia ramificada estimulam a secreção de insulina através da reação catalisada pela GDH (**Anno *et al.* 2004; Yang *et al.*, 2010**), promovendo assim, aumento de sinais metabólicos importantes para o controle da secreção de insulina, dentre os quais, ATP, NADPH e GLU (**Maechler & Wollhein, 1999; Rubi *et al.* 2001; Li *et al.* 2004; MacDonald *et al.*, 2008; Casimir *et al.*, 2009; Carobbio *et al.*, 2009; Odegaard *et al.*, 2010**).

Tem sido demonstrado que tratamentos com LEU aumentam a secreção de insulina estimulada por glicose, aminoácidos e agentes potencializadores da secreção. Neste sentido, a LEU aparece como principal secretagogo dentre os aminoácidos de cadeia ramificada (**MacDonald *et al.*, 2008**) e pode exercer seu mecanismo de ação por duas vias distintas: a primeira envolve a transaminação da LEU pela transaminase de aminoácidos de cadeia ramificada mitocondrial (BCATm) em α-cetoisocaproato (KIC) e sua consequente oxidação mitocondrial e produção de ATP (**Gao *et al.* 1999; Gao *et al.* 2003**); a segunda, promove liberação de insulina por ativação alostérica da GDH, aumentando a concentração de intermediários do ciclo do ácido tricarboxílico (ATC) (**Li *et al.* 2004; MacDonald *et al.*, 2008;**

Odegaard *et al.*, 2010) e resulta na produção de ATP (**Sener & Malaisse, 1980**) ou, ainda, na formação de glutamato (**Maechler & Wollhein, 1999; Casimir *et al.*, 2009**).

O aumento de ATP gerado via metabolismo da glicose ou via metabolismo da LEU causa a despolarização da membrana da célula β, por inibir os canais de potássio sensíveis à ATP (**Branstrom *et al.* 1998**). A despolarização leva à abertura dos canais de cálcio voltagem dependente aumentando a concentração de cálcio intracelular ($[Ca^{2+}]_i$), este participando do processo de exocitose dos grânulos de insulina (**Ohara-Imaizumi & Nagamatsu, 2006; Ashcroft, 2006**).

Ainda, em relação a sua ação na funcionalidade das células β pancreáticas, recentes estudos indicam que a LEU ou seu produto transaminado, KIC, podem regular a secreção de insulina por inibir diretamente o canal de potássio dependente de ATP, com magnitude idêntica àquela observada por glicose. Porém, o mecanismo pelo qual LEU e KIC estimulam a secreção de insulina ainda permanece obscuro (**Li *et al.*, 2004; Zhou *et al.*, 2010**).

Função da GDH em outros tecidos

A função mitocondrial no cérebro está associada com o metabolismo do GLU. O GLU é o principal aminoácido neurotransmissor excitatório no sistema nervoso central (**Baudry & Lynch, 1979**). Ele é liberado por sinapses ativas e, então, despolariza os neurônios alvo através de receptores específicos. O GLU também é precursor do ácido γ-aminobutírico nos neurônios e de GLN nos astrócitos (**Mayer *et al.*, 1984; Spreafico *et al.*, 1994; Johnson & Roberts, 1984**). No cérebro, a GDH é altamente expressa nos astrócitos. Tal expressão garante uma retirada eficiente do GLU liberado pelos neurônios e seu catabolismo (**Aoki *et al.*, 1987; Schousboe *et al.*, 1977**), evitando a neurotoxicidade induzida por este aminoácido (**Duchen, 2004**). Em condições de

necessidade de energia, os astrócitos metabolizam o GLU primariamente via GDH e os neurônios, via aspartato aminotransferase. A oxidação completa do GLU ocorre no ciclo do ATC, onde, após a ação de deaminação oxidativa da GDH, o GLU entra no ciclo ATC como α-cetoglutarato, produzindo ATP (**Yu *et al.*, 1982**).

O fígado é de fundamental importância na homeostase glicêmica, no metabolismo energético e lipídico (**Fritsche *et al.*, 2008**). Também é o órgão central no metabolismo de nitrogênio (**Kelly & Stanley, 2001; Stanley, 2004; Frigerio *et al.*, 2008**).

O metabolismo de nitrogênio efetuado no fígado tem como enzima chave a GDH, que produz α-cetoglutarato e amônia para a conversão de uréia (**Kelly & Stanley, 2001; Fang *et al.*, 2002; Li *et al.*, 2003; Stanley, 2004; Li *et al.*, 2004; Kawajiri *et al.*, 2006; Li *et al.*, 2006b; Hoe *et al.*, 2006; Frigerio *et al.*, 2008**). A importância desta enzima para a ureagênese é demonstrada pela síndrome congênita de HI-HA e pela deficiência de n-acetilglutamato sintase (**Kelly & Stanley, 2001; Stanley, 2004; Frigerio *et al.*, 2008**). O α-cetoglutarato também pode ser utilizado para a produção de ATP (**Shashidharan *et al.*, 1994; Kelly & Stanley, 2001; Plaitakis *et al.*, 2003; Kawajari *et al.* 2006; Frigerio *et al.*, 2008**) ou gliconeogênse (**Frigerio *et al.*, 2008**).

No fígado, a GDH é exclusivamente expressa nos hepatócitos e sua quantidade é diferenciada nas regiões do fígado. A GDH está mais expressa nas regiões perivenais que nas regiões periportais (**Geerts *et al.*, 1996; Frigerio *et al.*, 2008**). O RNA mensageiro (RNAm) da GDH é expresso em quantidades diferenciadas entre machos e fêmeas de ratos Wistar dependendo da região hepática avaliada (**Geerts *et al.*, 1996**).

O rim possui uma vasta gama de funções, dentre as quais: manutenção do metabolismo e

do equilíbrio ácido-base, balanço de eletrólitos e fluidos; regulação da hematopoiese e da excreção de produtos (**Van de Poll, 2004**). A GLN é quantitativamente o mais importante doador de grupamentos NH_3 no rim (**Newsholme *et al.*, 2003**). Ao contrário dos outros tecidos, nos quais o metabolismo da GLN é praticamente constitutivo, o catabolismo renal é ativado agudamente em resposta a acidose metabólica. Durante o balanço normal de ácido-base, o rim extrai e metaboliza muito pouco da GLN plasmática (**Curthoys, 2001**). A utilização da pequena fração extraída do plasma de GLN requer o seu transporte à matriz mitocondrial onde é deaminada pela glutaminase e depois deaminada por oxidação pela GDH. Durante a acidose metabólica crônica, o aumento de amoniagênese renal de ratos é sustentado, em parte, por aumentar a expressão da glutaminase mitocondrial e da GDH. O aumento dessas enzimas ocorre pela estabilização de seus RNAm (**Schoroeder *et al.* 2003**). Já na acidose aguda, o fluxo através da glutaminase dependente de fosfato (PDG) e da GDH é estimulado. Aproximadamente, 60% da amônia são derivadas da glutamina via fluxo PDG contra 15-25% da reação da GDH (**Nissim, 1999**).

O tecido muscular é o local de maior síntese de GLN e contém 90% de toda a GLN corpórea. Estudos quantitativos realizados em humanos demonstraram que, no estado pós-absortivo, alanina e GLN totalizam 60% dos aminoácidos liberados pelo tecido muscular (**Newsholme *et al.*, 2003**). Em um estudo realizado, há evidências de que da mesma maneira que no rim, a acidose metabólica resulta em um aumento da atividade da GDH no músculo esquelético de ratos (**Zhou & Thompson, 1996**). Há ainda a sugestão de que o músculo esquelético e os rins devem agir de maneira coordenada para garantir a retirada do H^+ durante a acidose metabólica (**Zhou & Thompson, 1996**).

Ações Biológicas da Leucina

Aminoácidos de cadeia ramificada que incluem LEU, isoleucina e valina são aminoácidos essenciais que não podem ser sintetizados pelos vertebrados e, portanto, devem ser obtidos pela dieta diariamente. Além de seu efeito descrito anteriormente sobre a estimulação da secreção de insulina, a LEU desempenha um papel crucial no controle da síntese protéica, modulando a iniciação da tradução em vários tipos de células (**Yang *et al.*, 2010**).

Na década passada, foi demonstrado que a LEU ativa a proteína alvo da rapamicina de mamíferos (mTOR), uma serina/treonina quinase sensível a nutrientes que regula a síntese protéica e o metabolismo celular (**McDaniel *et al.*, 2002**). Também foi demonstrado que a LEU é capaz de estimular a transcrição de genes e a síntese protéica em células β pancreáticas por vias dependentes e independentes de mTOR (**Kwon, 2004; Yoshizawa, 2004; Blomstrand *et al.*, 2006; Stipanuk, 2007**).

Igualmente, demonstrou-se que a suplementação com LEU em roedores induz modulações sistêmicas que resultam na melhora do controle glicêmico em diversos modelos experimentais. LEU aumenta os níveis circulantes de insulina e melhora o controle glicêmico em camundongos dB/dB ou diabéticos induzidos por dieta rica em gordura, promovendo restabelecimento da glicemia nestes animais (**Rozance *et al.*, 2006; Zhang *et al.*, 2007; Nilsson *et al.*, 2007**). Também, o aumento do consumo de LEU na dieta reduziu a obesidade induzida por dieta, hiperglicemia e hipercolesterolemia em humanos e roedores por múltiplos mecanismos (**Parker *et al.*, 2002; Layman *et al.*, 2006; Baum *et al.*, 2006**). A administração de LEU aumenta a síntese protéica no músculo, no tecido adiposo e fígado, além de inibir a proteólise neste último tecido (**Tremblay & Marette, 2001**). Portanto, a suplementação com LEU parece ser um importante aliado no combate a situações que provoquem alteração na homeostasia

glicêmica, devido a sua atuação multifatorial nas células dos diversos tecidos envolvidos neste processo.

Desnutrição e Secreção de Insulina

A desnutrição protéica atinge grandes contingentes populacionais, principalmente nos países em desenvolvimento. Apesar da tendência de queda nas taxas de desnutrição, uma alta prevalência tem sido encontrada em crianças nascidas com baixo peso (**Post *et al.*, 1996**). Segundo **Villar *et al.*** (**1982**), nos países em desenvolvimento, a mais importante causa de baixo peso ao nascer é a desnutrição intra-uterina. A associação entre desnutrição e prevalência de doenças durante a infância, principalmente infecções, está bem documentada (**Pelletier, 1994**). No que se referem às repercussões tardias da desnutrição, estudos epidemiológicos realizados na Europa, América do Norte e em países em desenvolvimento mostraram forte associação entre baixo peso em crianças nascidas a termo e o desenvolvimento da síndrome plurimetabólica (**Hales & Barker, 1992**).

Em nosso âmbito, temos estudado o papel da desnutrição na determinação da síndrome plurimetabólica, usando o modelo experimental animal de restrição protéica na vida intra-uterina, lactação e após desmame, avaliando os seus efeitos sobre a secreção e ação da insulina na vida adulta (**Latorraca *et al.*, 1998; Ferreira *et al.*, 2003, Ferreira *et al.*, 2004; Araujo *et al.*, 2004; Filiputti *et al.*, 2008; da Silva *et al.* 2010, Amaral *et al.* 2010; Batista *et al.*, 2010**). Estudos que avaliem o impacto imediato das repercussões tardias da má-nutrição são de interesse para a medicina e podem também contribuir para a elaboração de estratégias de tratamento e de prevenção de doenças crônicas.

A restrição protéica modifica o mecanismo de secreção de insulina alterando a resposta

secretória para diferentes agentes insulinotrópicos, tais como glicose, aminoácidos, potássio e também para agentes potencializadores da secreção de insulina (**Ferreira *et al.*, 2003; Araujo, *et al.*, 2004; Filiputti *et al.*, 2008; da Silva *et al.* 2010, Amaral *et al.* 2010; Batista *et al.*, 2010**). Esta mudança no padrão de secreção deve-se a alterações em proteínas envolvidas com o mecanismo de secreção, tais como proteína quinase C (PKC), proteína quinase A (PKA) e a GDH (**Ferreira *et al.*, 2003; Ferreira *et al.*, 2004; da Silva *et al.* 2010, Amaral *et al.* 2010; Batista *et al.*, 2010**). Além disso, existe um conjunto de genes e proteínas que participam da produção e secreção de insulina que estão modificados neste modelo experimental (**Ferreira *et al.*, 2002, Ferreira *et al.*, 2003; Delguingaro *et al.*, 2004; Filiputti *et al.*, 2008; da Silva *et al.* 2010, Amaral *et al.* 2010; Batista *et al.*, 2010**).

Igualmente, temos estudado a modulação do processo de secreção de insulina em animais desnutridos que foram suplementados com LEU. Observamos que este aminoácido aumenta a secreção de insulina, estimulado por diferentes agentes insulinotrópicos, tanto em animais controle como em desnutridos. Os animais desnutridos suplementados com LEU apresentam restabelecimento da secreção de insulina, voltando a apresentar níveis similares aos de animais controle quando estimuladas por de arginina (**Filiputti *et al.*, 2010**), alta glicose, (**Filiputti *et al.*, 2010; da Silva *et al.*, 2010; Amaral *et al.* 2010**), BCH, KIC (**da Silva *et al.*, 2010**) e carbacol (ativador do receptor muscarínico do tipo 3, M3) quando associado a de glicose (**Amaral *et al.* 2010**).

Hipóteses do Trabalho

Considerando os aspectos aqui levantados sobre GDH, secreção de insulina, LEU e desnutrição protéica, testamos, neste estudo, a hipótese de que a desnutrição pode alterar a expressão gênica da GDH em ilhotas, o conteúdo protéico da GDH em cérebro, fígado, rim, músculo e ilhotas de ratos machos *Wistar*, além de modular a secreção de insulina via GDH. De igual forma, verificamos a hipótese de que a suplementação com LEU pode restaurar as alterações induzidas pela dieta hipoprotéica nos parâmentros avaliados.

OBJETIVOS

Avaliar em ilhotas de Langerhans de ratos machos *Wistar* submetidos à restrição protéica e suplementados com LEU:

- A regulação, pela LEU, da expressão gênica e protéica da enzima GDH e seu envolvimento com o mecanismo de secreção de insulina em ratos desnutridos e suplementados com LEU;
- O envolvimento da GDH com o controle de $[Ca^{2+}]_i$ e a modulação do mecanismo de secreção de insulina em ratos desnutridos e suplementados com LEU;
- O mecanismo de ação da LEU e do KIC sobre a secreção de insulina em ratos desnutridos e suplementados com LEU.

