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REGULAÇÃO DA SECREÇÃO DE INSULINA EM ILHOTAS PANCREÁTICAS DE CAMUNDONGOS SUPLEMENTADOS COM TAURINA

Tese apresentada ao instituto de Biologia para a obtenção do título de Doutora em Biologia Funcional e Molecular, na área de Fisiologia.

Orientador: Prof. Dr. Everardo Magalhães Carneiro

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LISTA DE ABREVIATURAS

[Ca^{2+}]i – *concentração intracelular de Ca^{2+}*

AC - *adenilato ciclase*

ADP – *adenosina difosfato*

Akt - *thymona viral oncogene*

AMPc – *adenosina monofosfato cíclico*

AOA – *ácido aminooxiacético*

ATP – *adenosina trifosfato*

BCH - *2-aminobicyclo[2,2,1]-heptane-2-carboxylic acid*

Canal de Ca_v – *canal de Ca^{2+} sensível à voltagem*

Cch - *carbacol*

CSA - *ácido cisteinosulfinato*

CTL – *controle*

DAG - *diacilglicerol*

DM - *Diabetes mellitus*

DM 1 – *DM tipo 1*

DM 2 – *DM tipo 2*

ERK1/2 - *mitogen-activated protein kinase 1/2*

GCK - *glicoquinase*

GDH - *glutamato desidrogenase*

GIP - *peptídeo insulinotrópico*

GLP-1 - *glucagon like peptide 1*

GLUT – *transportador de glicose*

H89 - *N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide*

HOCl – *ácido hipocloroso*

IBMX – *3-isobutyl-1-methyl-xanthine*

IP_3 – *inositol trifosfato*

IR - *receptor de insulina*

IRS - *substrato do IR*

K_{ATP} – *canal de K^+ sensível ao ATP*

KIC - *ácido isocaproato*

Kir - *ATP-sensitive inwardly rectifying K^+ channel*

KRB - Krebs-Ringer bicarbonate

M3 – *receptor muscarínico 3*

mTOR - *serine/threonine kinase target of rapamycin in mamalian tissues*

NOD – *não-obesos diabéticos*

OLETF - *Otsuka Long-Evans Tokushima Fatty*

PDX-1 – *pancreatic duodenal homeobox*

PHAS-I - *phosphorylated heat- and acid-stable protein regulated by insulin*

PI - *fosfatidilinositol*

PK – *proteína quinase*

PKA – *proteína quinase A*

PKB – *proteína quinase B*

PKC – *proteína quinase C*

PLC – *fosfolipase C*

PMA – *phorbol 12-myristate 13-acetate*

SNAP25 - *synaptosomal-associated protein*

SNARE - *soluble N-ethylmaleimide-sensitive factor attachment protein receptor*

SUR - *receptor de sulfoniluréia*

TAU – *taurina*

RESUMO

Neste estudo, investigamos os efeitos da suplementação com taurina (TAU; 2% adicionada à água de beber) sobre a tolerância à glicose e a secreção de insulina frente a diferentes secretagogos em camundongos adultos. Camundongos suplementados apresentaram aumento da tolerância à glicose e da sensibilidade à insulina. Ilhotas isoladas destes animais secretaram mais insulina em resposta à glicose e L-leucina. A oxidação da L-leucina foi maior no grupo TAU, não havendo diferenças quanto ao consumo de glicose, concentrações de ATP e expressão do transportador da glicose (GLUT) 2 e da glicoquinase (GCK). A captação de Ca^{2+}, na presença de glicose, e a expressão protéica da subunidade β_2 do canal de Ca^{2+} sensível à voltagem foi maior no grupo TAU comparado ao controle (CTL). Ainda, a expressão protéica da PL (fosfolipase) $C_{\beta 2}$ e da PK (proteína quinase) Aα, bem como a secreção de insulina em resposta a agentes potencializadores tais como carbacol (Cch) e IBMX, foi maior nas ilhotas TAU. A mobilização intracelular de Ca^{2+} induzida por Cch foi também maior em ilhotas deste grupo, e observamos que a inibição da PKA reduziu a captação de Ca^{2+} em resposta à glicose no grupo suplementado. Além disso, ilhotas TAU secretaram mais glucagon em relação a ilhotas CTL, quando em presença de baixa concentração de glicose. Concluindo, a suplementação com TAU melhora a homeostase glicêmica e aumenta a secreção de insulina de ilhotas isoladas e incubadas na presença de nutrientes e agentes potencializadores da secreção. Os efeitos sobre a secreção estão relacionados ao melhor manejo dos íons Ca^{2+} pelas células insulares provenientes dos animais suplementados com TAU.

ABSTRACT

In this study, we investigated the effects of taurine (TAU)-supplementation (2% in the drinking water) on glucose tolerance and insulin secretion stimulated by different secretagogues in adult mice. TAU-supplemented mice showed enhanced glucose tolerance and insulin sensitivity when compared to controls (CTL). In addition, their islets secreted more insulin in response to high concentrations of glucose and L-leucine. L-[U-^{14}C]leucine oxidation was higher in TAU islets compared with CTL islets, whereas D-[U-^{14}C]glucose oxidation, ATP levels, and the protein expression of the glucose transporter (GLUT) 2 and of glucokinase (GCK) were similar. ^{45}Ca uptake induced by high glucose concentrations was increased in TAU islets as well as the expression of the β_2 subunit of the L-type Ca^{2+} channel. In addition, the insulin secretion induced by carbachol (Cch) and IBMX, but not, by forskolin and PMA was higher in TAU-supplemented compared with CTL islets. The higher insulin secretion in the presence of Cch is accompanied by an increase in the expression of PL (phospholipase) $C_{\beta 2}$ protein and a higher intracellular Ca^{2+} mobilization. Besides, TAU-supplemented islets showed increased PK (protein kinase) Aα expression. Since the increase in Ca^{2+} uptake induced by glucose in TAU islets was minimized by the presence of the PKA inhibitor, H89, this kinase seems to be important for the better Ca^{2+} handling in these islets. TAU-supplementation also turns the α-cells more sensitivity since these cells secreted more glucagon compared with CTL islets. In conclusion, TAU supplementation enhances glucose tolerance and insulin sensitivity in mice and turns the islets more sensitive to nutrients and to potentiators of secretion. The effect on insulin secretion seems to be linked to a better Ca^{2+} handling by β-cells.

APRESENTAÇÃO

A glicose é o principal estímulo para a secreção de insulina, porém a função das células β pancreática pode ser regulada por outros nutrientes, tais como: aminoácidos, ácidos graxos e cetoácidos (Gao *et al.*, 2003; Li *et al.*, 2004; Um *et al.*, 2004).

Com relação aos aminoácidos, a secreção de insulina pode ser estimulada por estes nutrientes via geração de ATP derivado do metabolismo do aminoácido, ou pela geração de compostos que ao serem degradados aumentam o fluxo de intermediários para o ciclo de Krebs (Wu e Morris, 1998). Evidências experimentais demonstraram que os aminoácidos ativam reguladores da síntese protéica, contribuem para o *turnover* protéico, altera o potencial de membrana devido ao seu cotransporte com o íon Na^+ (Sener *et al.*, 2000; Newsholme *et al.*, 2007). Além disso, Newsholme *et al.* (2005) e Corless *et al.* (2006) observaram que a L-glutamina e a L-alanina regulam a expressão gênica das células β. Por outro lado, aminoácidos não exercem somente efeitos positivos, Patterson *et al.* (2006) verificaram que a homocisteína inibe a secreção de insulina em resposta a nutrientes e potencializadores da secreção.

A taurina (TAU) é um aminoácido que está presente em altas concentrações tanto no interior das células como no plasma de mamíferos. Apesar de vários estudos demonstrarem a contribuição da TAU na prevenção do *Diabetes mellitus* (DM) do tipo 1 (DM 1) e 2 (DM 2) (Anuradha e Balakrishnan, 1999; Nakaya *et al*., 2000; Chang e Kwon, 2000; Nandhini e Anuradha, 2002; Arany *et al*., 2004; Di Leo *et al*., 2004; Tsuboyama-Kasaoka *et al*., 2006), seu papel sobre a secreção de insulina não está estabelecido. Bustamante *et al.* (2001) demonstraram que no pâncreas a TAU está presente em células α e δ da ilhota, sugerindo que a liberação de TAU por estas células participe da modulação da secreção em células β. Contudo, dados quanto à contribuição da TAU sobre a secreção de insulina são contraditórios, sendo verificado redução da liberação do hormônio em ilhotas perfundidas com solução contendo TAU (Bustamante *et al.*, 2001), e aumento em ilhotas

incubadas com o aminoácido (Cherif *et al.*, 1998). Por outro lado, o tratamento crônico com TAU demonstrou aumentar a secreção de insulina em diferentes modelos experimentais (Cherif *et al.*, 1996; Cherif *et al.*, 1998; Carneiro *et al.*, 2008). Neste estudo investigamos a secreção de insulina em resposta a nutrientes e agentes potencializadores da secreção, bem como os possíveis mecanismos envolvidos com a liberação de insulina regulados pela TAU em ilhotas pancreáticas de camundongos adultos que foram submetidos à suplementação com 2% de TAU por 30 dias.

INTRODUÇÃO

Secreção de Insulina

A secreção de insulina pelas células β é controlada continuamente de acordo com as flutuações da concentração de nutrientes circulantes, em especial, a glicose. Este açúcar é o regulador mais importante da secreção de insulina sendo que em resposta a glicose há um aumento rápido, ou pico da liberação do hormônio nos primeiros minutos da estimulação, o qual constitui a primeira fase da secreção. Enquanto a concentração de glicose permanecer elevada, um segundo aumento ou fase é observado, e embora seja de menor amplitude do que a primeira resposta, esta é sustentada até que a euglicemia seja estabelecida (Straub e Sharp, 2002; Hiriart e Aguilar-Bryan, 2008).