ARTIGO 1

Preliminary report: Leucine supplementation enhances glutamate dehydrogenase expression and restores glucose-induced insulin secretion in protein-malnourished rats

Artigo publicado em: ***Metabolism Clinical and Experimental 59****: 911–913 (2010) (ANEXOS)*

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The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work. The experiments with animals are in adherence with the institutional State University of Campinas Committee for Ethics in Animal Experimentation.

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Abstract

Low-protein diet impairs insulin secretion in response to nutrients and may induce several metabolic disorders including diabetes, obesity, and cardiovascular disease. In the present study, the influence of leucine supplementation on glutamate dehydrogenase (GDH) expression and glucose-induced insulin secretion (GIIS) was investigated in malnourished rats. Four groups were fed with different diets for 12 weeks: a normal-protein diet (17%) without or with leucine supplementation or a low (6%)-protein diet without (LP) or with leucine supplementation (LPL). Leucine (1.5%) was supplied in the drinking water. Western blotting analysis revealed reduced GDH expression in LP, whereas LPL displayed improved GDH expression, similar to control. The GIIS and leucine-induced insulin release were also enhanced in LPL compared with LP and similar to those observed in rats fed a normal-protein diet without leucine supplementation. In addition, GDH allosteric activators produced an increased insulin secretion in LPL. These findings indicate that leucine supplementation was able to increase GDH expression leading to GIIS restoration, probably by improved leucine metabolic pathways.

List of Abbreviations

BCH, 2-amino-2-norbornanecarboxylic acid; GDH, glutamate dehydrogenase; GIIS, glucose-induced insulin secretion, KIC; α-ketoisocaproate; LP, low-protein; LPL, low-protein supplemented with LEU; mTOR, mammalian target of rapamycin; NADPH, nicotinamide adenine dinucleotide phosphate; NP, normal-protein; NPL, normal-protein supplemented with LEU; PI3K, phosphoinositide 3-kinase; p70S6K, p70 S6 kinase; TCA, tricarboxylic acid cycle; TTBS, TRIS-tween buffer saline

1. Introduction

Protein malnutrition impairs glucose-induced insulin secretion (GIIS) by reducing the transcription of several genes related to β-cell signaling [1]. Actually, it has been reported that the insulin signaling pathway is impaired in protein-undernourished rats because of reduced PI3K and p70S6k expression in pancreatic islets [2]. In this process, leucine was demonstrated to improve insulin release [3], an effect that was associated with elevated mitochondrial energy production [4]. Although the mechanism remains unclear, leucine metabolism might supply the tricarboxylic acid cycle (TCA) with different anaplerotic substrates including α-ketoisocaproate (KIC), which can be further metabolized to acetyl-coenzyme A and acetoacetate [5]. Leucine may also allosterically activate glutamate dehydrogenase (GDH), the enzyme that converts glutamate to α-ketoglutarate, an important anaplerotic substrate for the second span of TCA. The observation that β-cells exhibit a high level of anaplerotic mitochondrial enzymes suggests that this process is particularly important for the insulin secretion mechanism [5]. In addition, leucine might activate mammalian target of rapamycin (mTOR) downstream events, affecting elongation and translation of intracellular signaling proteins [6]. In the β-cell line RINm5F, the leucine-induced GDH activation was shown to increase the p70S6k phosphorylation and mitochondrial activity [4], suggesting that leucine might be directly associated with improvement of insulin release. In addition, preliminary data from our group demonstrated that, after leucine supplementation, undernourished rats exhibit an increased PI3K/mTOR pathway activation as well as enhanced GIIS despite reduced glucose oxidation [7]. However, although leucine plays an important function as an allosteric activator of GDH, whether this enzyme has a role in the amelioration of islet function in malnourished rats has not been examined. Therefore, the purpose

of this work was to test the hypothesis that, in undernourished rats, leucine supplementation restores GIIS capacity via enhanced GDH expression.

2. Material and methods

2.1. Animals and treatment

The experiments were approved by the institutional Committee for Ethics in Animal Experimentation-UNICAMP. Male Wistar rats (21 days old) from the breeding colony at UNICAMP were housed at 24°C on a 12-hour light/dark cycle. Rats were separated into 4 groups of isocaloric diets and treated for 12 weeks with the following: normoprotein diet (NP) (17% protein), normoprotein diet plus leucine (NPL) (17% protein plus leucine supplementation during the last 4 weeks), low-protein diet (LP) (6% protein), and low protein diet plus leucine (LPL) (6% protein plus leucine supplementation during the last 4 weeks). The 2 isocaloric diets compositions were previously described elsewhere [2]. Leucine (1.5%) was supplied in the drinking water.

2.2. Insulin secretion

Islets were isolated by collagenase digestion of the pancreas [8]. For static incubation, 5 islets from each group were incubated with Krebs-Ringer-bicarbonate containing glucose 5.6 mmol/L for 45 minutes at 37°C as previously described [2]. This medium was then replaced with Krebs-Ringer-bicarbonate with the following secretagogues: glucose (22.2 mmol/L), leucine (20 mmol/L), 2-amino-2-norbornanecarboxylic acid (BCH) (20 mmol/L), and KIC (20 mmol/L) for 1 hour. Samples from independent experiments were collected on different days and stored at -80°C. Insulin concentration was determined by radioimmunoassay method.

2.3. Western blotting assay

Isolated islets were pelleted and resuspended in buffer containing protease inhibitors, as previously described [9]. Total protein content was determined by the Bradford method. Samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Glutamate dehydrogenase was detected in the membrane after 12 hours of incubation at −4° temperature with a rabbit polyclonal antibody against GDH (Rockland Immunochemicals, Gilbertsville, PA). Antibody was diluted in TRIS-Tween buffer saline (TTBS) containing 30 g/L dry skimmed milk. Detection was performed by chemiluminescence (SuperSignal West Pico; Pierce, Rockford, IL) after incubation with a horseradish peroxidase–conjugated secondary antibody. Band intensities were quantified by optical densitometry (Scion Image, Frederick, MD).

2.4. Statistical analysis

Results are expressed as means ± SEM of the indicated number (n) of experiments. Analysis of variance for unpaired groups, followed by Newman-Keuls post hoc test, was used for multiple comparisons of parametric data. The significance level adopted was $P < .05$.

3. Results

The LP group showed reduced GDH content ($P < .05$), whereas leucine supplementation (LPL) restored its expression to the same levels observed for NP and NPL groups (Fig. 1). Interestingly, LPL showed improved GIIS to the same level observed in NP. Fig. 2 presents insulin secretion stimulated by several secretagogues and gives clues about the possible metabolic pathways by which leucine supplementation could enhance GIIS. Although similar

levels of GDH expression were observed in NP, NPL, and LPL groups, our findings showed higher insulin release in response to glucose, leucine, and KIC in NPL ($P < .05$). In addition, insulin release presented similar profiles among groups. The secretory response to allosteric GDH activators such as leucine and BCH as well as KIC in LP was, as expected, significantly lower ($P < .05$) as compared with control. Remarkably, LPL islets showed improved insulin secretion in response to all other tested secretagogues compared with LP islets. Actually, the secretory response of LPL in the majority of cases was similar to that observed in control NP. Thus, these results indicate that leucine supplementation enhances GDH activity and leucine oxidation pathway in LPL, which may lead to the increased GIIS during protein undernourishment status.

4. Discussion

Direct evidence linking GDH and GIIS was previously reported by using adenovirus-mediated GDH overexpression and deletion, which increased and reduced GIIS, respectively [10]. Likewise, we showed here in protein malnutrition rats that the reduced β-cell GDH expression and GIIS were restored by leucine supplementation. Xu et al [4] demonstrated that the leucine-induced effect on GDH activity is coupled with higher levels of GIIS and is associated with mTOR pathway. Previous results from our group are in agreement with the aforementioned report suggesting that the leucine-induced increase in PI3K/mTOR activity [7] might be directly related to upper GDH expression also in protein-undernourished rats supplemented with leucine. Given that the LPL group exhibited both an increased GDH expression and an augmented secretory response to leucine, BCH, and KIC compared with islets from the LP rats, the improved GIIS with leucine supplementation may imply an enhanced allosteric activation of GDH as well as improved leucine oxidation pathway. Indeed, a previous study showed evidence for a possible

interaction between GDH and leucine oxidation pathways to enhance leucine induced insulin secretion in pancreatic islets of BTBR mice [11]. Thus, leucine metabolism would provide higher anaplerotic replenishment, supporting glucose metabolism to increase metabolic coupling factors including adenosine triphosphate, malonyl–coenzyme A, and nicotinamide adenine dinucleotide phosphate (NADPH) [5]. This statement is reinforced by previous results from our group that demonstrated that leucine supplementation did not enhance glucose oxidation in protein-malnourished rats [7]. Taken together, these data suggest that leucine might be the major anaplerotic substrate supplying oxidative substrates to TCA under protein undernourishment. In conclusion, our results showed that leucine supplementation could restore GIIS in low-protein-fed rats by enhanced GDH expression, activating amino acid-dependent anaplerotic routes. However, further studies are needed to also investigate leucine oxidation pathway alterations to better understand the mechanisms by which leucine supplementation enhances GIIS.

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Figure Legends

Fig. 1. Increased levels of GDH protein in islets lysates from LPL rats. Western blotting analysis of GDH expression in NP, NPL, LP and LPL. Values are means ± SEM indicated by vertical bars. Different letters indicate significant differences ($P < .05$) among all groups (n = 7-10).

Fig. 2. The LPL rats exhibited increased insulin secretion. Static insulin secretion in NP, NPL, LP and LPL in response to glucose 2.8 and 22.2 mmol/L, leucine 20 mmol/L, BCH 20 mmol/L, and KIC 20 mmol/L. Values are means ± SEM indicated by vertical bars. Different letters indicate significant differences ($P < .05$) among NP, NPL, LP and LPL in response to the same secretagogue condition (n = 10).

Figure 1

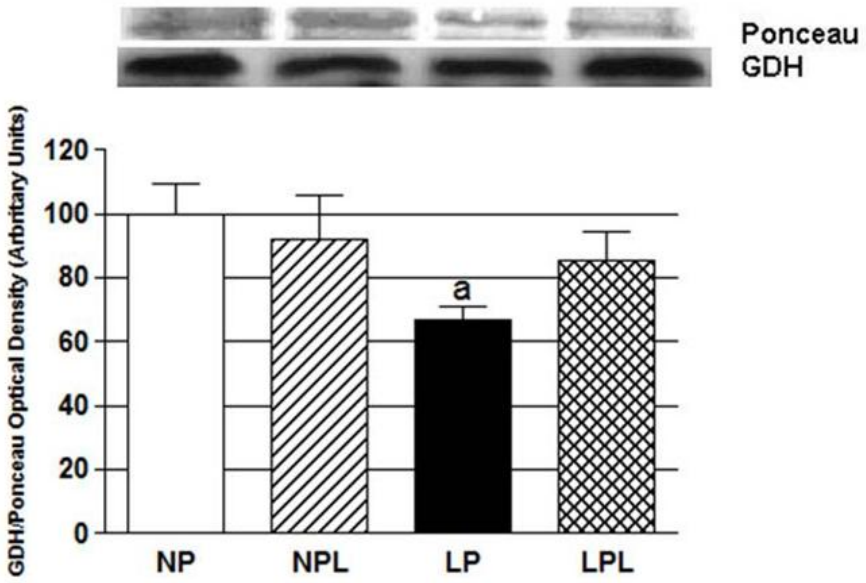
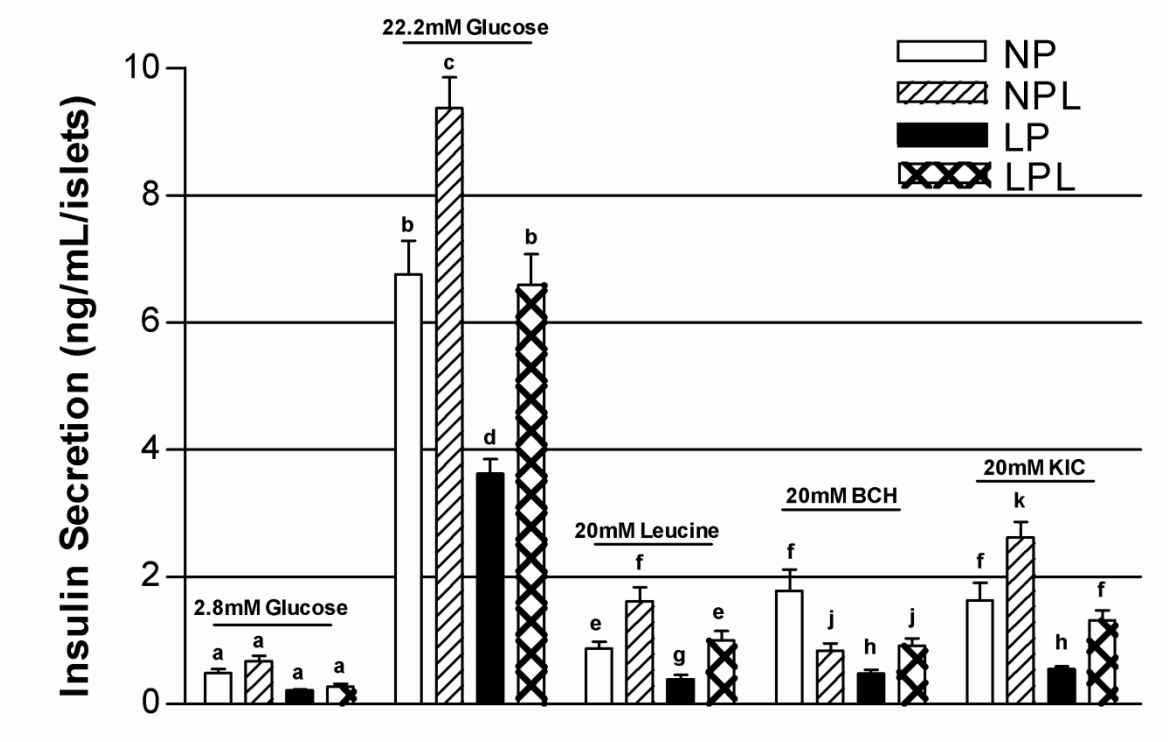

Ponceau
GDH
GDH/Ponceau Optical Density (Arbitrary Units)
120
100
80
60
40
20
0
a
NP
NPL
LP
LPL

Figure 2


Insulin Secretion (ng/mL/islets)
10
8
6
4
2
0
NP
NPL
LP
LPL
2.8mM Glucose
22.2mM Glucose
20mM Leucine
20mM BCH
20mM KIC
a
a
a
a
b
c
d
b
e
f
g
e
f
j
h
j
f
k
h
f

ARTIGO 2

Decreased Insulin Secretion in Protein Malnourished Rats is Associated with Impaired Glutamate Dehydrogenase Function: Effect of Leucine Supplementation

Artigo submetido ao periódico: ***Metabolism Clinical and Experimental*** *(ANEXOS)*

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Abstract

Glutamate dehydrogenase (GDH) is associated with insulin secretion. Low-protein diet impairs insulin secretion while leucine (LEU) supplementation seems to restore it. Herein, we studied the role of GDH in response to LEU supplementation upon insulin secretion of malnourished rats. Weaned male *Wistar* rats were fed normal (17%, NP) or low-protein diet (6%, LP) for eight weeks. Half of rats of each group were supplemented with LEU (1.5%) in drinking water for the following four weeks (NPL and LPL groups). GDH mRNA was 58% lower in LP islets and LEU supplementation augmented it in 28% in LPL. LP islets showed lower insulin secretion when exposed to LEU 20 mmol/L, LEU 20 mmol/L + GLN 2 mmol/L with or without AOA 5 mmol/L (branched chain aminotranferase inhibitor) or EGCG 20 μmol/L (GDH inhibitor), KIC 20 mmol/L (alpha-ketoisocaproate), GLN + BCH 20 mmol/L (GDH activator) and glucose 22.2 mmol/L (G22.2). LEU supplementation augmented insulin secretion to similar levels as NP in all conditions, an effect that was blunted when islets were only incubated with GDH inhibitor. LP islets showed lower $[Ca^{2+}]_i$ amplitude and total $[Ca^{2+}]_i$ when exposed to GLN+BCH. LEU supplementation augmented these patterns similar to NP. Impaired GDH function was associated with LP reduced insulin secretion and LEU supplementation was able to restore insulin secretion via GDH restoration. Likewise, GDH may contribute to insulin secretion through Ca^{2+} handling stimulus/secretion coupling.