Os mecanismos responsáveis pela secreção de insulina estimulada pela glicose iniciam-se com o transporte deste açúcar pelas células β pancreáticas, através de um transportador específico (GLUT 2); a glicose é fosforilada à glicose-6-fosfato pela enzima glicoquinase (GCK) e metabolizada gerando ATP. O resultado é o aumento da relação ATP/ADP, que provoca o fechamento de um canal de K^+ sensível ao ATP (K_{ATP}), presente na membrana da célula β. A redução do efluxo de K^+ das células leva à despolarização da membrana que, por sua vez, provoca a abertura de canais de Ca^{2+} sensíveis à voltagem (Ca_v), e influxo deste cátion (Yang e Berggren, 2006; Hiriart e Aguilar-Bryan, 2008).

A metabolização da glicose nas células e a subseqüente elevação da concentração intracelular de Ca^{2+} ($[Ca^{2+}]i$) pode ativar enzimas que produzirão outros mensageiros intracelulares que contribuem para a amplificação do sinal iniciado pela glicose. Uma destas enzimas é a adenilato ciclase (AC) que, ao clivar o ATP, produz adenosina monofosfato cíclico (AMPc) que, por sua vez, ativa a proteína quinase (PK) A (Delmeire *et al.*, 2003; Dyachok *et al.*, 2008). Além disso, a metabolização da glicose e o aumento da $[Ca^{2+}]i$ também estimula a hidrólise de fosfoinositídeos através da ativação da fosfolipase (PL) C (Thore *et al.*, 2007), resultando na formação do inositol-

1,4,5-trifosfato (IP_3) e diacilglicerol (DAG), que induz a liberação de Ca^{2+} de estoques intracelulares e ativa a PKC, respectivamente. Portanto, aumento da $[Ca^{2+}]i$ e ativação da PKA, PLC e PKC culminam com a exocitose dos grânulos de insulina (Nesher *et al.*, 2002; Seino e Shibasaki, 2005; Tengholm e Gylfe, 2009).

A secreção de insulina também é regulada pela concentração de aminoácidos, ácidos graxos e cetoácidos plasmáticos (Kahn *et al.*, 2006; Newsholme *et al.*, 2007). Com relação aos aminoácidos, sabe-se que os aminoácidos essenciais em condições que mimetizam as fisiológicas, aumentam a resposta secretória da célula β (Dura *et al.*, 2002), e em concentrações suprafisiológicas aminoácidos individuais exercem diversas ações sobre o processo de síntese e secreção de insulina (Newsholme *et al.*, 2007).

A L-glutamina, por exemplo, é utilizada para síntese de purinas e pirimidinas, e subseqüente produção de mRNA participando do *turnover* protéico nas células β (Newsholme *et al.*, 2005). O metabolismo da L-glutamina produz L-glutamato que ao ser oxidado amplifica a secreção de insulina pelo aumento do fluxo de substratos para o ciclo de Krebs (Sener e Malaisse, 1980); isto ocorre pela ação alostérica da L-leucina sobre a enzima glutamato desidrogenase (GDH). O L-glutamato também pode ser direcionado para a formação de glutationa que eleva a viabilidade e função das células β (Brennan *et al.*, 2003), ou ser secretado pelas células e inibir a secreção de insulina via receptores ionotrópicos e metabotrópicos (Corless *et al.*, 2006).

Além da ação alostérica sobre a enzima GDH, a L-leucina também contribui para a secreção de insulina por meio do seu metabolismo. A transaminação deste aminoácido ao ácido α-cetoisocaproato (KIC) alimenta o ciclo de Krebs com Acetil-CoA para produção de ATP (Sener e Malaisse, 1980). Estudos têm demonstrado que L-leucina pode ativar reguladores da síntese protéica como *PHAS-I* (*phosphorylated heat- and acid-stable protein regulated by insulin*), e a proteína quinase *p70S6* via mTOR (*serine/threonine kinase target of rapamycin in mamalian tissues*), via de

sinalização importante para a manutenção da massa de células β (Xu *et al.*, 2001; Briaud *et al.*, 2003).

Os aminoácidos L-alanina e L-arginina podem estimular a secreção por meio da geração de intermediários metabólicos, ou por contribuir na alteração do potencial de membrana devido ao seu cotransporte com Na^+ (Mcclenaghan *et al.*, 1998; Sener *et al.*, 2000). Estes aminoácidos também regulam a expressão de genes envolvidos no metabolismo, vias de transdução de sinal, regulação gênica, síntese protéica, estruturais e com apoptose das células β (Newsholme *et al.*, 2005; Corless *et al.*, 2006).

Por fim, a secreção de insulina pode ser modulada por neurotransmissores e hormônios peptídicos. O sistema nervoso parassimpático, por exemplo, via nervo vago, através de receptores muscarínicos na célula β, potencializa a secreção da insulina, enquanto o sistema nervoso simpático, agindo por meio de receptores α-adrenérgicos, inibe a secreção deste hormônio (Gilon e Henquin, 2001; Gautam *et al.*, 2007). Hormônios intestinais também participam da regulação da liberação de insulina. O hormônio GLP-1 (*glucagon like peptide 1*), e o peptídeo insulinotrópico (GIP), são importantes durante a ingestão alimentar. Eles são liberados do intestino para circulação e potencializam a secreção de insulina. Outros hormônios produzidos pelas ilhotas pancreáticas como o glucagon e a somatostatina exercem ação estimulatória e inibitória, respectivamente, sobre a secreção das células β (Nesher *et al.*, 2002; Mcclenaghan, 2007; Hiriart e Aguilar-Bryan, 2008).

Íons Ca^{2+}, Canais de Ca^{2+} e a Célula β Pancreática

A elevação da $[Ca^{2+}]i$ na célula β é essencial para secreção de insulina, pois este cátion regula a ancoragem ("*docking*") e início da fusão dos grânulos secretórios com a membrana plasmática, num processo mediado pelas proteínas SNARE (*soluble N-ethylmaleimide-sensitive factor*

attachment protein receptor). Além disso, o influxo de Ca^{2+} na célula β participa de vários outros eventos celulares, como a transcrição gênica, fosforilação e ativação de enzimas, mitose, proliferação celular, diferenciação, e apoptose (Yang e Berggren, 2006).

Os principais canais responsáveis pelo influxo de Ca^{2+} para a célula β pancreática são os canais de Ca_v. Estes canais, conforme o tipo da subunidade "α1" que forma o poro do canal, podem ser classificados em: Ca_v1, Ca_v2 e Ca_v3 (Ertel *et al.*, 2000). É necessário ressaltar que além da subunidade α1, os canais de Ca_v apresentam subunidades auxiliares que são denominadas "β, γ, $\alpha_2\delta$" e que influenciam a atividade e a translocação dos canais de Ca_v para a membrana plasmática (Yang e Berggren, 2006).

Atualmente existe um consenso de que os canais do tipo L, que apresentam uma alta condutividade ao cátion e são sensíveis à diidropiridinas, são os canais que contribuem com o maior influxo de Ca^{2+} para as células β, sendo que os canais $Ca_v1.2$ e 1.3 são os subtipos mais expressos nestas células (Ohta *et al.*, 1993; Davalli *et al.*, 1996; Schulla *et al.*, 2003). Além disso, o canal de Ca_v não contribui apenas para a entrada do cátion; evidências experimentais demonstraram que o canal $Ca_v1.2$ interage com proteínas envolvidas no processo exocitótico da insulina (Wiser *et al.*, 1999; Trus *et al.*, 2007), sendo que a sua deleção na célula β reduz a exocitose dos grânulos de insulina da primeira fase da secreção do hormônio (Schulla *et al.*, 2003).

O influxo de Ca^{2+} na célula β também está relacionado com o desenvolvimento celular. Foi observado que camundongos *knockout* para o canal $Ca_v1.3$ apresentam baixo peso ao nascer, e este canal parece essencial para o desenvolvimento pancreático pós-natal pois o número e o tamanho das ilhotas no animal adulto está reduzido (Namkung *et al.*, 2001). Além disso, o influxo de Ca^{2+} pelos canais Ca_v ativa a ERK1/2 (*mitogen-activated protein kinase 1/2*) que contribui para o controle da massa de células β, regulação da transcrição do gene da insulina, e fosforilação de proteínas

envolvidas com o processo de secreção da insulina (Benes *et al.*, 1999; Longuet *et al.*, 2005; Lawrence *et al.*, 2008).

Os canais de Ca_v são regulados por diferentes mecanismos que alteram a condutividade do Ca^{2+} conforme as condições fisiológicas. Dentre estes reguladores tem-se a participação de vários hormônios, mensageiros intracelulares, e quinases como PKA, PKC, Ca^{2+}/Calmodulina, PKG, Akt/PKB (*thymona viral oncogene*) (Yang e Berggren, 2006).

Portanto, Ca^{2+} é importante para o acoplamento estímulo/secreção, desenvolvimento, sobrevivência, crescimento e morte das células β. Em condições patológicas o fluxo de Ca^{2+} é diferencialmente regulado nestas células, sendo evidenciado em diferentes modelos experimentais de DM que a expressão gênica e a atividade dos canais de Ca_v apresentam-se alterados (Wang *et al.*, 1996; Wang *et al.*, 1999; Iwashima *et al.*, 2001; Yang e Berggren, 2006).