Keywords: Low-protein diet, leucine, glutamate dehydrogenase, insulin secretion, calcium handling

List of Abbreviations:

$[Ca^{2+}]_i$, intracellular calcium concentration; ADP, adenosine diphosphate; Anova, analysis of variance; AOA, aminooxyacetic acid; ATP, adenosine triphosphate; AUC, area under the curve; BCATm, mitochondrial branched chain aminotransferase; BCH, β-2-aminobicyclo[2.2.1]heptane-2-carboxylic acid; BSA, bovine serum albumin; BW, body weight; CHOL, cholesterol; EGCG, epigallocatequin gallate; ER, reticulum endoplasmatic; FFA, free fatty acids; GDH, glutamate dehydrogenase; GLN, glutamine; GLU, glutamate; GLUD1, glutamate dehydrogenase 1 gene; GLUD2, glutamate dehydrogenase 2 gene; GTP, guanosine triphosphate; INS-1E, insulinoma-1E β cells; ip, intraperitoneal; ipITT, intraperitoneal insulin tolerance test; KIC; α-ketoisocaproate; kITT, constant ratio for glucose disappearance*;* KRBB, Krebs-Ringer bicarbonate buffer; LEU, leucine; LP, low-protein; LPL, low-protein supplemented with LEU; mTOR, mammalian target of rapamycin; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; NP, normal-protein; NPL, normal-protein supplemented with LEU; RIA, radioimmunoassay; mRNA, messenger ribonucleic acid; RPS29, ribosomal protein S29; SDS, sodium duodecil sulfate; TCA, tricarboxylic acid cycle; TG, triglyceride

Introduction

Glutamate dehydrogense (GDH) is a homohexameric enzyme with two isoforms encoded by two distinct genes, *GLUD1* and *GLUD2*, and it is located in the mitochondrial matrix [1-3]. GDH catalyses the reversible reaction alpha-ketoglutarate + NH_3 + NAD(P)H ↔ glutamate + $NAD(P)^+$ [1, 4-6]. This enzyme is allosterically regulated by leucine (LEU) and ADP, as positive effectors, whereas GTP acts as a negative allosteric modulator [7-10]. GDH is highly expressed

in the brain by *GLUD2*, liver, kidney and pancreas by *GLUD1* [2, 5, 6]. In the brain, GDH plays a key role in the cycling of the neurotransmitter glutamate (GLU) between neurons and astrocytes [11]. GDH is also of major importance for ammonia metabolism and detoxification, mainly in the liver and kidney [12]. In pancreatic β-cells, GDH is recognized as a key enzyme in the regulation of insulin secretion [7]. Inhibition of GDH activity was shown to decrease insulin release [6, 8, 9, 13], while activating mutations have been associated with a hyperinsulinism syndrome [14, 15].

Pancreatic GDH role in the insulin secretion process has been well documented since 70's. In this sense, *in vitro* studies with the non-metabolizable LEU analogue, beta-2-aminobicyclo[2.2.1]heptane-2-carboxylic acid (BCH) provided some evidence for the involvement of this enzyme with insulin secretion [7,16]. In addition, mutations in GDH inhibitory GTP binding domain are associated with the hyperinsulinaemia-induced hypoglycemia in children (hyperinsulinism/hyperammonemia syndrome) [14,15,17]. Recently, studies using GDH inhibitors like green tea poliphenol, epigallocatechin gallate (EGCG), and 5'-deoxypyridoxal showed reduced LEU or BCH-induced insulin secretion [13,18]. In the same way, GDH knockout in mice pancreas was associated with lower insulin release stimulated with glucose and BCH [19]. On the other hand, GDH superexpression in rat islets and INS-1E β-cell line were associated with augmented glucose-induced insulin secretion [20].

Although it is widely accepted that GDH takes part of insulin secretion signaling, it still remains unclear how this modulation occurs [21]. GDH may regulate glucose-induced insulin secretion by generation of GLU [22, 23], pointed as a metabolic coupling factor, or by the production of anaplerotic compounds that amplify the insulin secretion response [19, 24-26].

Branched chain amino acids stimulate insulin release through the reaction catalyzed by GDH [27, 28] or by mitochondrial branched chain aminotransferase (BCATm) [30], producing

important metabolic signals such as ATP and NADPH, which in turn, control insulin secretion [19, 22-26]. Specifically, LEU is thought to perform its action by two different ways: the first involves the transfer of the amino group to α-ketoglutarate resulting in the production of α-ketoisocaproato (KIC) and GLU [31-33]; the second, promotes insulin secretion by GDH allosteric activation augmenting the tricarboxylic acid cycle (TCA) intermediates [24, 26], ATP [7] or GLU content [22, 23].

Early life protein restriction reduces insulin secretion [34-42]. Our group and others, provided evidence that the lower insulin secretion observed in this model is related to a variety of alterations in pancreatic islets secretory machinery. It was reported that proteins involved in insulin secretion as glucokinase [35], protein kinase C α [38, 41], protein kinase A α [41] and synapotosomal associated protein of 25 kDa [43] are altered in malnourished rodents. In addition, we have recently showed that GDH content was reduced in islets from protein malnourished rats [protein content, as well as GDH content, leading to insulin secretion improvement, which was formerly altered by protein malnutrition [40-42]. Keeping in mind that LEU could act by two specific pathways upon insulin release, we hypothesized that protein restriction affects insulin secretion by altering GDH function and LEU supplementation is capable to restore metabolic GDH action and, therefore, insulin secretion. In this way we decided to investigate how LEU supplementation exerts this effect upon insulin secretion control in protein malnourished rats analysing the metabolic pathways by which LEU could act.

Materials and Methods

Chemicals

Human recombinant insulin (^{125}I) was purchased from PerkinElmer (Boston, MA, USA). Human recombinant insulin Biohulin N was purchased from Biobrás (Montes Claros, MG, Brazil). Standard commercial kits were used for measurement of plasma total proteins, albumin (both from Laborlab, Guarulhos, SP, Brazil), total cholesterol (CHOL), triglycerides (TG) (both from Roche Diagnostics; Mannheim, Germany) and free fatty acids (FFA; Wako Chemicals, Neuss, Germany). GDH antibody was from Rockland Immunochemical (Gilbertsville, PA, USA). Visualization of specific protein bands was obtained with SuperSignal West Pico from Pierce (Rockford, IL, USA). TRIzol, horseradish peroxidase-conjugated secondary antibody and Fura-2 AM were purchased from Invitrogen (Carlsbad, CA, USA). Fast SYBR Green technology was from Applied Biosystems (Foster City, CA, USA). Primers were purchased from Integrated DNA technologies (Coralville, IA, USA)**.** All others reagents were purchased from Sigma Chemical (St Louis, MO, USA).

Animals, Diet and LEU Supplementation

All experiments were approved by the University's Committee on Ethics in Animal Experimentation (CEUA/UNICAMP). Weaned Male Wistar rats (21 day-old) from the breeding colony at UNICAMP were housed in standard cages and maintained on a 12 h light/dark cycle (lights on 06-18 h) and controlled temperature (22 ± 1°C). The rats were fed a normal (17%, NP) or low-protein isocaloric diet (6%, LP) for 60 days. Then, half of the rats of each group were supplemented with LEU (1.5%) in the drinking water for the next 30 days (NPL and LPL groups). The two isocaloric diets compositions were detailed elsewhere [39].

Animals Features

Body weight and food intake were measured throughout experimental period (from 21 to 105 days of life). Food intake was expressed by feed efficiency (food intake/body weight) [44]. At the end of the diet treatment and supplementation period, rats were decapitated, their blood was collected and plasma stored at -20°C. Heart, liver, spleen, kidney, perigonadal and retroperitoneal fat pad weights were measured.

Blood glucose was measured using a handheld glucose analyzer (Accu-Chek Advantage, Roche Diagnostic, Switzerland) and insulin was measured by radioimmunoassay (RIA) as previously reported [45]. Total plasma protein, plasma albumin, cholesterol (CHOL), triglycerides (TG) and free fatty acids (FFA) were measured using standard commercial kits, according to the manufacturer's instructions.

For ip insulin tolerance test (ipITT), blood glucose (time 0) was measured as described above in independent groups of 6 h fasted rats. The rats received 2 U/kg body weight of human recombinant insulin in the peritoneal cavity. Blood glucose concentrations were then determined at 4, 8, 12 and 16 min after insulin administration. The constant ratio for glucose disappearance (kITT) was calculated using the analysis of the square fall of glucose concentration during the linear phase decay, as detailed elsewhere [46].

Pieces of about 150 mg from brain, liver, kidney and muscles were solubilized in homogenization buffer containing protease inhibitors, as previously described [47]. Total protein content was determined by the Bradford method [48]. Samples with 30 μg of protein were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Equal loading was confirmed with Ponceau staining [37, 49]. The nitrocellulose membranes were treated overnight with a blocking buffer (5% non-fat dried milk, 10 mmol/L Tris, 150 mmol/L NaCl, and 0.02%

Tween 20) and were subsequently incubated with a polyclonal antibody against GDH (1:5,000). Detection was performed using enhanced chemiluminescence after 2 h incubation with a horseradish peroxidase-conjugated secondary antibody (1:10,000). Band intensities were quantified by optical densitometry (Scion Image, Frederick, MD).

Islets isolation and insulin static secretion

Islets were isolated, as previously described, by collagenase digestion of the pancreas [50]. For static incubation, five islets from each group were first incubated for 45 min at 37°C in Krebs-Ringer bicarbonate buffer (KRBB) with the following composition (in mmol/L): 115 NaCl, 5 KCl, 2.56 $CaCl_2$, 1 $MgCl_2$, 10 $NaHCO_3$, 15 HEPES, supplemented with 5.6 glucose, 3 g/L of bovine serum albumin (BSA) and equilibrated with a mixture of 95% O_2/5% CO_2 to give pH 7.4. This medium was then replaced with fresh buffer and the islets were incubated for 1 h with (in mmol/L): 2.8 and 22.2 glucose, 2 glutamine (GLN), 20 LEU, 10 2-amino-2-norbornanecarboxylic acid (BCH), 20 alfa-cetoisocaproato (KIC). Aminooxyacetic acid (AOA) 5 mmol/L was also used in combination with GLN + LEU. For the experiments with epigallocatechin gallate (EGCG), islets were pre-incubated with 20 µmol/L EGCG, and then the medium was replaced with 20 µmol/L EGCG plus some of the others compounds cited above. At the end of the incubation period, samples from independent experiments were collected on different days and stored at -80°C. Insulin concentration of the medium was determined by RIA method.

Cytoplasmatic Ca^{2+} Oscillations

Fresh pancreatic islets were incubated with fura-2/AM (5 μmol/l) for 1 h at 37°C in KRBB buffer containing 5.6 mmol/L glucose, 0.3% BSA and pH 7.4. After this period, the islets were washed with the same medium and placed in a chamber that was thermostatically regulated at 37°C on the stage of an inverted microscope (Nikon UK, Kingston, UK). Islets were then perifused with Ca^{2+}-free KRBB continuously gassed with 95% O_2/5% CO_2, pH 7.4 containing glucose 2.8 mmol/L, GLN 2 mmol/L and BCH 10 mmol/L. A ratio image was acquired approximately every 5s with an ORCA-100 CCD camera (Hammamatsu Photonics Iberica, Barcelona, Spain), in conjunction with a Lambda-10-CS dual filter wheel (Sutter Instrument Company, CA, USA), equipped with 340 and 380 nm, 10 nm bandpass filters, and a range of neutral density filters (Omega opticals, Stanmore, UK). Data were obtained using the ImageMaster3 software (Photon Technology International, NJ, USA).

Real-time PCR

Total cellular RNA was extracted from groups of 500 islets using TRIzol reagent. One microgram of total RNA was reverse transcribed using a reverse transcriptase and random hexamer primers. Real-time PCR reactions were performed in a total volume of 15 μL using the Fast SYBR Green technology. Samples were denatured at 94°C for 10 min followed by 40 PCR cycles at 95°C/60°C. PCR amplifications were performed in duplicate. The purity of the amplified PCR products was verified by melting curves. The expression of the target genes was normalized against the expression levels of the housekeeping gene RPS-29. Sequence of the primers used were (5'-3'): GDH forward: GCCACTACAGCGAAGCGG, DH reverse:

CGGGTCTTCAGGTCTTCCAC; RPS-29 forward: TTTTTCCTTGGGCGTCTG, RPS-29 reverse: ACGGAAGCACTGTCGGCACA.

Statistical Analysis

Results are expressed as means ± SE of the indicated number (*n*) of experiments. Two-way analysis of variance (two-way ANOVA) followed by Newmans-Kewels *post hoc* test was used for comparisons among groups, one-way analysis of variance (one-way ANOVA) followed by Newmans-Kewels *post hoc* test was used for multiple comparisons within a group and a two-tailed nonpaired *t* test was used to assess the difference between two groups. The significance level adopted was $P < 0.05$.