O Aminoácido TAU e Suas Ações Biológicas

A TAU é um aminoácido que está presente em altas concentrações tanto no interior das células como no plasma de mamíferos. Este aminoácido é obtido pela ingestão de leite e carne (principalmente peixe e frutos do mar), mas também pode ser biossintetizado a partir de metionina e cisteína (Tappaz, 2004). No que diz respeito à síntese de TAU, a cisteína é oxidada ao ácido cisteinosulfinato (CSA) numa reação catalizada pela enzima cisteína desoxigenase. O CSA, por sua vez, pode ser metabolizado por dois mecanismos que são catalizados ou pela aspartato aminotransferase ou pela CSA descarboxilase, que produzem o sulfato inorgânico e a hipotaurina, respectivamente (Huxtable, 1992; Nakamura *et al.*, 2006). Nesta fase do metabolismo da cisteína postula-se que exista uma descarboxilase que catalisa a conversão de hipotaurina a TAU (Huxtable, 1992; Tappaz, 2004).

Uma vasta literatura demonstra que o aminoácido TAU abrange inúmeras ações biológicas sendo que o mecanismo de ação da TAU pode ocorrer pela combinação do aminoácido com vários tipos de canais iônicos, transportadores e enzimas. Estudos demonstram que TAU possui a propriedade de reagir com o ácido hipocloroso (HOCl) gerando taurocloraminas, e desta forma ser um antioxidante (Franconi *et al.*, 2004). A TAU participa do controle da atividade de canais iônicos, sendo verificado que este aminoácido inibe canais K_{ATP} no músculo esquelético (Tricarico *et al.*, 2000) e em fibras cardíacas (Satoh, 1996). A TAU contribui para a regulação da $[Ca^{2+}]i$, sendo observado que este aminoácido em células cardíacas promove estimulação da abertura do canal de Ca^{2+} do tipo L na presença de baixa $[Ca^{2+}]i$; por outro lado, inibição da abertura do canal em alta $[Ca^{2+}]i$ (Satoh, 1998). TAU aumenta a atividade do trocador de Na^{+}/Ca^{2+} nos miócitos atriais (Satoh e Sperelakis, 1998). Além disso, em neurônios é evidenciado um papel protetor da TAU contra a excitotoxicidade desencadeada por glutamato, onde este aminoácido reduz a captação de Ca^{2+} e amplitude do aumento da $[Ca^{2+}]i$ estimulada pelo neurotransmissor (Chen *et al.*, 2001). Contudo, em hepatócitos TAU aumenta a atividade do uniporte de Ca^{2+} para a mitocôndria, o que eleva a atividade mitocondrial destas células por aumentar o aporte de Ca^{2+} para a ativação de desidrogenases (Palmi *et al.*, 1999).

TAU e Secreção de Insulina

No pâncreas a TAU está compartimentalizada em células da ilhota contendo glucagon e somatostatina, sugerindo que a liberação de TAU por estas células exerça uma possível ação parácrina sobre o processo de secreção de insulina pelas células β (Bustamante *et al.*, 2001).

Uma das ações mediadas pela TAU nas células β pancreática é a de inibir a atividade dos canais de K_{ATP} (Park et al., 2004). O canal de K_{ATP} nestas células é composto pelas subunidades

Kir6.2/SUR1 (Aguilar-Bryan *et al.*, 1995; Inagaki *et al.*, 1995). A subunidade Kir (*ATP-sensitive inwardly rectifying K^+ channel*) forma um poro, enquanto a SUR (receptor de sulfoniluréia) é uma subunidade regulatória que favorece a sensibilidade da Kir a drogas como as sulfoniluréias e estimuladores da abertura do canal de K_{ATP}. Park *et al.* (2004) ao investigarem as propriedades dos canais K_{ATP} em células β, quando aplicada a TAU via intracelular em presença da glibenclamida que tem a propriedade de interagir com duas regiões de alta afinidade, os sítios sulfoniluréia e benzamida da subunidade SUR, verificaram que a TAU aumenta a sensibilidade do canal de K_{ATP} em resposta a esta sulfoniluréia. Além disso, células pré-tratadas com TAU apresentam aumento da secreção de insulina e da $[Ca^{2+}]i$ na presença de glibenclamida. Portanto, a TAU modula a atividade do canal de K_{ATP} nas células β, efeito este relacionado à sua interação com o sítio benzamida na subunidade SUR1, resultando em aumento da $[Ca^{2+}]i$, e consequentemente da secreção de insulina.

Neste sentido, estudos desenvolvidos pelo nosso grupo de pesquisa demonstraram que o tratamento *in vivo* e *in vitro* de ilhotas pancreáticas com a TAU aumenta a sensibilidade das células β à glicose e ao K^+, e eleva a expressão do PDX-1 (*pancreatic duodenal homeobox*), fator de transcrição importante para o desenvolvimento pancreático e regulação da expressão do gene da insulina (Carneiro *et al.*, 2008).

Ação da Insulina, Diabetes e TAU

A elevação da concentração de insulina em resposta ao aumento da concentração de nutrientes no plasma ativa processos anabólicos e inibe os catabólicos. A insulina age principalmente no músculo e tecido adiposo estimulando a captação da glicose, e no fígado inibindo a produção deste açúcar (Whiteman *et al.*, 2002; Youngren, 2007).

As ações da insulina nos tecidos alvo ocorrem por meio da sua ligação com a subunidade α do receptor de insulina (IR), que apresenta atividade tirosina quinase intrínseca. O resultado da interação entre o hormônio e o IR é uma mudança conformacional, que estimula a autofosforilação de vários resíduos tirosinas nas subunidades β do IR e a fosforilação em tirosina dos substratos do IR (IRS), os quais servem de ancoragem para proteínas que possuem um sítio específico para o acoplamento com outros sinalizadores intracelulares que apresentam o domínio SH2 (assim denominados devido à homologia com o produto do oncogene *src*). A ligação dos domínios SH2 da fosfatidilinositol-3-quinase aos resíduos fosfotirosinas do IRS-1 ativa esta enzima, que produz fosfolipídeos de fosfatidilinositol (PI), dentre eles o PI(3,4,5)P3, que ativa a proteína quinase dependente de PI, e subsequentemente a Akt. Esta serina/treonina quinase fosforila várias proteínas, dentre as quais estão proteínas relacionadas à translocação de GLUT4 para a membrana plasmática (como a proteína alvo da Akt: AS160), modula a atividade dos componentes da tradução, contribuindo para a síntese protéica; fosforila e inativa a enzima glicogênio sintase quinase 3; e estimula a transcrição de enzimas relacionadas com a lipogênese e, em contrapartida, inibe a lipólise por ativar fosfodiesterases, inibindo a lipase hormônio sensível (Whiteman *et al.*, 2002; Youngren, 2007).

O DM caracteriza-se por distúrbio na homeostase da glicose, resultante ou da destruição autoimune das células β (DM 1), ou pela resistência periférica à ação da insulina, que pela sobrecarga secretória pode levar a disfunção e falência das células β (DM 2) (Cnop *et al.*, 2005).

Apesar de várias evidências demonstrarem o envolvimento do aminoácido TAU com o controle da concentração plasmática de glicose (Kulakowski e Maturo, 1984; Franconi *et al.*, 1995; Anuradha e Balakrishnan, 1999; Aerts e Van Assche, 2001; Colivicchi *et al.*, 2004; Tsuboyama-Kasaoka *et al.*, 2006; Loizzo *et al.*, 2007; Carneiro *et al.*, 2008; Xiao *et al.*, 2008), o mecanismo de ação da TAU em prevenir ou restaurar distúrbios da homeostase da glicose não está completamente

compreendido. Contudo, evidências sugerem uma interrelação entre a redução da concentração plasmática de TAU e a ocorrência de distúrbios no controle da glicemia (Anuradha e Balakrishnan, 1999; Nakaya *et al*., 2000; Chang e Kwon, 2000; Nandhini e Anuradha, 2002; Arany *et al*., 2004; Di Leo *et al*., 2004; Tsuboyama-Kasaoka *et al*., 2006).

Em pacientes com DM 1 e DM 2, as concentrações plasmáticas e plaquetárias de TAU apresentam-se reduzidas (Franconi *et al.*, 1995). Deficiência de TAU é também descrita em vários modelos animais como em ratos com DM induzido por estreptozotocina (Aerts e Van Assche, 2001; Colivicchi *et al.*, 2004), e em roedores com resistência à insulina (Anuradha e Balakrishnan, 1999; Tsuboyama-Kasaoka *et al.*, 2006). Baixos níveis plasmáticos de TAU no diabetes relaciona-se diretamente com hiperglicemia e distúrbios na osmorregulação celular (Hansen, 2001).

Maturo e Kulakowski (1988) verificaram a ligação do aminoácido TAU com o IR. Além disso, Carneiro *et al.* (2008) observaram aumento da fosforilação do IR no fígado e no músculo de camundongos que receberam injeção ip de TAU. Portanto, este aminoácido parece potencializar os efeitos da insulina, e a suplementação com TAU em animais com resistência à insulina normaliza não somente a concentração plasmática de insulina e glicose, mas também sensibilidade à insulina, hipertensão e hiperlipidemia (Anuradha e Balakrishnan, 1999; Nakaya *et al.*, 2000; Nandhini e Anuradha, 2002; Tsuboyama-Kasaoka *et al.*, 2006). Exerce também efeito protetor contra a destruição de células β de ratos com DM 1 induzido por estreptozotocina, e em camundongos não obesos diabéticos (NOD) (Chang e Kwon, 2000; Arany *et al.*, 2004), e a longo prazo aumenta a taxa de sobrevivência nestes animais (Arany *et al.*, 2004; Di Leo *et al.*, 2004).

Desta forma, suplementos nutricionais que reduzem a necessidade da terapia com insulina, e que impeçam o desenvolvimento de complicações patológicas no DM, são uma importante linha de investigação em busca de biomoléculas que possam ser utilizadas para finalidades terapêuticas. Conhecer o mecanismo pelo qual a TAU exerce seu papel fisiológico, seja em controlar direta ou

indiretamente a concentração plasmática da glicose, regular a atividade de canais iônicos, a $[Ca^{2+}]i$, ou aumentar a expressão de genes e diminuir a apoptose de células β, demonstra ser uma linha de pesquisa promissora para a obtenção de novas formas de tratamento e/ou prevenção de enfermidades endócrinas em especial o DM.

OBJETIVOS

Neste estudo avaliamos a secreção de insulina estimulada por diferentes nutrientes e potencializadores da secreção, bem como os possíveis mecanismos envolvidos com a liberação de insulina regulados pela TAU, em ilhotas pancreáticas de camundongos adultos que foram submetidos à suplementação com 2% de TAU por 30 dias.

OBJETIVOS ESPECÍFICOS

- Investigar o efeito da suplementação com TAU sobre a tolerância à glicose e sensibilidade à insulina;

- Avaliar a secreção de insulina estimulada pela glicose e L-leucina, bem como a oxidação destes nutrientes, a captação de Ca^{2+}, e expressão do canal de Ca_v;

- Verificar a secreção de insulina em resposta à estimulação colinérgica e ativação da PKA, e o possível envolvimento destes mecanismos com o movimento e captação de íons Ca^{2+}.

Os resultados alcançados durante a realização deste estudo estão apresentados a seguir sob a forma de dois artigos:

1. *Taurine supplementation enhances nutrient-induced insulin secretion in pancreatic mice islets (Submetido e aceito para publicação - Diabetes/Metabolism Research & Reviews);*
2. *Taurine supplementation: involvement of Cholinergic/Phospholipase C and PKA pathways in potentiation of insulin secretion and Ca^{2+} handling.*

ARTIGO 1

TAURINE SUPPLEMENTATION ENHANCES NUTRIENT-INDUCED INSULIN SECRETION IN PANCREATIC MICE ISLETS

Artigo submetido e aceito para a publicação - Diabetes/Metabolism Research and Reviews (ver anexos)

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Keywords: glucose homeostasis; insulin secretion; taurine supplementation

Abstract

Background: Taurine (TAU), a naturally occurring sulfur-containing amino acid, is found at high concentrations in plasma and mammalian tissues and regulates osmolarity, ion channel activity, and glucose homeostasis. Several reports have shown that physiological plasma TAU levels seem to be important for adequate beta (β)-cell function and insulin action, since low concentrations of TAU in the plasma have been reported in the prediabetic and diabetic states.

Methods: Glucose tolerance and insulin sensitivity were investigated in mice supplemented with 2% (w/v) TAU in the drinking water for 30 days, as well as the insulin secretion from isolated islets stimulated by glucose or L-leucine.

Results: TAU-supplemented mice demonstrated improved glucose tolerance and higher insulin sensitivity, compared to controls (CTL). In addition, their islets secreted more insulin in response to high concentrations of glucose and L-leucine. L-[U-^{14}C]leucine oxidation was higher in TAU than in CTL islets, whereas D-[U-^{14}C]glucose oxidation, ATP levels, glucose transporter (GLUT) 2 and glucokinase (GCK) protein expressions were similar in both types of islets. The L-type β_2 subunit voltage-sensitive Ca^{2+} channel protein, as well as ^{45}Ca uptake, was significantly higher in TAU-supplemented than CTL islets. In addition, islets from TAU-supplemented mice secreted more glucagon than CTL islets at low glucose.

Conclusions: TAU supplementation improves glucose tolerance and insulin sensitivity in mice, as well as insulin secretion from isolated islets. The latter effect seems to be, at least in part, dependent on a better Ca^{2+} handling by the islets.

Introduction

Glucose is the main stimulus for insulin secretion from pancreatic β-cells. The stimulus-secretion coupling induced by glucose culminates with increased intracellular Ca^{2+} concentration

([Ca^{2+}]i), which together with metabolic signals results in a complete biphasic insulin secretion (Straub and Sharp, 2002). As with glucose, amino acids exert insulinotropic activity in β-cells. There are three different mechanisms by which amino acids may increase insulin secretion: direct depolarization of the plasma membrane by transport of cationic amino acids, such as L-arginine; cotransport with Na^+, which also results in an increase in [Ca^{2+}]i; and metabolism of the amino acid, resulting in an increase in ATP and in the ATP-to-ADP ratio (Newsholme *et al.*, 2007).

TAU (2-aminoethanesulphonic acid), a naturally occurring sulfur-containing amino acid, which is not incorporated into protein and not used for energy production, is found at high concentrations in pancreatic islet cells (Bustamante *et al.*, 2001). Previous studies have shown that this amino acid in mammalian tissues regulates several biological processes, including osmolarity (Schaffer *et al.*, 2000), Ca^{2+} binding and transport (Satoh, 1998; Palmi *et al.*, 1999; Foos and Wu, 2002; Lee *et al.*, 2004; Park *et al.*, 2004) and ion channel activity (Lim *et al.*, 2004; Park *et al.*, 2004). TAU also prevents tissue injury by oxidative stress (Huxtable, 1992; Tas *et al.*, 2007), maintains the structural integrity of the plasma membrane (Moran *et al.*, 1987), and demonstrates hypoglycemic properties (Kulakowski and Maturo, 1984; Maturo and Kulakowski, 1988; Kaplan *et al.*, 2004).

A relationship between low concentrations of TAU in the plasma and the prediabetic and diabetic states has also been suggested (Cherif *et al.*, 1998; Nandhini *et al.*, 2005; Tsuboyama-Kasaoka *et al.*, 2006). Thus, restoring the normal plasma TAU levels by supplementation may help in the prevention of diabetes mellitus (Arany *et al.*, 2004; Tsuboyama-Kasaoka *et al.*, 2006). In this regard, the maintenance of physiological plasma TAU concentrations seems to be an important factor for adequate β-cell function and insulin action (Boujendar *et al.*, 2002; Arany *et al.*, 2004; Nandhini *et al.*, 2005; Kaniuk *et al.*, 2007; Tas *et al.*, 2007; Xiao *et al.*, 2008). However, the physiological role of this amino acid in the endocrine pancreas has not yet been completely

elucidated. Conflicting results concerning the effects of TAU on isolated islets have been found in the literature. It has been shown that TAU does not affect the first phase insulin secretion induced by high concentrations of glucose in isolated rat islets, but instead reduces the rate of insulin secretion during the second phase (Bustamante *et al.*, 2001). In contrast, TAU plus physiological concentrations of glucose significantly stimulated insulin secretion (Cherif *et al.*, 1996; Cherif *et al.*, 1998). The ability of TAU to ameliorate insulin secretion, stimulated by glucose and other fuels in fetal and adult isolated islets, was also observed when the plasma TAU of rodents was increased or at least restored by TAU supplementation (Cherif *et al.*, 1996; Cherif *et al.*, 1998; Carneiro *et al.*, 2008).

In this study, we confirm previous observations that TAU supplementation improves glucose tolerance and insulin sensitivity in mice (Carneiro *et al.*, 2008). In addition, we observed that TAU supplementation improves the islet hormone secretion, represented by higher insulin release stimulated by glucose or L-leucine and higher glucagon at low glucose levels. These islets also metabolized more L-leucine. Finally TAU-supplemented islets accumulated more Ca^{2+} in the presence of high glucose concentrations and showed an increased expression of the L-type β_2 subunit Ca^{2+} channel.

Materials and Methods

Materials

D-[U-^{14}C]glucose, L-[U-^{14}C]leucine and $^{45}CaCl_2$ were purchased from Amersham International (Little Chalfont, Bucks, UK). Routine reagents, L-leucine, L-glutamine, 2-aminobicyclo[2,2,1]-heptane-2-carboxylic acid (BCH), aminooxyacetic acid (AOA) and ATP were purchased from Sigma Chemical (St Louis, MO, USA).

Animals

All experiments were approved by the ethics committee at UNICAMP. Three-week-old Swiss mice were obtained from the colony at UNICAMP. The mice were maintained on a 12 hour light/dark cycle (lights on 06:00-18:00 h), controlled temperature (22 ± 1ºC), and allowed free access to water and standard laboratory chow (Rodent chow; Nutrilab, Colombo, Brazil) *ad libitum*. At 60 days, mice were distributed into two groups: mice that received 2% of TAU in their drinking water for 30 days (TAU group) and those that received only water (CTL group).

General nutritional parameters

Body weight, food and water intake were measured during the experimental period (30 days) and expressed by the area under the curve (AUC) of total body weight and food and water intake, respectively, during the 30 days of the treatment. At the end of the supplementation period, fasted and fed mice were decapitated, their blood collected and plasma was stored at -20 °C. Total plasma protein and plasma albumin were measured using standard commercial kits, according to the manufacturer's instructions (Laborlab, Guarulhos, SP, Brazil). Plasma glucose was measured using a glucose analyzer (Accu-Chek Advantage, Roche Diagnostic, Switzerland), insulin was measured by RIA, and glucagon plasma concentration was quantified by commercial kit (Linco Research, St. Charles, MO, USA).

TAU plasma levels

To measure TAU plasma concentrations in fed and fasted conditions, 200 µl of plasma were deproteinized by adding 200 µl of 25% trichloroacetic acid solution and were then centrifuged at 21,000 g for 10 min. Supernatant (100 µl) was collected and mixed with sample loading buffer (100 µl) (Biochrom 20 reagent kit, Cambridge, UK). An aliquot of 25 µl of the mixture was then resolved

by liquid chromatography on a Biochrom 20 plus amino acid analyzer (Amersham Pharmacia, Piscataway, NJ, USA). Amino acid standards were analyzed first, followed by the samples. Amino acids were quantified using Biochrom 20 control software, version 3.05.