Results

Characterization of malnourishment

Figure 1A shows that body weight in LP rats was significantly lower soon after 8 days of the treatment onset, when compared to NP rats ($P < 0.001$). This difference was accompanied throughout the period and LEU supplementation did not alter this parameter in both groups ($P < 0.001$). LP rats showed increased food efficiency compared with NP and LEU supplementation did not change this parameter (Fig. 1C, $P < 0.0002$, $P < 0.0003$, respectively). This was expected since the total body weight (BW) from LP rats was less than 50% from NP demonstrated by AUC from BW (Fig. 1B, $P < 0.0001$).

Table 1 shows the weight of heart, liver, spleen, kidney, retroperitoneal and perigonadal fat pads (RPF and PGF, respectively) corrected by body weight (BW). Since LP rats have almost 50% of the BW observed in NP rats, the weight presented by heart, liver, spleen and kidney and

fat pads from LP rats were lower than in NP rats (data not shown). However, when we corrected tissues weight with respectively BW, tissues showed similar values among groups, except heart and fat pads that were higher and lower, respectively, when compared with NP rats ($P < 0.004$ and $P < 0.04$, respectively).

Rats that were fed with LP diet showed decreased fasted total protein, albumin and fed insulin plasma levels, whereas fasted FFA plasma levels were significantly higher in LP compared with NP rats (Tab. 2, $P < 0.0003$, $P < 0.02$, $P < 0.02$ and $P < 0.01$, respectively). LEU supplementation augmented fasted albumin and fed insulin plasma levels in LP rats (Tab. 2, $P < 0.004$ and $P < 0.02$, respectively).

LP rats showed higher insulin sensitivity (Tab2). LEU supplementation diminished insulin sensitivity in LP rats ($P < 0.009$).

Low protein Diet and LEU supplementation did not exert effects on tissues GDH protein content

Figure 2 illustrates GDH protein content in the brain (A), liver (B), kidney (C) and skeletal muscles (D) in NP, NPL, LP, LPL rats. GDH content was not different in any of the tissues evaluated. However, we have recently reported that GDH was lowered in LP islets and LEU supplementation restored GDH to similar levels observer in NP islets [42]. Taken together, these results indicate that the protein restriction effect to GDH was related only to endocrine pancreas.

Low protein diet disrupts GDH mRNA expression and LEU supplementation restores it

As we have previously shown with GDH protein content [42], GDH mRNA expression was 58% lower in LP islets than with NP and LEU supplementation augmented it in 28% (Fig. 3, $P < 0.0002$ and $P < 0.03$, respectively).

GDH pathway activation is responsible for augmented insulin secretion observed in LP islets

To evaluate insulin secretion induced by LEU, that acts as a positive GDH allosteric effector and can also be converted to KIC by BCATm, we incubated isolated islets with LEU 20 mmol/L plus GLN 2 mmol/L combined with AOA 5 mmol/L or EGCG 20 µmol/L. AOA and EGCG are respectively, BCATm and GDH inhibitors and were used to evaluate these two pathways of LEU action, separately. Glutamine by itself does not induce insulin secretion [51]. However, it may be converted to GLU by a phosphate-dependent glutaminase in islets, acting as a supply of GLU to GDH or BCATm reactions [31].

As we previously reported [40, 42], LEU 20 mmol/L induced higher insulin secretion in NPL islets and LP islets showed lower insulin secretion as compared to NP (Fig. 4, $P < 0.0001$, $P < 0.005$, respectively), whereas LPL showed similar levels of insulin secretion as NP. When islets were incubated with LEU 20 mmol/L plus GLN 2 mmol/L, insulin secretion was augmented approximately 2-fold compared with the levels induced only by LEU. Moreover, the insulin secretion pattern observed by the groups' islets was the same when using both incubations. When we added AOA 5 mmol/L, the islets from all groups showed similar values of insulin secretion observed in the last condition without this inhibitor. In addition, it was not observed differences of insulin secretion in NP, NPL and LP islets when added EGCG 20 µmol/L to LEU+GLN compared with LEU+GLN U alone. However, LPL islets showed similar insulin secretion when

exposed to LEU+GLN+EGCG compared with LP islets and diminished when compared with incubation with LEU+GLN or LEU+GLN+AOA ($P < 0.01$). KIC-stimulated insulin secretion was similar to LEU+GLN-stimulated insulin secretion in all groups. Taken together, these results indicate that for NP rats, both metabolic pathways of LEU action are capable of induce similar levels of insulin secretion. In contrast, it seems that for the LP rats both pathways of LEU action are impaired and LEU supplementation improves the insulin secretion resulting from allosteric GDH activation by LEU.

To access the GDH function specifically, we used GLN 2 mmol/L and, the non-metabolizable LEU analogue, BCH (20 mmol/L) with or without EGCG (20 µmol/L) (Fig.5). GLN+BCH stimulated insulin secretion in the same pattern induced by LEU+GLN (Fig. 5 and Fig. 4, respectively). LEU supplementation augmented insulin secretion induced by GLN+BCH (Fig. 5, $P < 0.009$). Yet, in all groups, insulin secretion induced by GLN+BCH was 2-fold lower than LEU+GLN secretion (Fig. 5 and Fig. 4, respectively). In opposite to the observed when added AOA or EGCG to LEU+GLN condition, NP and NPL islets showed almost 50% reduced insulin secretion when added EGCG 20 µmol/L to GLN+BCH (Fig. 5, $P < 0.05$, $P < 0.01$, respectively). LP insulin secretion was not reduced by the addition of EGCG, but LPL islets showed similar reduction that was observed with NP and NPL rat islets and similar insulin levels to LP ($P < 0.01$ and $P > 0.05$, respectively). It seems that LP loses GDH control of insulin secretion and thus, the EGCG inhibitory effect is not observed in LP.

To evaluate whether GDH content and GDH pathway activation were significantly important to glucose stimulated insulin secretion, we combined this nutrient to EGCG 20 µmol/L (Fig. 6). As previously described [40, 42], LP islets insulin secretion was lower and LEU supplementation augmented insulin secretion ($P < 0.0001$ and $P < 0.005$, respectively). When

added EGCG, nor NP and NPL, neither LP insulin secretion was affected, however LPL group showed almost 40% lower insulin secretion ($P < 0.0001$) becoming similar to LP. In this way, GDH pathway activation seems to be important for insulin secretion when stimulated by glucose as well.

LEU supplementation augments intracellular Ca^{2+} handling in LP islets

Figure 7 shows the involvement of GDH pathway activation in the Ca^{2+} handling in NP, NPL, LP and LPL islets stimulated with BCH 10 mmol/L.

LP islets showed lower intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) amplitude compared with NP islets (Fig. 7E, $P < 0.007$) and LEU supplementation augmented $[Ca^{2+}]_i$ amplitude similar to NP islets ($P < 0.04$). Regarding $[Ca^{2+}]_i$ curve profile, between 0-10min of perfusion it was showed a reduction in $[Ca^{2+}]_i$ islets of all groups studied (Fig. 7A, 7B, 7C e 7D). This reduction is resulting from cation sequestration by endoplasmatic reticulum (ER) stimulated by ATP production [52]. Total $[Ca^{2+}]_i$ in this period (demonstrated by AUC, Fig. 7E) was higher in LP islets indicating that ER from LP islets was not able to sequester the cation or maybe producing less ATP than NP islets ($P < 0.001$). However, LPL islets showed similar levels of $[Ca^{2+}]_i$ to NP islets. After 10 min of perfusion, total $[Ca^{2+}]_i$ was lower in LP islets ($P < 0.02$) and LEU supplementation augmented the total $[Ca^{2+}]_i$ to similar NP levels.

Discussion

In agreement with previous studies [34-40, 42, 43], LP rats showed reduced plasma total protein, albumin and fed insulinemia; augmented FFA and insulin sensitivity; and reduced insulin secretion. Moreover, we enlarged the evidences about pancreatic function impairment in LP

animals and showed that LEU supplementation restores plasmatic albumin and fed insulinemia, insulin secretion response to nutrients, Ca^{2+} handling and GDH mRNA expression.

It is well established that LEU is an insulinotropic agent [25, 53], and stimulates insulin secretion by its metabolism via BCATm or by allostericaly activating GDH. To date, there are several metabolic intermediates thought to amplify insulin secretion signaling, the so called metabolic coupling factors. Among others, the main proposed factors are ATP, NADPH, reactive oxygen species, GLU and long chain acyl-CoAs [54]. It is still under discussion which compound resulting from LEU metabolism is more important to insulin secretion signaling, and consequently the main LEU-induced insulin secretion pathway.

There are evidences that GDH reaction modulates insulin release preferentially through the production of GLU, which might be exported from mitochondria as a coupling factor. Maechler and Wollhoeim (1999) [22] showed that in permeabilized INS-1 cells, under conditions of fixed $[Ca^{2+}]_i$, GLU addition directly stimulates insulin exocytosis, independently of mitochondrial function. Moreover, rat islets knockdown for mitochondrial glutamate carrier 1 had reduced insulin secretion when stimulated with glucose [23]. However, some authors suggest that GLU would not be a second messenger to insulin secretion [55, 56]. In this sense, experiments performed elsewhere did not find an increase in cellular GLU content upon glucose stimulated insulin secretion in either INS-1 cells or isolated rat islets [55]. In addition, rat islets cultured for 4–5 days with glucose 10 mmol/L showed higher GLN oxidation when incubated with BCH 10 mmol/L plus AOA 5 mmol/L than just with BCH or AOA [33]. In the same way, rat islets incubated with GLN 10 mmol/L and LEU 10 mmol/L or BCH 10 mmol/L augmented GLN oxidation [25]. Furthermore, GDH transgenic mice were generated to express the human GDH H454Y mutation that causes significant perturbations in enzyme kinetics and manifests clinically

as a severe form of hyperinsulinism/hyperammonemia syndrome. Stimulation of insulin released by the H454Y GDH mutation or by LEU activation is associated with increased oxidative deamination of GLU via GDH [57].

Besides GDH activation, LEU catabolism producing KIC is considerate the major direction of the reaction catalyzed by BCATm [31]. KIC may be converted to acetyl-CoA and acetoacetate by branched-chain ketoacid dehydrogenase complex signaling insulin secretion [25]. KIC and glucose have similar effects upon insulin secretion. Nevertheless, there are studies showing the importance of alpha-ketoglutarate production by BCATm to insulin secretion. Rabaglia *et al.* (2005) [51] showed that increased transamination of GLU to α-ketoglutarate leads to hyperinsulinemia. Mice islets knockout to BCATm showed reduced insulin secretion when stimulated with KIC+GLN and normal levels of insulin secretion when stimulated with dimethyl-α-ketoglutarate + GLN or LEU+GLN [31]. Furthermore, α-ketoglutarate production is associated with NADPH and malonyl-CoA increase, regarded as important metabolic coupling factors for insulin secretion [54].

In our study, we reported that NP islets exhibit similar insulin secretion when stimulated with LEU+GLN even at the presence of AOA or EGCG. Thus, GDH activation by LEU is capable to induce insulin secretion as LEU catabolism. This result is in agreement with others that show the importance of GDH activation and α-ketoglutarate production, enhancing the TCA flux, for insulin secretion [13, 31, 57]. In addition, we observed that when LPL islets were stimulated with GLN+BCH, the addition of EGCG resulted in insulin secretion reduction, similar to LP islets levels. It was expected, since LP diet disrupted GDH islets mRNA and protein content [42] and LEU supplementation restored it, allowing GDH inhibition by EGCG.

When we exposed LPL islets to high glucose in addition to EGCG, we observed that insulin secretion was inhibited. Previous report showed impairment of fully insulin secretory response to high glucose in mice islets with specific GDH knockout [19]. In agreement, we reported here for the first time, that insulin secretion impairment induced by LP diet when stimulated by high glucose is also due to GDH control of insulin secretion disruption. In addition, it was reported by our results that LPL islets insulin secretion restoration to both LEU and glucose stimuli arises from LEU supplementation-induced improved GDH function.

In addition, our findings concerning Ca^{2+} handling corroborate the GDH metabolic control of insulin release data. In this sense, it was previously reported that mice islets cultivated with AOA showed higher $[Ca^{2+}]_i$ when perifused with the combination LEU+GLN [32]. Moreover, hyperinsulinemic mice with GDH mutation in the inhibitory GTP binding domain showed higher $[Ca^{2+}]_i$ compared with control islets when stimulated with GLN [57]. Therefore, such evidences suggest that GDH may modulate the Ca^{2+} handling. Besides, there are reports showing that LP islets had reduced $[Ca^{2+}]_i$ when stimulated with glucose and other insulinotropic agents [43, 59, 58]. Broadening these studies, we also found that LP islets when perifused with GLN+BCH had also reduced Ca^{2+} oscillation amplitude, ER Ca^{2+} sequester capacity and $[Ca^{2+}]_i$. Moreover, we observed that LPL islets showed similar Ca^{2+} handling to NP and NPL islets when stimulated with GLN+BCH. Thus, it seems that GDH and, LEU supplementation are relevant in this process since we were specifically stimulating GDH and LEU supplementation restored the pattern of Ca^{2+} oscillation that was altered by LP diet.

In conclusion, LP diet affects islets GDH content and function, reducing insulin secretion signaling (Fig. 9 A). Probably, lowered GDH function reduces α-ketoglutarate, ATP production

and Ca^{2+} handling. LEU supplementation restores insulin secretion to nutrients by restoration of GDH function (Fig. 9 B).

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Disclosure Statement

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work. The experiments with animals are in agreement with the institutional University of Campinas Committee for Ethics in Animal Experimentation (CEUA/UNICAMP n° 2011-11).

Author Contributions

Da Silva, PMR, Batista, TM and Ribeiro, RA contributed with the acquisition of data. Da Silva, PMR, Ribeiro, RA, Zoppi, CC and Carneiro, EM contributed to the analysis of data and designed the experiments. Zoppi, CC, Boschero, AC and Carneiro, EM contributed to the discussion of the study. Da Silva, PMR wrote the manuscript.

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Table 1 Body weight (BW) and tissues weight at the end of 105 day old NP, NPL, LP and LPL rats

	NP (n=12)	**NPL (n=11)**	**LP (n=11)**	**LPL (n=12)**
BW (g)	466,4±9,2[a]	467,5±13,0[a]	299,5±11,83[b]	301,9±13,8[b]
Heart (mg/g of BW)	2,5±0,06[a]	2,8±0,1[a]	3,3±0,2[b]	3,2±0,1[b]
Liver (mg/g of BW)	32,5±1,1	32,0±1,2	30,6±1,3	30,9±1,7
Spleen (mg/g of BW)	2,0±0,06	2,2±0,1	2,4±0,1	2,2±0,1
Kidney (mg/g of BW)	5,6±0,1	5,7±0,2	5,5±0,2	5,6±0,2
RPF (mg/g of BW)	21,4±1,3[a]	19,9±3,5[a]	13,0±1,4[b]	13,5±1,4[b]
GPF (mg/g of BW)	20,9±1,1[a]	18,0±2,1[a]	12,5±1,2[b]	13,7±1,5[b]

Data are means ± SEM. Different letters indicate significant difference ($P<0.05$*).*

Table 2 Total plasma protein, albumin, glucose, insulin, triglyceride (TG), cholesterol (CHOL) and free fatty acids (FFA) concentrations and the kITT from fasted and fed (*) 105 day-old NP, NPL, LP and LPL rats

	NP (n)	**NPL (n)**	**LP (n)**	**LPL (n)**
Total protein, g/dL	7.6 ±0.3 (14)[a]	7.6±0.15 (14)[a]	6.3±0.2 (11)[b]	6.2±0.2 (11)[b]
Albumin, g/dL	2.6 ±0.04 (12)[a]	2.7±0.1 (13)[a]	2.4±0.1 (9)[b]	2.6±0.1 (10)[a]
Glucose, mg/dL	87.3±2.6 (15)	85.7±3.3 (14)	87.3±3.1 (12)	81.9±3.1 (13)
Glucose*, mg/dL	102.7±3.1 (13)	99.1±3.7 (11)	96.3± 1.9 (17)	100.3±2.4 (13)
Insulin, ng/mL	1.1±0.2 (14)	1.2±0.3 (12)	0.9±0.1 (14)	0.9±0.1 (11)
Insulin*, ng/mL	2.9±0.3(8)[a]	2.9±0.5 (8)[a]	1.1± 0.1 (12)[b]	2.5±0.6 (7)[a]
TG, mg/dL	74.6±13.9 (4)	60.50±5.33 (4)	76.50±7.84 (5)	97.80±7.31 (4)
CHOL, mg/dL	78.90±4.24 (4)	83.0±2.72 (4)	87.40±2.63 (5)	87.0±2.06 (4)
FFA, mmol/L	0.64±0.08 (5)[a]	0.68±0.07 (4)[a]	1.10±0.09 (6)[b]	0.97±0.09 (5)[b]
kITT (%/min)	1,95±0,33 (7)[a,b]	1,39 ±0,42 (6)[b]	3,91±0,24 (8)[c]	2,72±0,21 (7)[a]

Data are means ± SEM. Different letters indicate significant difference ($P<0.05$*).*

Figure Legends

Figure 1. Body weight (A and B) and food efficiency (C) during the period were measured weekly in rats fed on a NP or a LP diet without or with LEU supplementation. Values are means ± SEM; n = 15 rats. *Significant difference of LP and LPL compared with NP and NPL groups, $P < 0.05$. Different letters over the bars represent significant differences among the groups, $P < 0.05$.

Figure 2. GDH protein expressions in brain (A), liver (B), kidney (C) and muscle (D) from NP, NPL, LP and LPL rats. The bars represent the means ± SEM of the values, determined by optical densitometry; n=4. Different letters indicate significant difference, $P < 0.05$.

Figure 3. Real-time PCR determination of GDH mRNA expression in isolated islets from NP, NPL, LP, LPL rats. The data were corrected for RPS-29 expression and are shown as relative to NP. Values are means ± SEM; n = 5. Different letters over the bars represent significant differences among the groups, $P < 0.05$.

Figure 4. Insulin secretion induced by LEU 20 mmol/L, LEU 20 mmol/L plus GLN 2 mmol/L with AOA 5 mmol/L or EGCG 20 μmol/L and KIC 20 mmol/L in islets from NP, NPL, LP and LPL rats. All stimuli had glucose 2.8 mmol/L. Each bar represents means ± SEM from 12-24 groups of islets. Different letters over the bars represent significant differences, $P < 0.05$.

Figure 5. Insulin secretion induced by GLN 2 mmol/L plus BCH 20 mmol/L with or without EGCG 20 μmol/L in islets from NP, NPL, LP and LPL rats. There was no addition of glucose.

Each bar represents means ± SEM from 12-24 groups of islets. Different letters over the bars represent significant differences, $P < 0.05$.

Figure 6. Insulin secretion induced by glucose 2.8 mmol/L (G2.8), glucose 22.2 mmol/L (G22.2) with or without EGCG 20 μmol/L in islets from NP, NPL, LP and LPL rats. Each bar represents means ± SEM from 12 groups of islets. Different letters over the bars represent significant differences, $P < 0.05$.

Figure 7. Representative curves of BCH 10 mol/L induced internal Ca^{2+} mobilization in islets isolated from NP (A), NPL (B), LP (C) and LPL (D) rats. (E) Amplitude and AUC during 0-10 min of stimulation (F) and after 10 min of stimulation (E) of the $[Ca^{2+}]i$ in response to BCH. The experiments were performed with a Krebs buffer containing: 2.8 mmol/L (G2.8), GLN 2 mmol/L. Values are the ratio of F340/F380 registered for each group. Data are means ± SEM obtained from 3 independent experiments. Different letters indicate significant differences ($P < 0.05$).

Figure 8. Overview of insulin secretion mediated by GDH in LP (A) and LPL (B) β cells. LP diet diminished GDH content, Ca^{2+} handling and impaired insulin secretion (A). LEU supplementation restores insulin secretion to nutrients by restoration of GDH function (B). Dotted arrows indicate impaired mechanisms by LP diet. Full arrows indicate restored mechanism by LEU supplementation. LEU in large letters in the panel indicate the LEU supplementation. LEU: leucine; GLN: glutamine; TCA: tricarboxilic acid; GDH: glutamate dehydrogenase.

Figure 1

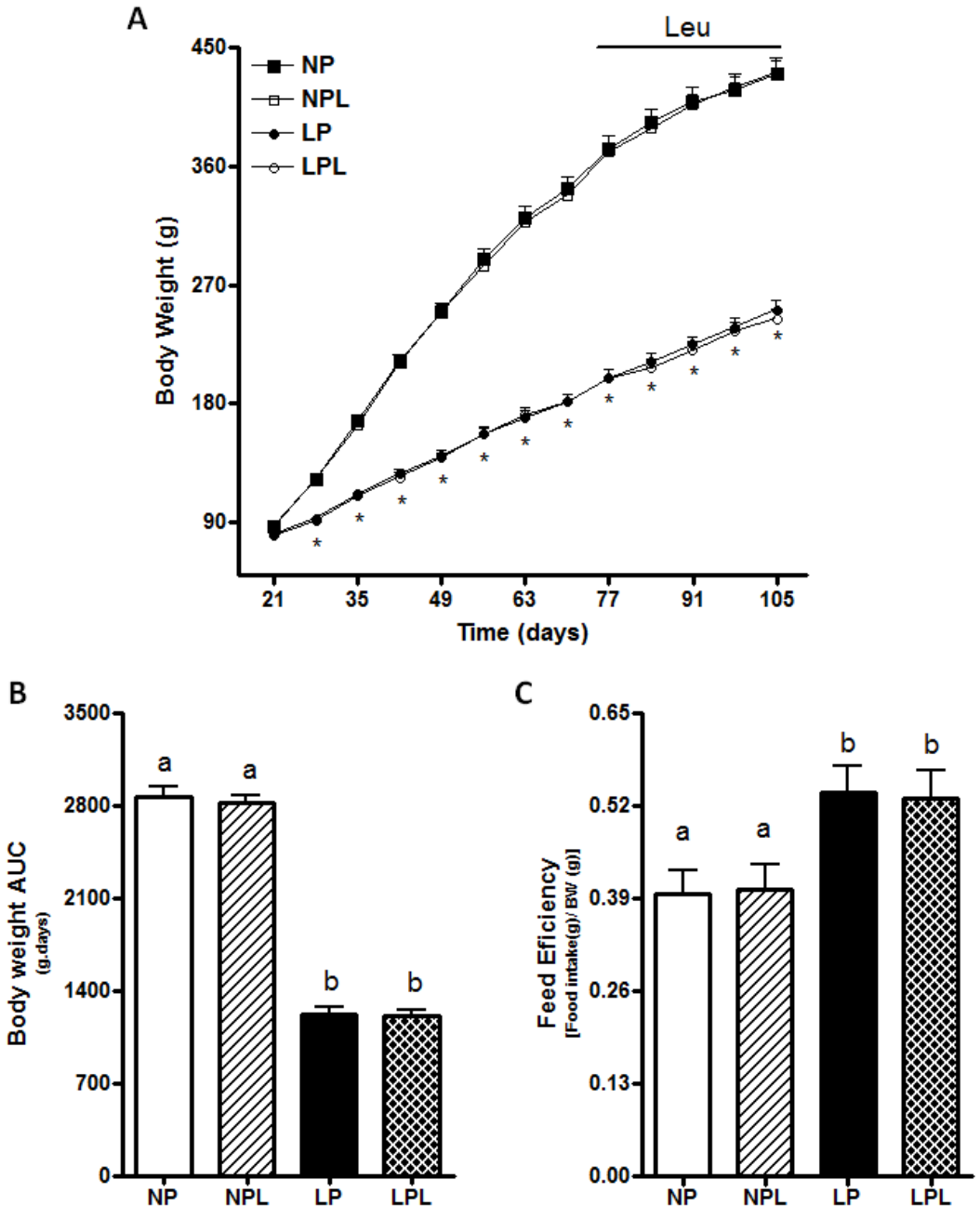

A
Leu
NP
NPL
LP
LPL
Body Weight (g)
450
360
270
180
90
21
35
49
63
77
91
105
Time (days)
B
Body weight AUC
(g.days)
3500
2800
2100
1400
700
0
a
a
b
b
NP
NPL
LP
LPL
C
Feed Eficiency
[Food intake(g)/ BW (g)]
0.65
0.52
0.39
0.26
0.13
0.00
a
a
b
b
NP
NPL
LP
LPL

Figure 2

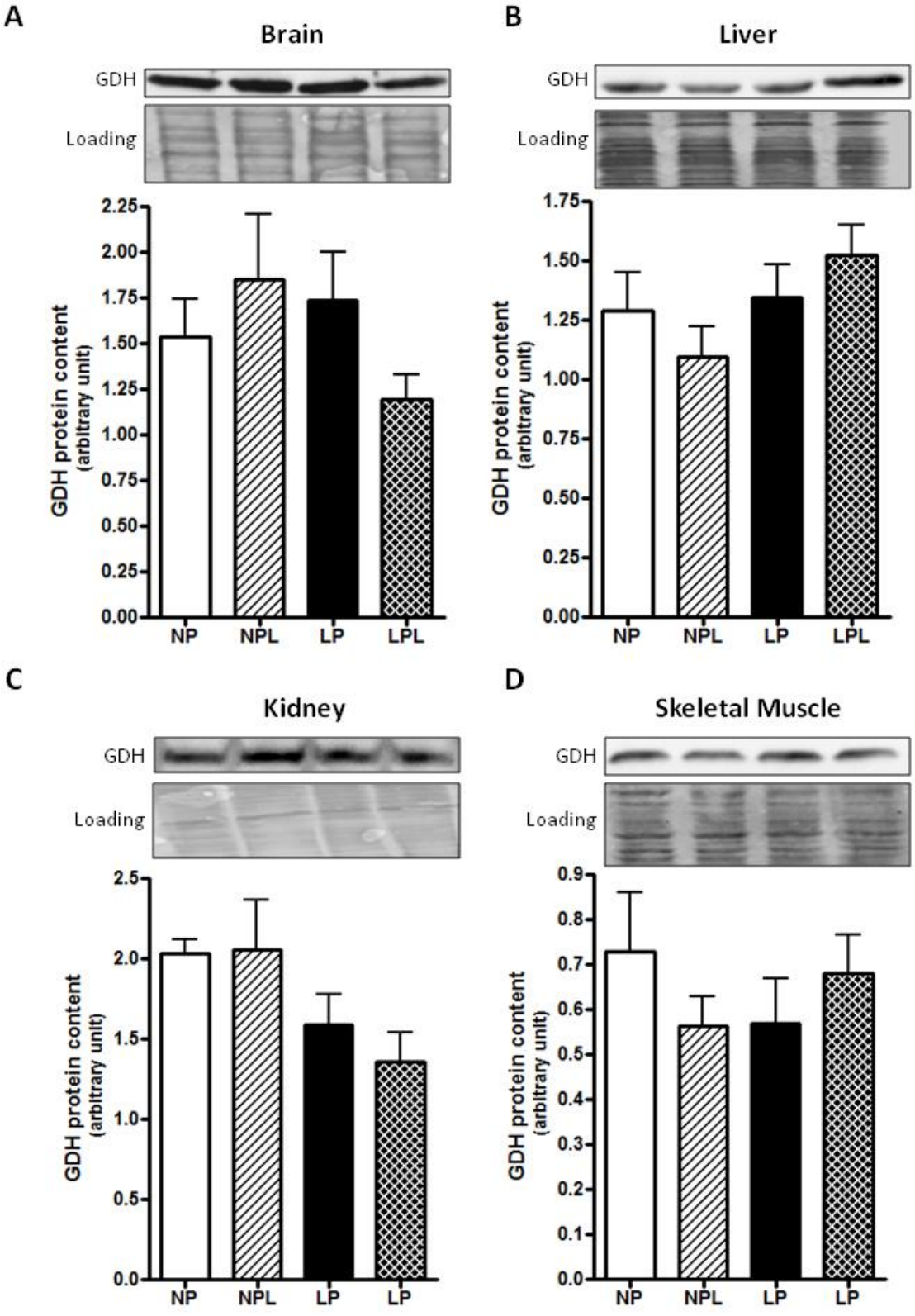

A
Brain
GDH
Loading
GDH protein content (arbitrary unit)
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
NP
NPL
LP
LPL
B
Liver
GDH
Loading
GDH protein content (arbitrary unit)
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
NP
NPL
LP
LPL
C
Kidney
GDH
Loading
GDH protein content (arbitrary unit)
2.5
2.0
1.5
1.0
0.5
0.0
NP
NPL
LP
LP
D
Skeletal Muscle
GDH
Loading
GDH protein content (arbitrary unit)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
NP
NPL
LP
LP