Intraperitoneal glucose (ipGTT) and insulin tolerance test (ipITT)

For ipGTT, blood glucose levels (time 0) were measured (as previously described) in overnight fasted mice. A glucose load of 2 g/kg body weight was then administered by ip injection and additional blood samples were collected at 15, 30, 60, 120 and 180 min. For ipITT, fed mice were injected with 0.75 U/Kg body weight of human insulin (Biohulin ®R, Biobrás, Brazil). Blood samples were collected before insulin injection and at the times 15, 30, 60 and 90 min for glucose analysis. The glucose disappearance rate (K_{itt}) was calculated using the formula, $0.693/t_{1/2}$. Glucose $t_{1/2}$ was calculated from the slope of the least-squares analysis of plasma glucose concentrations during the linear decay phase (Bonora *et al.*, 1987).

Islet isolation, static insulin and glucagon secretion

Islets were isolated by collagenase digestion of the pancreas, as described (Bordin et al., 1995). For static incubations, groups of four islets were first incubated for 30 min at 37 ºC in Krebs-Ringer bicarbonate (KRB) buffer with 2.8 mmol/l glucose and 3 g of BSA/l, and equilibrated with a mixture of 95% O2/5% CO2 to give pH 7.4. This medium was then replaced with fresh buffer and the islets incubated for 1 h under the following conditions: glucose 2.8 mmol/l alone or with L-leucine (10 mmol/l), BCH (10 mmol/l; a non-metabolized analog of L-leucine), L-glutamine (10 mmol/l), AOA (10 mmol/l), and ATP (10 μmol/l); and glucose (22.2 mmol/l) with or without glucagon (43 pmol/l). At the end of the incubation period, the insulin content of the medium was measured by RIA. For glucagon analyses, groups of 15 islets were incubated for 30 min in 250 μl of

KRB buffer with 5.6 mmol/l glucose. This medium was then replaced with 250 µl of fresh buffer and the islets were further incubated with 0.5 mmol/l glucose for 1 h. Glucagon release was measured as previously described and results were expressed as pg/15 islets.h (Hoy et al., 2001).

Dynamic insulin secretion studies

Groups of 50 freshly isolated islets were placed on Millipore SW 1300 filters (8.0 µm pore) and perifused in a KRB buffer at a flow rate of 1ml/min for 30 min in the presence of 2.8 mmol/l glucose (basal conditions). After this period, the islets were perifused with 22.2 mmol/l of glucose, or 10 mmol/l of L-leucine, or 10mmol/l of L-leucine and 10 mmol/l of L-glutamine, as indicated in the figure legends and Results section. Perifusion solutions were gassed with 95% O_2/5% CO_2 and maintained at 37 ºC. Insulin release was measured by RIA.

Islet insulin, total protein and DNA content

Groups of 4 islets were collected and transferred to tubes of 1.5 ml. Deionized water (1 ml) was added to the samples, followed by sonication of the pancreatic cells (3 times, 10 sec pulses), and the islet insulin content was measured by RIA. For protein quantification, groups of 15 islets were transferred to tubes of 0.6 ml, 25 µl of NaOH 1 mol/l were added to the tubes, and the samples were stored at -20 ^{o}C. On the day of the protein assay, 25 µl of HCl 1N were added to the samples, and islet total protein was quantified by the Bradford method (Bradford, 1976). DNA was measured using fluorimetric probes and a standard curve of known DNA concentrations.

ATP and ADP levels

To determine ATP and ADP concentrations, groups of 150 islets were preincubated for 30 min at 37 ºC in a KRB solution with 2.8 mmol/l glucose, the solution was then replaced by fresh buffer

containing 22.2 mmol/l glucose (gassed with a mixture of 95% O_2/5% CO_2) and incubated for 15 min. At the end of the incubation period, the supernatant was discarded and the islets were frozen in liquid nitrogen. Before chromatographic analysis, islets were resuspended in 1 ml of a solution containing 50 mmol/l KH_2PO_4 and 25 mmol/l citric acid (pH 4.5). The mixture was kept in a water bath (85ºC) for 2 min and the pellet of islets was then lysed by mechanical stress and filtered through a 0.45 μm Millex filter (Millipore, Milford, MA, USA). Subsequently, 200 μl of filtrate were mixed with 20 μl of 2-chloroacetaldehyde solution and heated at 80°C for 20 min (Kawamoto *et al.*, 1998; Katayama *et al.*, 2001). An aliquot of 25 μl of the reaction mixture was then resolved by liquid chromatography.

Chromatography: Chromatographic analyses were carried out on a Waters Alliance equipment series 2695 (Milford, MA, USA) equipped with a quaternary pump, a sampler manager, a degasser, and a Waters 2475 fluorescence detector model. The fluorescence of derivative compounds (ATP, ADP) was monitored with excitation and emission wavelengths set at 280 and 420 nm, respectively. Chromatographic separations of the compounds were achieved at room temperature, using a reversed-phase Cosmosil 5C18-MS column (150 x 4.6 mm id.; 5 μm particle size) with a Cosmosil guard column (5C18-MS 10 x 4.6 mm) purchased from Phenomenex (Torrance, CA, USA). The column was equilibrated and eluted under gradient conditions using a flow rate of 1 ml/min. The standards and samples were separated using a gradient mobile phase consisting of methanol (A) and a solution of 50 mmol/l KH_2PO_4 and 25 mmol/l citric acid (pH 4.5) (B), which was prepared immediately before use and filtered through a 0.45 μm filter (Millipore, Milford, MA, USA). The gradient conditions were: 0–4 min, 2% A; 4–12 min linear gradient, 2–15% A; 17-18 min reconditioning step of column was 2% A isocratic for 2 min. The chromatographic run time for each analysis was 20 min. Aliquots of 25 μl were injected into the HPLC system. System control, data acquisition, and processing were performed with a PC-Pentium IV Processor personal computer

from Dell, operated with Microsoft Windows XP version 2003 and Waters Empower 2002 chromatography software.

Glucose and L-leucine oxidation

Glucose and L-leucine oxidation were measured by the rate of formation of $^{14}CO_2$ from D-[U-^{14}C]glucose (20 μCi/ml) or L-[U-^{14}C]leucine (8 μCi/ml) by the islets. Groups of 25 islets were incubated for 2 h at 37 ºC in 30 μl of KRB supplemented with 22.2 mmol/l of glucose or 10 mmol/l of L-leucine. After incubation, metabolism was stopped with HCl for cell cleavage. $^{14}CO_2$ liberated was absorbed by NaOH and the $NaH^{14}CO_3$ obtained was measured by liquid scintillation in a β radiation counter.

Uptake of 45Ca by isolated islets

Groups of 150 to 200 islets, derived from the same batch of islets, were preincubated for 30 min at 37 °C in a KRB buffer containing 2.8 mmol/l of glucose, pH 7.4. The islets were then incubated for 5 min and 1h in 200 μl of the same medium containing $^{45}CaCl_2$ (60 μCi/ml) and 22.2 mmol/l glucose. At the end of the incubation period, 800 μl of ice-cold medium containing 2 mmol/l of $LaCl_3$ (pH 7.4) was added to stop the reaction. The medium was then removed and an aliquot was saved to determine the amount of ^{45}Ca in the solution. The islets were subsequently washed three times with fresh ice-cold La^{3+}-containing medium and islets were then placed in a Petri dish and groups of ten islets were transferred to counting vials containing 1 ml of EGTA 0.5 mmol/l. The uptake of ^{45}Ca was expressed as pmol Ca^{2+} per islet per time of the incubation.

Western blotting

Pools of islets were transferred to tubes containing protease inhibitor buffer (Amaral *et al.*, 2003). Samples were sonicated (3 times, with 10s pulses) and the protein was determined by the Bradford method using BSA as the standard. For SDS gel electrophoresis and Western blot analysis, the samples were homogenized with a loading buffer containing DTT. After heating to 95 °C for 5 min, the proteins were separated by electrophoresis (55 μg protein/lane, 10% gels). Following electrophoresis, proteins were transferred to nitrocellulose membranes. The membranes were subsequently blotted with specific polyclonal antibodies to the β_2 subunit of the voltage sensitive Ca^{2+} channel (1:1,000; Sigma Chemicals, St Louis, MO, USA), GLUT 2 (1:1,000; Calbiochem, San Diego, CA, USA) or GCK (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Visualization of specific protein bands was performed by incubating the membranes with goat anti-rabbit secondary antibody (1:10,000; Zymed Laboratories, Inc., San Francisco, CA, USA) and detecting with 1 ml enhanced chemiluminescence reagents (Pierce Biotechnology, USA) followed by exposure to X-ray film (Kodak, AM, Brazil). The band intensities were quantified by optical densitometry (Scion, Image, Frederick, MD, USA). After assay of the target proteins, Western blotting was repeated using β-actin (1:10,000, Abcam, Cambridge, MA, USA) antibody as an internal control.

Statistical analysis

The results are presented as means ± SEM for the number of determinations (n) indicated. The statistical analyses were performed using unpaired Student's t-test, and the level of significance was set at $P < 0.05$.