Figure 3

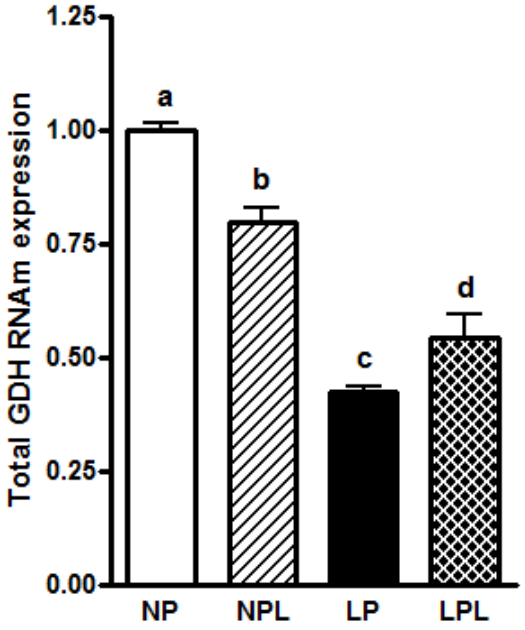

Total GDH RNAm expression
1.25
1.00
0.75
0.50
0.25
0.00
a
b
c
d
NP
NPL
LP
LPL

Figure 4

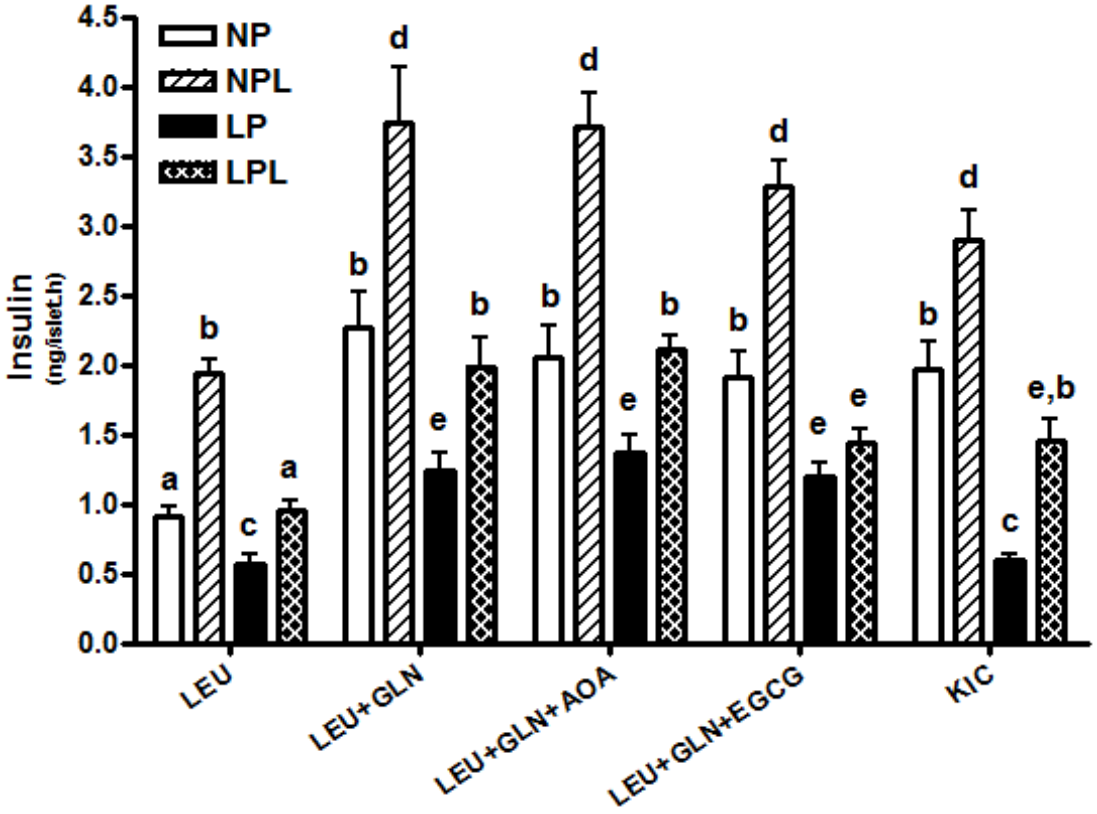

NP
NPL
LP
LPL
Insulin
(ng/islet.h)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
b
c
a
b
d
e
b
b
d
e
b
b
d
e
e
b
d
c
e,b
LEU
LEU+GLN
LEU+GLN+AOA
LEU+GLN+EGCG
KIC

Figure 5

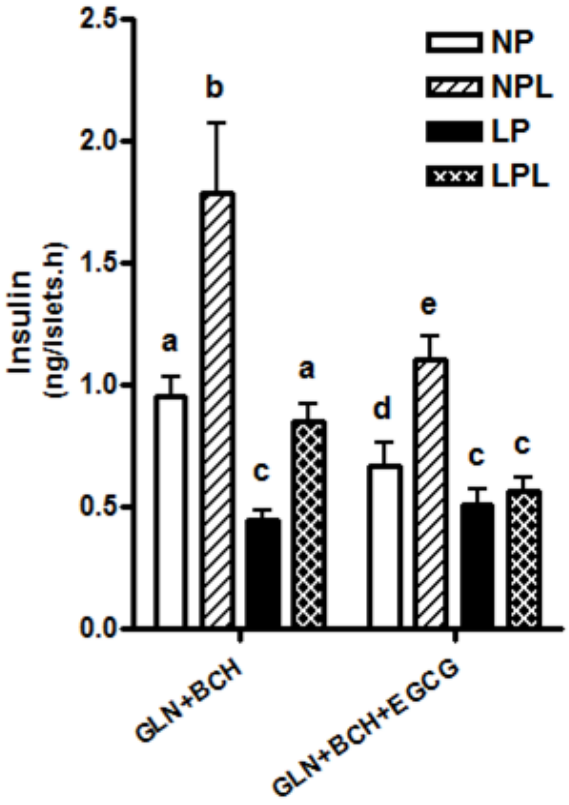

2.5
2.0
1.5
1.0
0.5
0.0
Insulin
(ng/Islets.h)
NP
NPL
LP
LPL
a
b
c
a
d
e
c
c
GLN+BCH
GLN+BCH+EGCG

Figure 6

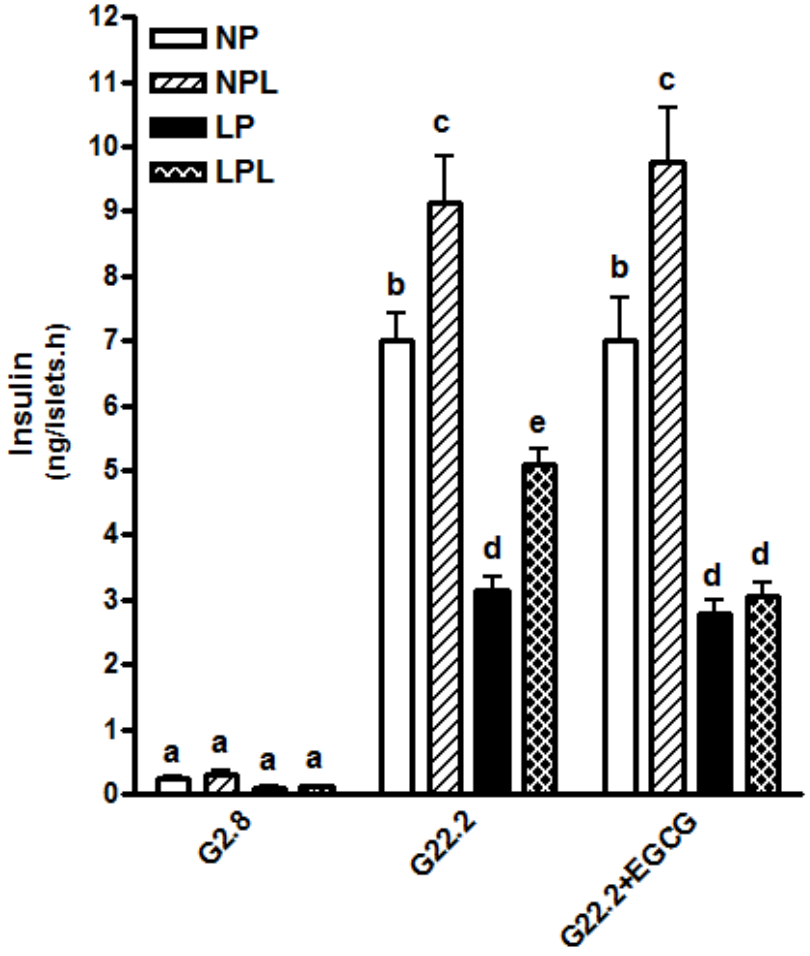

NP
NPL
LP
LPL
Insulin
(ng/Islets.h)
12
11
10
9
8
7
6
5
4
3
2
1
0
a
a
a
a
b
c
d
e
b
c
d
d
G2.8
G22.2
G22.2+EGCG

Figure 7

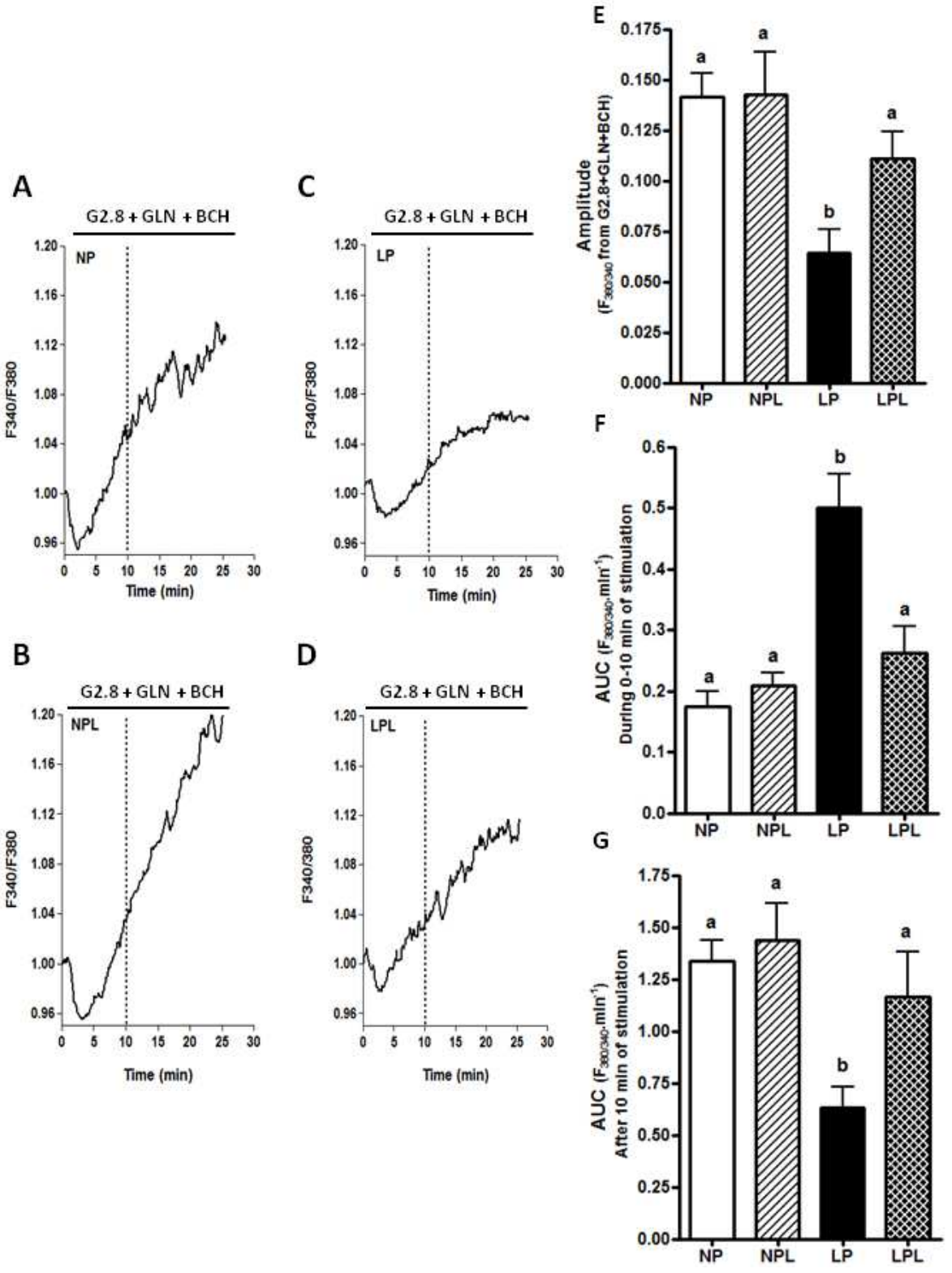
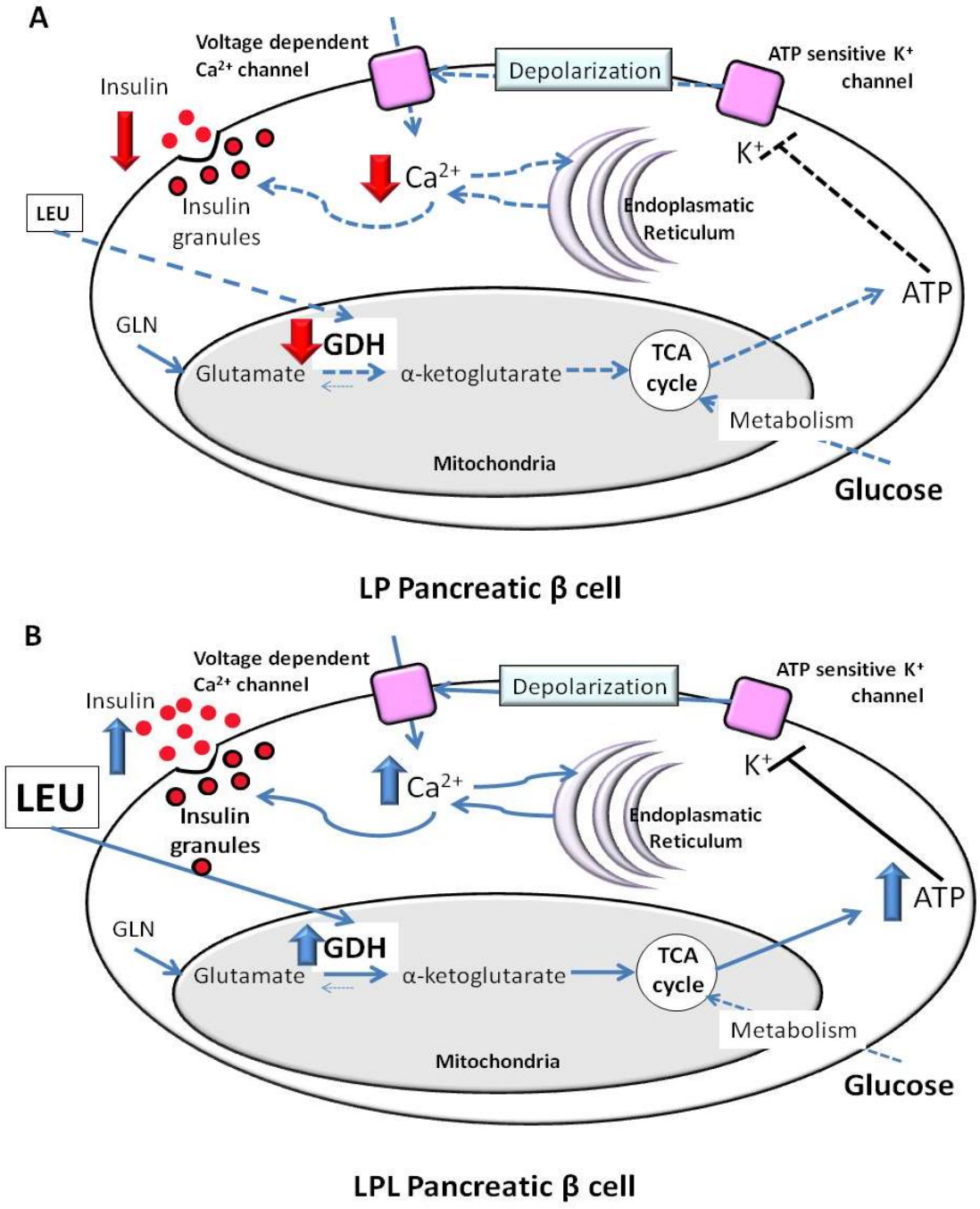