Results

Mice features

Food and water intakes were similar between the two groups during the period of TAU supplementation (data not shown). At the end of this period, body size and weight, as well as various organ weights (liver, spleen, heart, kidneys, and retroperitoneal and periepididimal fats) were not different between TAU-supplemented and CTL mice (data not shown). Plasma parameters, such as fasted and fed insulin, fasting glucose, albumin and total protein were also similar between the two groups (Table 1). Glucagon plasma levels from fasted, but not fed, TAU-supplemented mice were 35% higher than CTL ($P < 0.005$) (Table 1). Table 1 shows that the supplementation methodology applied in our study efficiently increased plasma TAU concentrations, since TAU plasma levels in fasted and fed supplemented mice were approximately 3 times higher, compared with CTL mice ($P < 0.05$).

Glucose homeostasis

At the end of the experimental period, CTL and TAU-supplemented mice were submitted to an ipGTT and ipITT. After glucose load, plasma glucose concentration reached maximal levels at 30 min in both groups. However, the TAU-supplemented mice showed lower glucose values at 15, 30, 60 and 120 min, compared with CTL mice (Figure 1 (A)). The area under the curve (AUC) of the glucose plasma levels during the ipGTT in TAU was 37% lower than the CTL group ($P < 0.01$). After insulin administration (ipITT), plasma glucose showed a constant decrease between min 15 to min 60 in both mice groups (Figure 1 (B)). The glucose decrease was significantly higher in TAU-supplemented than in CTL mice with a K_{itt} of 1.9 ± 0.4 and of 0.4 ± 0.2 %/min for TAU-supplemented and CTL mice, respectively ($P < 0.02$).

Insulin and Glucagon secretion

Figure 2 (A) shows that the increase in glucose concentrations from 2.8 to 22.2 mmol/l induced a biphasic insulin secretion in both types of islets (min 25 to 90). This biphasic secretion is represented by a first prominent phase, followed by a period where the secretion was lower than the first phase, but still higher than the adaptation period (min 0 to 25). During the stimulatory period with 22.2 mmol/l glucose, the insulin secretion was significantly higher in TAU-supplemented than CTL islets. The AUC for insulin released during min 25 to 90 of perifusion was 319 ± 24 and 125 ± 11 ng/50 islets.min^{-1}, respectively ($P < 0.0001$). When dynamic insulin release induced by 10 mmol/l of L-leucine was analyzed (Figure 2 (B)), a similar biphasic secretion was observed in both types of islets. Again, the insulin secretion was significantly higher in islets from TAU-supplemented compared with CTL mice. The sum of insulin released (AUC) during the stimulatory period with L-leucine was 129 ± 20 and 44 ± 7 ng/50 islets.min^{-1}, respectively ($P < 0.008$).

L-leucine is known to stimulate insulin secretion by two mechanisms; firstly, by L-leucine catabolism and secondly by allosteric activation of glutamate dehydrogenase (GDH), resulting in an increase of the catabolism of L-glutamate synthesized from L-glutamine (Sener and Malaisse, 1980). As such, to discriminate whether the higher L-leucine-induced insulin secretion in TAU-supplemented islets was due to a possible alteration in GDH activity and/or L-leucine metabolism, dynamic and static insulin release was analyzed in the presence of GDH activators and L-leucine metabolism inhibitors.

The L-leucine-induced dynamic insulin secretion in TAU and CTL islets was similar when the perifusion medium contained 10 mmol/l L-glutamine (Figure 2 (C)). In static experimental conditions, increased insulin secretion in response to L-leucine in TAU islets was confirmed. Figure 3 shows that, at 2.8 mmol/l glucose, the increment in the insulin secretion induced by 10 mmol/l L-leucine was significantly higher in TAU than CTL islets ($P < 0.03$). Although significantly higher than basal conditions (2.8 mmol/l glucose alone), the insulin secretion induced by 10 mmol/l BCH

(a stimulator of GDH), or the combination of BCH and 10 mmol/l L-glutamine (a substrate for GDH), was similar in both types of islets. The L-leucine induced secretion was also similar in both groups when the islets were incubated in the presence of BCH and L-glutamine. Finally, the addition of AOA (a transaminase inhibitor) significantly reduced the insulin secretion to similar levels to those obtained when only GDH activity was present.

Glucagon release in response to 0.5 mmol/l glucose was higher in islets from TAU-supplemented mice, compared with CTL islets (491 ± 16; 375 ± 34 pg/15 islets.h, respectively; $P < 0.01$). To verify the stimulatory effect of glucagon on insulin secretion, islets were incubated in the presence of 22.2 mmol/l glucose and 43 pmol/l glucagon (a concentration close to that observed in the plasma of fasted mice). Glucagon-induced insulin secretion was 43% higher in TAU-supplemented than CTL islets (16.7 ± 1.6 vs 11.6 ± 1.7 ng/islet.h, respectively; $P < 0.05$).

D-[U-^{14}C]glucose and L-[U-^{14}C]leucine oxidation, ATP and ADP islet levels, GLUT 2 and GCK protein expression

D-[U-^{14}C]glucose oxidation in response to 22.2 mmol/l glucose was similar between groups (35.3 ± 4.1; 40.2 ± 5.9 pmol/islet/2h^{-1}, for TAU and CTL, respectively), whereas L-[U-^{14}C]leucine oxidation in the presence of 10 mmol/l of L-leucine was 28% higher in TAU compared with CTL islets (22.9 ± 1.6 and 17.8 ± 0.8 pmol/islet/2h^{-1}, respectively; $P < 0.008$). The ATP concentration and the ATP-to-ADP ratio in the presence of 22.2 mmol/l of glucose were similar in TAU and CTL islets after 15 min of incubation (Table 2). In addition, the GLUT 2 and GCK protein expressions were similar in the TAU and CTL groups (Figure 4 (A) and (B)).

Islet insulin and total protein content

Total insulin content was 48% higher in TAU compared to CTL islets (130 ± 9; 87 ± 10 ng/islet, respectively; $P < 0.005$). Protein and DNA content did not differ between TAU and CTL islets (0.28 ± 0.01 and 1.14 ± 0.1 vs 0.25 ± 0.01 μg/islet and 1.02 ± 0.07 ng/islet, respectively), indicating that TAU supplementation did not alter islet cell mass.

^{45}Ca uptake and Ca^{2+} channel protein expression

^{45}Ca uptake by TAU-supplemented and CTL islets at 22.2 mmol/l glucose after 5 min incubation was similar (1.54 ± 0.21 vs 1.32 ± 0.12 pmol ^{45}Ca/islet.5 min, n = 37 - 33; respectively). However, after 1 h of incubation (representing the steady state), ^{45}Ca uptake by TAU islets was significantly higher than CTL islets (Figure 5, $P < 0.009$). Western blotting analysis revealed that the expression of the β_2 subunit of the L-type voltage sensitive Ca^{2+} channel was significantly higher in TAU-supplemented, compared with CTL islets ($P < 0.04$) (Figure 4 (C)). β-actin protein expression, measured in parallel, did not differ.

Discussion

The current work highlights the importance of TAU supplementation for pancreatic islet functionality. In agreement with other experimental models (Anuradha and Balakrishnan, 1999; Nakaya *et al.*, 2000; Tsuboyama-Kasaoka *et al.*, 2006; Loizzo *et al.*, 2007; Carneiro *et al.*, 2008), our study also demonstrates that TAU supplementation enhances glucose tolerance and insulin sensitivity in vivo.

Hypoglycemic properties of TAU have been suggested to be mediated by the interaction of TAU with the insulin receptor (Maturo e Kulakowski, 1988). Since neonatal TAU-treated mice demonstrate lower basal glucose plasma levels and increased glucose tolerance, it has been suggested that TAU programs glucose metabolism (Loizzo *et al.*, 2007). It has also been reported

that this amino acid amplifies insulin action and, in insulin-resistant rodents, TAU supplementation normalizes plasma insulin and glucose levels, insulin sensitivity, blood pressure, and improves hypercholesterolemia and energy expenditure in obesity (Anuradha and Balakrishnan, 1999; Nakaya *et al.*, 2000; Nandhini and Anuradha, 2002; Nandhini *et al.*, 2005; Tsuboyama-Kasaoka *et al.*, 2006).

TAU supplementation in our study increased fasting plasma glucagon levels, a hormone released during hypoglycemia that induces hepatic glucose output (Barg, 2003; Gromada *et al.*, 2007). Thus, by enhancing glucagon levels, TAU exerts a possible dual effect on glucose homeostasis; firstly in the fed state, by increasing glucose uptake, utilization, and/or storage, and secondly under fasting conditions, where it increases hepatic glucose mobilization, avoiding undesirable hypoglycemia.

High glucose tolerance in supplemented mice may account for a possible alteration in glucose-induced insulin secretion by pancreatic islets. Despite the fact that TAU supplementation did not alter insulin plasma levels, insulin secretion analyzed in vitro showed that supplementation improved β-cell sensitivity to fuel secretagogues.

In dynamic studies, the islets from TAU-supplemented mice showed an increase in the first phase and in the total insulin secretion in response to glucose (Figure 2). These alterations are not due to modifications in GLUT 2 and GCK expression, glucose oxidation and ATP content, nor the ATP-to-ADP ratio; however, they may be due to high ^{45}Ca uptake after 1 h of incubation in the presence of glucose. This enhanced Ca^{2+} influx probably results from the high Ca^{2+} voltage-dependent channel expression in the plasma membrane of the TAU islets.

Glucose-stimulated biphasic insulin secretion depends, at least, on two signaling pathways: the ATP-sensitive K^+ (K_{ATP}) channel-dependent pathway that involves glucose metabolism and an increase in $[Ca^{2+}]i$ that results in the first phase of insulin secretion, and the K_{ATP} channel-

independent mechanisms that work synergistically with the former and involve an increase in $[Ca^{2+}]i$ together with intracellular signals originating from glucose metabolism, culminating with second-phase release (Straub e Sharp, 2002). The glucose-induced increase in $[Ca^{2+}]i$ in pancreatic β-cells is usually manifested as slow oscillations that, in turn, provoke oscillations in insulin plasma levels, which are important for the action of insulin in peripheral tissues (Ahmed *et al.*, 1999; Tengholm and Gylfe, 2008). Thus, we suggest that increased insulin secretion and the high oscillatory insulin release profile in the TAU group (Figure 2) could be due to a better handling of Ca^{2+}, in combination with elevated insulin content in these islets.