A
G2.8 + GLN + BCH
NP
F340/F380
Time (min)
B
G2.8 + GLN + BCH
NPL
F340/F380
Time (min)
C
G2.8 + GLN + BCH
LP
F340/F380
Time (min)
D
G2.8 + GLN + BCH
LPL
F340/380
Time (min)
E
Amplitude
(F380/340 from G2.8+GLN+BCH)
NP
NPL
LP
LPL
F
AUC (F380/340-min-1)
During 0-10 min of stimulation
NP
NPL
LP
LPL
G
AUC (F380/340-min-1)
After 10 min of stimulation
NP
NPL
LP
LPL

Figure 8


A
Voltage dependent
Ca2+ channel
Depolarization
ATP sensitive K+
channel
Insulin
Ca2+
K+
Insulin
granules
LEU
Endoplasmatic
Reticulum
ATP
GLN
GDH
Glutamate
α-ketoglutarate
TCA
cycle
Metabolism
Mitochondria
Glucose
LP Pancreatic β cell
B
Voltage dependent
Ca2+ channel
Depolarization
ATP sensitive K+
channel
Insulin
LEU
Ca2+
K+
Insulin
granules
Endoplasmatic
Reticulum
ATP
GLN
GDH
Glutamate
α-ketoglutarate
TCA
cycle
Metabolism
Mitochondria
Glucose
LPL Pancreatic β cell

DISCUSSÃO

Iniciamos o trabalho caracterizando nosso modelo de estudo, avaliando a influência da restrição protéica sobre parâmetros biométricos, consumo alimentar e hídrico, parâmetros bioquímicos plasmáticos, tolerância à glicose, sensibilidade à insulina e ainda, avaliamos se a suplementação com LEU alteraria os padrões induzidos pela restrição protéica.

A restrição protéica influenciou a evolução do peso corpóreo dos animais. Trabalhos anteriores já caracterizaram bem as alterações corpóreas que a desnutrição protéica pós-desmame causa nos animais, ou seja, redução dos parâmetros biométricos quando comparados aos animais controle (**Okitolonda *et al.*, 1987; Ferreira *et al.*, 2003; Ferreira *et al.*, 2004; Filiputti *et al.*, 2008; Amaral *et al.*, 2010; Batista *et al.* 2010; Soriano *et al.*, 2010**). Existem ainda outros modelos de desnutrição (intra-uterina e durante a lactação) que relatam efeitos ainda mais marcantes sobre o peso corpóreo de animais submetidos à restrição protéica (**Latorraca *et al.*, 1998; Zambrano *et al.*, 2006; Fagundes *et al.*, 2007; Chen *et al.* 2009**). Ainda, nossos resultados sugerem que a suplementação com LEU não é capaz de reverter os padrões biométricos induzidos pela restrição protéica.

Estudo realizado em animais que receberam dieta LP (4,5% de proteína) mostrou que os animais apresentaram massa relativa do coração aumentada quando comparada com animais que receberam dietas normais (20% de proteína) (**Debski *et al.*, 2006**). Cunha *et al.* (2002) realizaram estudos com humanos com má-nutrição crônica, relatando que a massa relativa do coração está aumentada quando comparada com humanos que possuem nutrição normal. Esse estudo sugere que isso ocorra como uma possível preservação do miocárdio com relação a intensidade de perda de peso associado com o provável aumento de tecido conectivo cardíaco e de veias. Da mesma maneira, o coração dos animais desnutridos, aqui estudados, apresenta massa relativa aumentada quando comparada com os animais controles e a suplementação com LEU não altera esse

parâmetro. Este efeito observado no coração, da mesma maneira que o observado no estudo acima citado, talvez seja uma forma de preservar o miocárdio da perda de massa. Porém, se faz necessário, ainda, avaliar o tecido conectivo e as veias para verificar se há contribuição destes para esse aumento.

Estudos realizados com humanos ingerindo dietas hiperprotéicas comparadas com dietas isocalóricas de carboidratos ou de gordura indicam que as hiperprotéicas oferecem maior sensação de saciedade. No entanto, os mecanismos pelos quais a dieta hiperprotéica aumenta a saciedade ainda não estão bem estabelecidos (**Kushner & Doefler, 2008; Halton & Hu, 2004; Paddon-Jones *et al.*, 2008**). Sabe-se também que a diminuição do tecido adiposo pode contribuir com o aumento da ingestão, pois o tamanho do tecido adiposo afeta a produção de leptina, que contribui na regulação do comportamento de ingestão ao interagir com sistema neuroendócrino (**Trayhurn & Beattie, 2001**). Nossos animais desnutridos apresentaram consumo alimentar aumentado (indicado pelo índice de eficiência alimentar) quando comparado com os animais controles. A alteração no comportamento alimentar dos animais desnutridos pode estar sendo ocasionado pela ingestão de dieta LP, que não deve produzir saciedade da mesma maneira que a dieta normoprotéica. Além disso, a diminuição da gordura retroperitonial e perigonadal pode estar contribuindo na diminuição da produção de leptina, levando a uma alteração do controle relativo do consumo alimentar.

A avaliação do estado nutricional é realizada clinicamente pela análise da albumina circulante (**Pencharz, 2008**). A redução de seus valores em modelos de desnutrição em animais já foi verificada e estabelecida (**Ferreira *et al.*, 2003; Ferreira *et al.*, 2004; Filiputti *et al.*, 2008**). Em nosso estudo reproduzimos esses dados, em adição, demonstramos que a

suplementação com LEU restabelece os níveis de albumina plasmática de animais desnutridos, melhorando, desta forma, o estado nutricional dos animais.

Já foi descrito que a glicemia de animais de jejum e alimentados não apresentam diferenças entre animais controle e desnutridos; isso fica demonstrado em nossos dados e a suplementação com LEU não os altera. Por outro lado, os animais desnutridos apresentam menor insulinemia no estado alimentado e a suplementação com LEU restabelece os níveis de insulina circulante neste estado. Essas modificações já haviam sido observadas em outros estudos indicando a importância da suplementação com LEU para o restabelecimento da insulinemia em animais desnutridos (**Filiputti *et al.*, 2010; Amaral *et al.*, 2010**). Também demonstramos que a desnutrição protéica altera os níveis circulantes de ácidos graxos livres, porém a suplementação com LEU, em nosso estudo, não foi capaz de normalizar esses níveis.

Em acordo com estudos anteriores (**Levine *et al.*, 1983; Okitolonda *et al.*, 1988; Escriva *et al.*, 1991; Picarel-Blanchot *et al.*, 1995; Reis, *et al.*, 1997; Giozzet *et al.* 2008; Filiputti *et al.* 2010; Amaral *et al.* 2010**), no presente trabalho, também observamos que a desnutrição aumenta a tolerância a glicose e a sensibilidade a insulina. Este efeito é decorrente da adaptação ao pobre ambiente nutricional que resulta em aumento da fosforilação em tirosina do receptor de insulina (IR), do substrato do receptor de insulina - 1 (IRS-1) (**Filiputti *et al.*, 2008**), bem como da maior associação da subunidade de 85kDa (p85) da PI3K com o IRS-1 no músculo, fígado e adipócitos de ratos desnutridos (**Ozanne *et al.*, 1997; Reis *et al.*, 1997; Latorraca *et al.*, 1998**). Além disso, demonstramos que a suplementação com LEU não afeta a tolerância à glicose, porém diminui a sensibilidade a insulina nos ratos desnutridos. Esta diminuição de sensibilidade demanda maior liberação de insulina pelas células β para que a glicemia se mantenha em níveis de normalidade. De fato, alguns estudos mostraram que a LEU induz hiperinsulinemia e resistência à insulina em

músculo esquelético de humanos (**Schwenk & Haymond, 1987; Krebs *et al.*, 2002; Drummond *et al.*, 2008**) e a suplementação com LEU em animais desnutridos aumenta a secreção de insulina frente aos nutrientes (**Filiputti *et al.*, 2010; da Silva *et al.* 2010; Amaral *et al.* 2010**).

Está bem estabelecido que a GDH exerce funções essenciais no cérebro, fígado e rim. Entretanto, em nosso modelo animal, nós não observamos diferenças no conteúdo protéico da GDH nesses tecidos e no músculo, diferentemente do que observamos com relação às ilhotas, onde a GDH foi alterada pela desnutrição e pela suplementação com LEU. Portanto, podemos sugerir que a desnutrição protéica altera focalmente o pâncreas endócrino, semelhante a modelos de *knockdown* (**Carobbio *et al.*, 2004**).

Sabe-se que a secreção de insulina estimulada por glicose está prejudicada em animais desnutridos. Propostas de nosso grupo de pesquisa e outros laboratórios são feitas para tentar explicar a causa da menor sensibilidade dessas ilhotas frente à glicose. Dentre estas propostas estão: menor capacidade de metabolização da glicose (**Dixit & Kaung, 1985**), diminuição na mobilização do íon cálcio (**Latorraca *et al.*, 1999**), menor expressão de diversas proteínas como: PDX-1 (**Arantes *et al.*, 2002; Martin *et al.*, 2004**), do transportador de glicose GLUT-2 (**Dixit & Kaung, 1985**), da glicoquinase, da glicerolfosfato desidrogenase (**Rasschaert *et al.*, 1995**), da PKCα (**Ferreira *et al.*, 2004**), da PKAα (**Milanski *et al.*, 2005**), S6K-1(**Filiputti *et al.*, 2008**).

Além disso, está bem estabelecido ser a LEU, um agente insulinotrópico (**Fahien *et al.*,1988; MacDonald *et al.*, 2008**), estimulando ou potencializando a secreção de insulina por sua transaminação ou pela ativação alostérica da GDH. Estudos anteriores relataram que a suplementação com LEU em ratos desnutridos restabelece a secreção de insulina aos níveis do controle quando as ilhotas isoladas foram estimuladas com glicose e outros agentes insulinotrópicos (**Filiputti *et al.*, 2010; da Silva *et al.* 2010; Amaral *et al.* 2010**). Da mesma

maneira, nós observamos que animais desnutridos secretavam menos insulina frente a alta glicose e quando suplementados com LEU, apresentavam níveis similares ao do controle. Considerando que a LEU possui duas vias de atuação direta na secreção de insulina e as evidências da relação da GDH com a secreção de insulina, procuramos, neste trabalho, estudar o papel da GDH em ilhotas isoladas de animais submetidos à restrição protéica e suplementados com LEU. Para tal, analisamos a expressão gênica e protéica da GDH, realizamos secreções estáticas de insulina utilizando um ativador não metabolizável específico da GDH, o BCH, na combinação ou não de GLN (**da Silva *et al.*, 2010; da Silva *et al.*, 2011, submetido**), pois este último aminoácido é convertido em glutamato aumentando, assim, a disponibilidade de substrato para a GDH (**Fahein *et al.*,1988**). Além disso, investigamos o efeito do inibidor específico da GDH, o EGCG, sobre a secreção de insulina e avaliamos o manejo do cálcio estimulado com GLN+BCH.

Observamos que o conteúdo protéico da GDH está diminuído nas ilhotas de ratos desnutridos e a suplementação com LEU restabelece os níveis protéicos da enzima. Além disso, a secreção de insulina dos animais desnutridos está diminuída quando estimulada com 20 mM de LEU, 20 mM de BCH e 20 mM de KIC, semelhante ao que ocorreu quando as ilhotas foram estimuladas com 22,2 mM de glicose. Já a suplementação com LEU restabelece a secreção de insulina aos níveis do controle em resposta a todos esses estímulos. Estes dados estão de acordo com estudo de silenciamento da GDH (**Carobbio *et al.*, 2009**) que demonstrou que a GDH é importante para a resposta de insulina estimulada com GLN+BCH. Porém, nós evidenciamos pela primeira vez que animais desnutridos suplementados com LEU recuperam a secreção de insulina não só por melhorar a via da PI3K/mTOR (**Filiputti *et al.*, 2010**) ou o conteúdo protéico de M3, PKCα, entre outras (**Amaral *et al.*, 2010**), mas também por recuperar o conteúdo protéico da GDH (**da Silva *et al.*, 2010**). Em adição, é provável, ainda, que a melhora na atividade da

GDH seja através da ativação da via da mTOR, tendo em vista os dados apresentados por **Xu *et al.* (2003)**. Neste trabalho, os autores demonstraram que o efeito induzido pela LEU na atividade da GDH estava associado com a via da mTOR. Além disso, pudemos sugerir que a via de oxidação da LEU no grupo suplementado também possui importância no restabelecimento da secreção de insulina em ratos desnutridos. De fato, nossos dados estão de acordo com estudo prévio, o qual mostrou evidências de uma possível interação entre as vias da GDH e da oxidação da LEU para aumentar a secreção de insulina estimulada por LEU em ilhotas pancreáticas de camundongos BTBR (**Rabaglia *et al.*, 2005**).

Para investigarmos melhor a importância da GDH e da oxidação da LEU na secreção de insulina em animais desnutridos e suplementados com LEU, decidimos então utilizar inibidores específicos para enzimas chaves das vias envolvidas. Escolhemos o EGCG, pois foi demonstrado recentemente que este composto inibe a secreção de insulina por inibir a GDH (**Li *et al.*, 2006**), por permitir o trabalho com as ilhotas intactas e por ser um composto que está sendo muito estudado devido as suas supostas propriedades benéficas para o organismo (**Butt & Sultan, 2009**). Já para inibir a BCATm, foi utilizado um inibidor clássico, o AOA, empregado desde a década de 80 para estudos de secreção de insulina estimulada com aminoácidos ramificados (**Malaisse *et al.*, 1982**).

A secreção de insulina estimulada com LEU+GLN ou GLN+BCH apresentou padrão semelhante ao que havíamos observado quando as ilhotas de todos os grupos foram estimuladas com LEU, BCH e KIC. Porém, a incubação com LEU+GLN estimulou uma liberação maior de insulina quando comparada às secreções estimuladas somente com LEU e BCH. De fato, esse aumento na secreção de insulina era esperado uma vez que havíamos utilizado a GLN, precursor do glutamato, substrato da GDH e estimulávamos a via da oxidação da LEU, aumentando assim o

fluxo de ambas vias. Essa diferença na capacidade estimulatória da secreção de insulina entre LEU e BCH, combinados ou não, foi demonstrada também por MacDonald *et al.* (2008) em células INS-1 e ilhotas pancreáticas de ratos.

Ao incubarmos as ilhotas com AOA, inibindo, portanto, a oxidação da LEU, verificamos que a secreção de insulina não era inibida em nenhum dos grupos, apresentando um padrão semelhante a LEU+GLN. Já ao incubarmos com EGCG, a secreção de insulina era inibida somente no grupo LPL. Quando incubamos as ilhotas com GLN+BCH+EGCG, verificamos que a secreção de insulina era inibida em todos os grupos, menos no LP, sendo tal inibição mais eficiente no grupo LPL. Estes resultados nos permitem sugerir que a ativação da GDH é capaz de induzir a secreção de insulina similarmente à oxidação de LEU, pois, mesmo havendo a inibição da via da oxidação de LEU, a secreção de insulina ocorreu eficientemente. Este resultado está de acordo com anteriores que demonstraram a importância da ativação da GDH e da produção de α-cetoglutarato, aumentando o fluxo do ciclo do ATC para a mitocôndria e, conseuqentemente, para a secreção de insulina (**Rabaglia, *et al.*, 2005; Li *et al.*, 2006a; Li *et al* 2006b; Odegaard 2010; Zhou *et al.*, 2010**). Além disso, este resultado confirma os nossos resultados anteriores (**da Silva *et al.*, 2010**), pois evidencia que a secreção de insulina estimulada com LEU é dependente da GDH em animais LP suplementados com LEU .

Estudos anteriores relataram a diminuição da secreção de insulina em ilhotas de animais *knockout* ou em células INS-1 silenciadas para GDH quando expostas a glicose (**Carobbio *et al.*, 2004**). Porém sugere-se que a GDH seria importante somente para que a resposta secretória fosse completa não comprometendo a homeostase glicêmica em camundongos tratados com dietas isocalórica (**Carobbio *et al.*, 2009**). Desta forma, o passo subsequente foi investigar se a GDH estava relacionada com a melhora da secreção de insulina observada quando estas ilhotas de

animais LPL eram incubadas com alta glicose. Para tal, associamos a glicose com o EGCG e verificamos que a secreção de insulina foi inibida nos animais LPL. Nossos resultados estão de acordo com Li *et al.* (2006b), onde houve a combinação de EGCG com glicose em ilhotas pancreáticas de ratos e verificaram que o EGCG não é capaz de inibir a secreção de insulina estimulada por glicose. Desta maneira, podemos sugerir que a secreção de insulina estimulada por glicose observada nas ilhotas de animais LP suplementados com LEU é dependente da GDH, corroborando nossos dados previamente publicados (**da Silva *et al.*, 2010**).

A secreção de insulina é dependente ainda, do manejo de Ca^{2+}. Foi demonstrado que ilhotas de camundongos cultivadas com AOA apresentaram maior $[Ca^{2+}]_i$ quando perfundidas com LEU+GLN. Além disso, ilhotas de camundongos hiperinsulinêmicos com mutação na GDH no sítio de ligação do GTP possuem maior $[Ca^{2+}]_i$ comparadas com ilhotas controle quando estimuladas com GLN (**Li *et al.*, 2006a**). Portanto, tais evidências sugerem que a GDH pode modular o manejo de Ca^{2+}. Além disso, estudos anteriores demonstraram que ilhotas de roedores LP possuem redução do $[Ca^{2+}]_i$ quando estimuladas com glicose ou outros agentes insulinotrópicos (**Latorraca *et al.*, 1999; Carneiro, *et al.*, 2008; Batista *et al.*, 2010; Soriano *et al.* 2010; Oliveira *et al.*, dados não publicados**). Semelhantemente a esses estudos, nós também evidenciamos que ilhotas LP quando perfundidas com GLN+BCH também possuem diminuídos a amplitude da oscilação de Ca^{2+}, o sequestro de Ca^{2+} pelo retículo endoplasmático e a $[Ca^{2+}]_i$. Além disso, a suplementação com LEU foi capaz de restaurar todos esses parâmetros. Desta forma, é provável que tanto a reação catalisada pela GDH quanto a suplementação com LEU sejam relevantes para o manejo de Ca^{2+}.

Em resumo, podemos concluir que dieta hipoprotéica afeta a expressão e consequentemente a função da GDH, prejudicando a secreção de insulina. Provavelmente, isto é

causado pela diminuição na produção de α-cetoglutarato e do manejo de Ca^{2+} (Fig 1A). Por outro lado, a suplementação com LEU restaura a secreção de insulina frente a nutrientes, restaurando o conteúdo protéico, bem como a funcionalidade da GDH (fig. 2B).

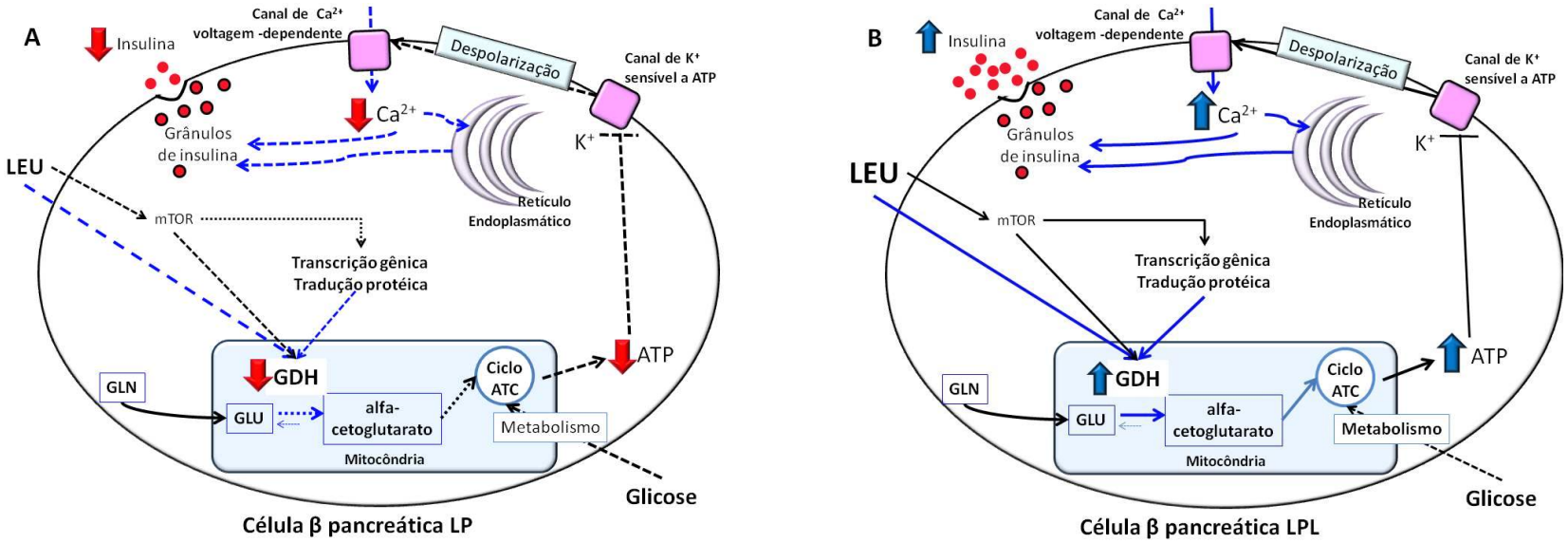


Figura 1. Esquema simplificado de mecanismos de secreção de insulina por célula B pancreática de animais desnutridos (A) e suplementados com LEU (B). As setas pontilhadas indicam os mecanismos que estão diminuídos com a desnutrição e as setas cheias indicam os mecanismos restaurados com a suplementação com LEU. *LEU*: leucina; *mTOR*: proteína alvo da rapamicina em mamíferos; *GLN*: glutamina; *GLN*: glutamato; *ATC*: ácido tricarboxílico.

CONCLUSÕES

Com este trabalho, podemos concluir:

- A desnutrição protéica modula a expressão gênica e protéica da GDH somente no pâncreas endócrino permitindo o estudo desta enzima e de sua importância especificamente para a secreção de insulina;
- A suplementação com LEU é capaz de modular a expressão de genes e proteínas no pâncreas endócrino, incluindo a expressão da GDH, restaurando o seu conteúdo protéico a valores do controle;
- A restauração da secreção de insulina em animais LPL frente a diferentes secretagogos é dependente da expressão e função da GDH e de seu conteúdo protéico;
- A ativação da GDH é capaz de induzir a secreção de insulina similarmente à transaminação da LEU.
- A GDH parece estar envolvida com o manejo de Ca^{2+} intracelular e, portanto, a suplementação com LEU é capaz de reverter os padrões de manejo de Ca^{2+} intracelular alterados pela dieta LP.

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ANEXOS

Publicação do artigo 1

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Preliminary report: Leucine supplementation enhances glutamate dehydrogenase expression and restores glucose-induced insulin secretion in protein-malnourished rats

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Abstract

Low-protein diet impairs insulin secretion in response to nutrients and may induce several metabolic disorders including diabetes, obesity, and cardiovascular disease. In the present study, the influence of leucine supplementation on glutamate dehydrogenase (GDH) expression and glucose-induced insulin secretion (GIIS) was investigated in malnourished rats. Four groups were fed with different diets for 12 weeks: a normal-protein diet (17%) without or with leucine supplementation or a low (6%)-protein diet without (LP) or with leucine supplementation (LPL). Leucine (1.5%) was supplied in the drinking water. Western blotting analysis revealed reduced GDH expression in LP, whereas LPL displayed improved GDH expression, similar to control. The GIIS and leucine-induced insulin release were also enhanced in LPL compared with LP and similar to those observed in rats fed a normal-protein diet without leucine supplementation. In addition, GDH allosteric activators produced an increased insulin secretion in LPL. These findings indicate that leucine supplementation was able to increase GDH expression leading to GIIS restoration, probably by improved leucine metabolic pathways.

1. Introduction

Protein malnutrition impairs glucose-induced insulin secretion (GIIS) by reducing the transcription of several genes related to β-cell signaling [1]. Actually, it has been reported that the insulin signaling pathway is impaired in protein-undernourished rats because of reduced PI3K and p70S6k expression in pancreatic islets [2]. In this process, leucine was demonstrated to improve insulin release [3], an effect that was associated with elevated mitochondrial energy production [4]. Although the mechanism remains unclear, leucine metabolism might supply the tricarboxylic acid cycle (TCA) with different anaplerotic substrates including α-ketoisocaproate (KIC), which can be further metabolized to acetyl–coenzyme A and acetoacetate [5]. Leucine may also allosterically activate glutamate dehydrogenase (GDH), the enzyme that converts glutamate to α-ketoglutarate, an important anaplerotic substrate for the second span of TCA. The observation that β-cells exhibit a high level of anaplerotic mitochondrial enzymes suggests that this process is particularly important for the insulin secretion mechanism [5]. In addition, leucine might activate mammalian target of rapamycin (mTOR) downstream events, affecting elongation and translation of intracellular signaling proteins [6]. In the β-cell line RINm5F, the leucine-induced GDH activation was shown to increase the p70S6k phosphorylation and mitochondrial activity [4], suggesting that leucine might be directly associated with

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work. Priscilla M.R. da Silva and Claudio C. Zoppi participated equally to manuscript drafting.

The experiments with animals are in adherence with the institutional State University of Campinas Committee for Ethics in Animal Experimentation.

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Carta de submissão do Artigo 2

Campinas, March 1st, 2011.

To the Editor-in-chief

Att. Dr. Christos S Mantzoros

Dear Dr. Mantzoros,

According to Metabolism – Clinical and Experimental publishing policy, we are submitting for appreciation as a Original Research Paper the present manuscript entitled "Decreased Insulin Secretion in Protein Malnourished Rats is Associated with Impaired Glutamate Dehydrogenase Function: Effect of Leucine Supplementation", authored by Priscilla M. R. da Silva, Thiago M. Batista, Rosane A. Ribeiro, Claudio C. Zoppi, Antonio Carlos Boschero and Everardo M. Carneiro.

This article is composed by final observations and conclusions that were firstly reported in this journal as a Preliminary Report entitled "Leucine supplementation enhances glutamate dehydrogenase expression and restores glucose-induced insulin secretion in protein malnourished rats", published at Metabolism Clinical and Experimental 59 (2010) 911-913. All authors listed have contributed to the work and agreed to submit the manuscript to Metabolism. No part of the study has been published before and it is not currently being considered by another journal.

Finally, all animal studies have been reviewed by the appropriated ethics committees. Looking forward to have our paper accepted for publication in this prestigious journal I remain,

Sincerely,

Priscilla Muniz Ribeiro da Silva

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Dr. Everardo Magalhães Carneiro

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DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha dissertação/tese de Mestrado/Doutorado intitulada CARACTERIZAÇÃO MOLECULAR E FUNCIONAL DA ENZIMA GLUTAMATO DESIDROGENASE (GDH) EM ILHOTAS DE RATOS SUBMETIDOS À RESTRIÇÃO PROTÉICA E SUPLEMENTADOS COM LEUCINA:

( ) não se enquadra no § 3º do Artigo 1º da Informação CCPG 01/08, referente a bioética e biossegurança.

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Aluno: Prisicilla Muniz Ribeiro da Silva

Orientador: Everardo Magalhães Carneiro

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CERTIFICADO

Certificamos que o Protocolo nº **2011-1**, sobre **"Caracterização molecular e funcional da enzima glutamato desidrogenase (GDH) em ilhotas de ratos e camundongos submetidos à restrição proteica e suplementados com leucina"**, sob a responsabilidade de **Prof. Dr. Everardo Magalhães Carneiro / Priscilla Muniz Ribeiro da Silva**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética no Uso de Animais – CEUA/Unicamp em **09 de novembro de 2009**.

CERTIFICATE

We certify that the protocol nº **2011-1**, entitled **"Functional and molecular characterization of glutamate dehydrogenase in pancreatic islets of protein-restricted rats and mice supplemented with leucine"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - Unicamp) on **November 9, 2009**.

Campinas, 16 de novembro de 2009.

Profa. Dra. Ana Maria A. Guaraldo
Presidente

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