The relationship between TAU and Ca^{2+} regulation has been documented. In many tissues, TAU markedly affects the kinetics of Ca^{2+} movement across both cellular and subcellular membranes. In cardiac cells, TAU is an agonist of the Ca^{2+} current and delayed rectified K^+ current at low $[Ca^{2+}]i$, whereas at high $[Ca^{2+}]i$, TAU inhibits both types of currents resulting in the prolongation of the duration of the action potential (Satoh, 1998). In isolated rat liver mitochondria, TAU enhanced Ca^{2+} uptake via the uniport system (Palmi *et al.*, 1999). In rat pancreatic β-cells, TAU increased insulin secretion and Ca^{2+} oscillation via inhibition of the K_{ATP} channels due to its interaction with a benzamido-binding site on SUR1 (Park *et al.*, 2004). TAU also restores impaired glucose-induced insulin secretion in uncoupling protein 2-overexpressing β-cells by inducing Ca^{2+} sequestration into the mitochondrial matrix (Lee *et al.*, 2004). Our work is the first to show that this amino acid increases the expression of the β_2 subunit of the Ca^{2+} voltage-dependent channels, in parallel with an increase in Ca^{2+} handling by the islets, in accordance with recent observations (Carneiro *et al.*, 2008).

Furthermore, these data also provide new information regarding TAU effects upon islet hormone secretion. We observed that, at low glucose concentration, TAU-supplemented islets secreted more glucagon than CTL. It is known that the control of glucagon secretion is multifactorial

and involves direct effects of nutrients, hormones and neurotransmitters. In alpha(α)-cells the closure of K_{ATP}-channels, due to an increased ATP-to-ADP ratio, leads to membrane depolarization and activation of voltage-dependent Na^+ and *N*-type Ca^{2+} channels that contribute to the generation of action potentials, increased $[Ca^{2+}]i$, and finally glucagon secretion (Gromada *et al.*, 2007). In addition to the glucagon action in hepatic glucose output, a possible paracrine effect of this hormone in β-cell increasing insulin release is suggested (Kawai *et al.*, 1995; Cabrera *et al.*, 2006). This hypothesis is supported by studies that demonstrate the presence of the glucagon receptor in β-cells (Moens *et al.*, 1996; Moens *et al.*, 1998), and a decreased glucose-stimulated insulin secretion in human islets in the presence of a glucagon receptor antagonist (Huypens *et al.*, 2000). Thus, the observations that TAU-supplemented islets release more glucagon at low glucose and that these islets secreted more insulin when incubated at high glucose in the presence of glucagon suggests that TAU supplementation improves whole endocrine hormone sensing to nutrients and possible glucagon paracrine effects on insulin secretion.

In β-cells, glucagon acts via a G protein-coupled receptor, increasing intracellular cAMP levels that culminate with protein kinase A (PKA) activation (Moens *et al.*, 1996). PKA contributes to enhance insulin secretion activating the exocytotic machinery and Ca^{2+} flux (Dyachok and Gylfe, 2004; Wan *et al.*, 2004; Hatakeyama *et al.*, 2006). It is known that PKA phosphorylates Ca^{2+} channel subunits and may increase open channel probability (Gao *et al.*, 1997; Felix, 2005). As such, a combination between increased glucagon paracrine action in the β-cell, PKA activation, and increased Ca^{2+} channel protein expression may contribute to enhance Ca^{2+} uptake and insulin secretion in the presence of high glucose in TAU-supplemented islets.

L-leucine is the most potent amino acid secretagogue of insulin and this is due to its ability to activate GDH, associated with the generation of ATP by its own metabolism (Sener and Malaisse,

1980). We observed that TAU islets secreted more insulin than CTL when stimulated by L-leucine, and that this alteration is probably due to high L-leucine catabolism without alterations in GDH activity in the TAU group. This hypothesis is supported by the fact that insulin secretion was not different between groups when carried out in the presence of a non-metabolizable analog of L-leucine, BCH (Liu *et al.*, 2003), or with the combination of BCH with L-glutamine. This is also supported by static and dynamic studies when islets were perifused or incubated with L-glutamine plus L-leucine with or without BCH, a condition in which L-leucine-induced insulin release occurs predominantly via GDH alosteric activity, since L-glutamine decreases L-leucine oxidation (Macdonald *et al.*, 2008). These results together with the similar insulin release between groups in the presence of the stimulus, BCH/L-glutamine/L-leucine plus AOA, when L-leucine metabolism was abolished, indicate that TAU supplementation may enhance L-leucine catabolism in islets. Thus, TAU supplementation modifies L-leucine-induced insulin secretion by increasing substrate flux to feed the Krebs cycle, in turn, enhancing L-leucine degradation in islet cells.

In conclusion, we demonstrate that increasing plasma TAU levels by supplementation modifies β-cell sensitivity to glucose and L-leucine. In glucose-induced insulin secretion, TAU supplementation may alter Ca^{2+} handling. However, the increase in the islet catabolism of L-leucine amino acid may be the main alteration provoked by TAU regarding L-leucine-induced release. In parallel, glucagon release was increased in TAU-supplemented islets and may also account for the increased insulin secretion from β-cells. These data support the presumable role of TAU in improving β-cell function in type II diabetes and pre-diabetic states that present a decreased ability to release insulin in response to glucose and L-leucine.

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Table 1. Plasma glucose, insulin, glucagon, TAU, albumin and total protein concentrations in fasted and fed CTL and TAU-supplemented mice.

		CTL	**TAU**
Glucose (mg/dl)	*Fasted*	83.8 ± 6.5	86.6 ± 3.6
	Fed	124.1 ± 4.3	125.3 ± 4.3
Insulin (ng/ml)	*Fasted*	0.34 ± 0.07	0.31 ± 0.05
	Fed	3.0 ± 0.4	3.0 ± 0.5
Glucagon (pg/ml)	*Fasted*	68.6 ± 7.4	105.3 ± 4.3*
	Fed	95.5 ± 8.3	107.1 ± 10.5
TAU (μmol/ml)	*Fasted*	2.7 ± 0.4	8.4 ± 0.8*
	Fed	3.5 ± 0.5	9.6 ± 2.0*
Albumin (g/dl)		2.4 ± 0.1	2.5 ± 0.1
Total proteins (g/dl)		4.9 ± 0.2	4.8 ± 0.1

Data are means ± SEM (n=3-12). * P <0.05 *vs* CTL.

Table 2. ATP, ADP concentrations and ATP-to-ADP ratio in islets from CTL and TAU-supplemented mice. Islets were incubated with 22.2 mmol/l glucose for 15 min.

	CTL	**TAU**
ATP (pmol/islet)	1.40 ± 0.17	1.54 ± 0.16
ADP (pmol/islet)	2.22 ± 0.17	2.77 ± 0.25
ATP-to-ADP ratio	0.59 ± 0.04	0.58 ± 0.02

Data are means ± SEM of 10 values from three independents experiments.

Figure Legends

Figure 1. *Effect of TAU supplementation on glucose tolerance and insulin sensitivity*.

Figures **A** and **B** represent changes in plasma glucose levels during ipGTT and ipITT, respectively. Data are means ± SEM obtained from 8 mice. * $P < 0.05$ indicates significant difference vs CTL.

Figure 2. Dynamic insulin release induced by 22.2 mmol glucose (G) **(A);** 2.8 mmol/l glucose and 10 mmol/l L-leucine **(B);** and by 2.8 mmol/l glucose, 10 mmol/l L-leucine and 10 mmol/l L-glutamine **(C)** in islets from TAU-supplemented and CTL mice. Data are means ± SEM obtained from 4 independent experiments. * $P < 0.05$ indicates significant difference vs CTL.

Figure 3. *Comparative effects of the GDH activity and L-leucine metabolism on insulin secretion in response to L-leucine*.

Islets were incubated with 2.8 mmol/l glucose (G) and/or stimulated with a combination of L-leucine, BCH, L-glutamine and AOA (10 mmol/l for all stimuli). Data are means ± SEM (n = 14 for each group). * $P < 0.05$ indicates significant difference vs CTL.

Figure 4. **(A)** GLUT 2, **(B)** GCK, **(C)** β_2 subunit of voltage-dependent Ca^{2+} channel ($Ca_{v\beta2}$) and β-actin protein expressions. * $P < 0.05$ indicates significant difference vs CTL.

Figure 5. Islet ^{45}Ca uptake after 1 h of incubation in the presence of 22.2 mmol/l glucose. Data are means ± SEM of 25 - 21 observations for TAU-supplemented and CTL islets, respectively. * $P < 0.05$, significant difference vs CTL.

Figure 1

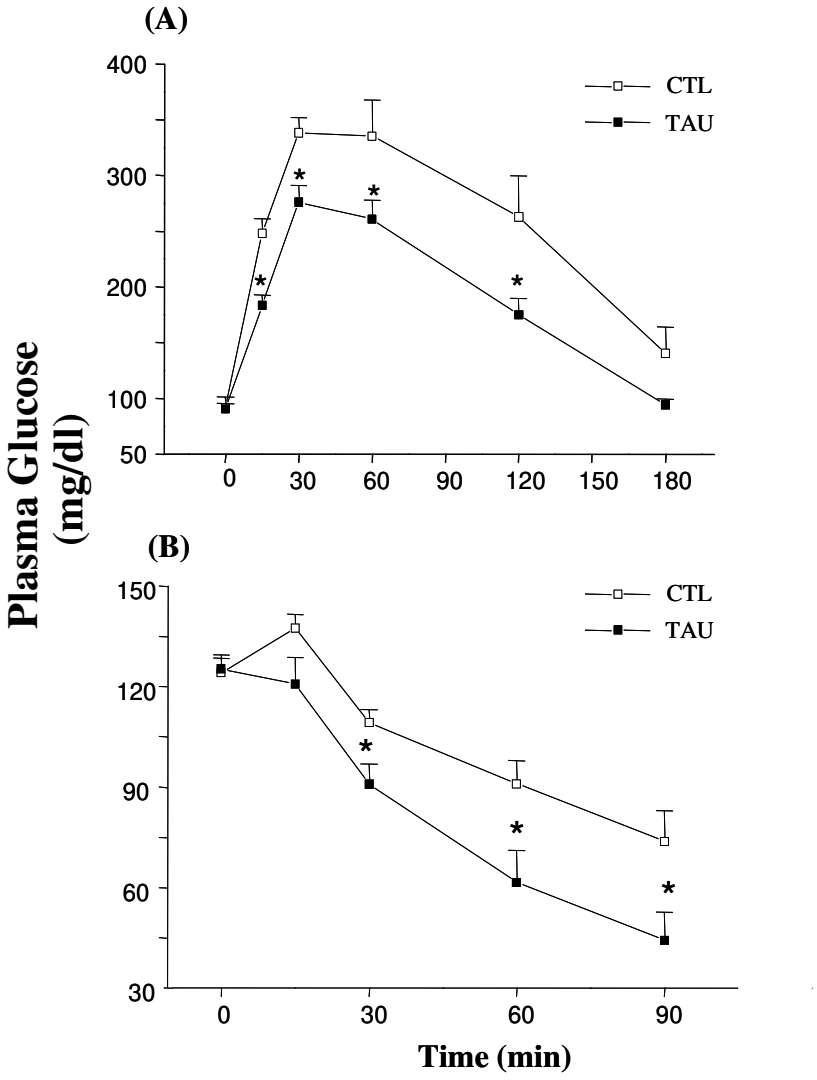

(A)
400
300
200
100
50
CTL
TAU
*
*
*
*
0
30
60
90
120
150
180
(B)
150
120
90
60
30
CTL
TAU
*
*
*
0
30
60
90
Plasma Glucose (mg/dl)
Time (min)

Figure 2

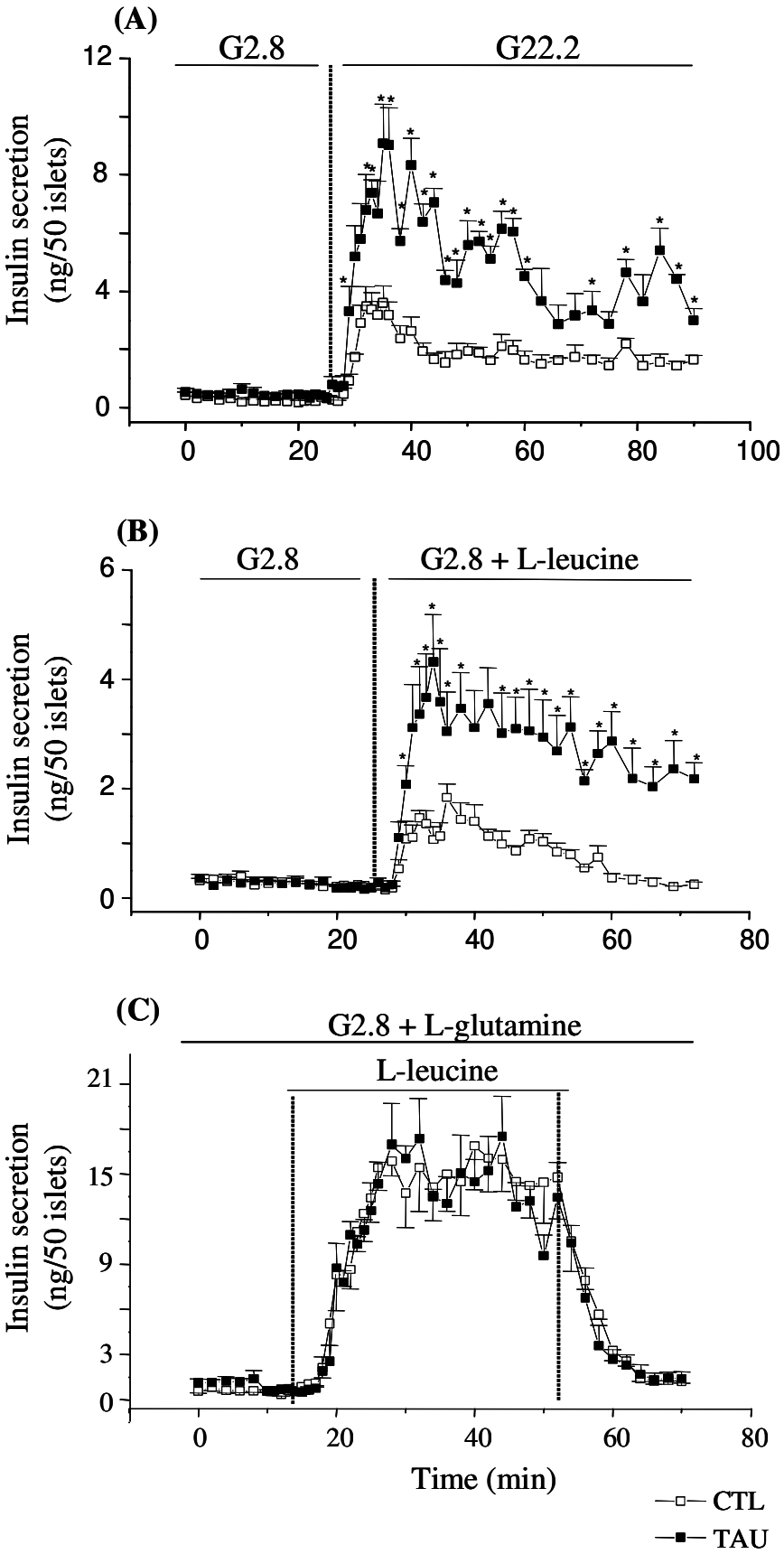

(A)
G2.8
G22.2
Insulin secretion (ng/50 islets)
12
8
4
0
0
20
40
60
80
100
(B)
G2.8
G2.8 + L-leucine
Insulin secretion (ng/50 islets)
6
4
2
0
0
20
40
60
80
(C)
G2.8 + L-glutamine
L-leucine
Insulin secretion (ng/50 islets)
21
15
9
3
0
0
20
40
60
80
Time (min)
CTL
TAU

Figure 3

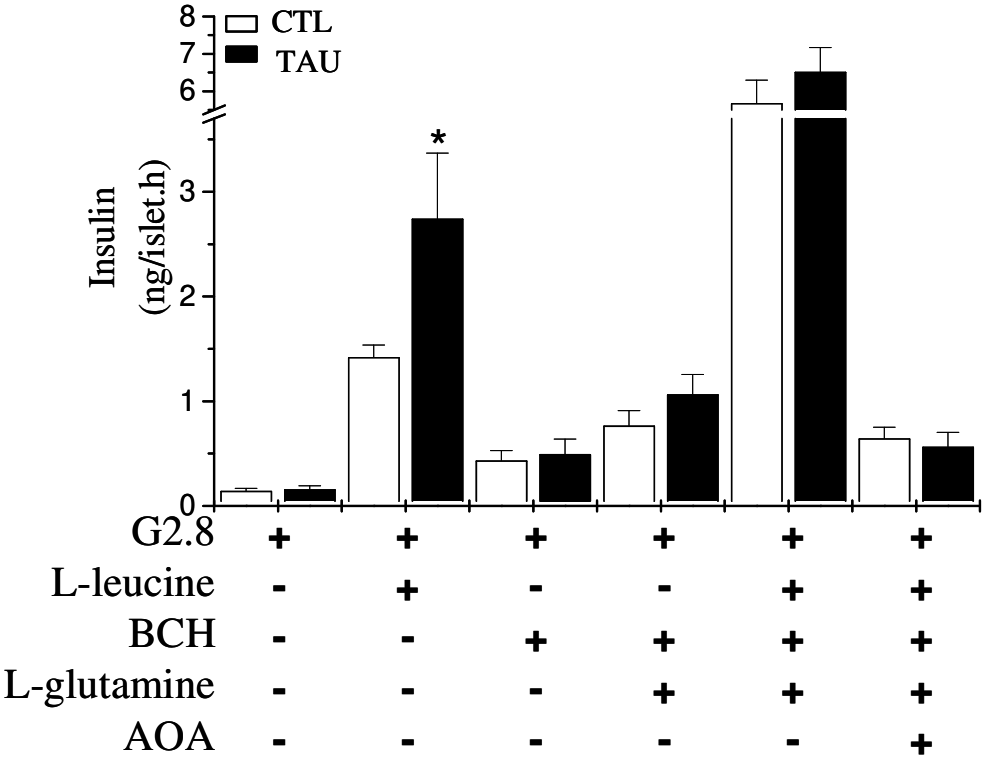

CTL
TAU
Insulin (ng/islet.h)
8
7
6
3
2
1
0
*
G2.8 + + + + + +
L-leucine - + - - + +
BCH - - + + + +
L-glutamine - - - + + +
AOA - - - - - +

Figure 4

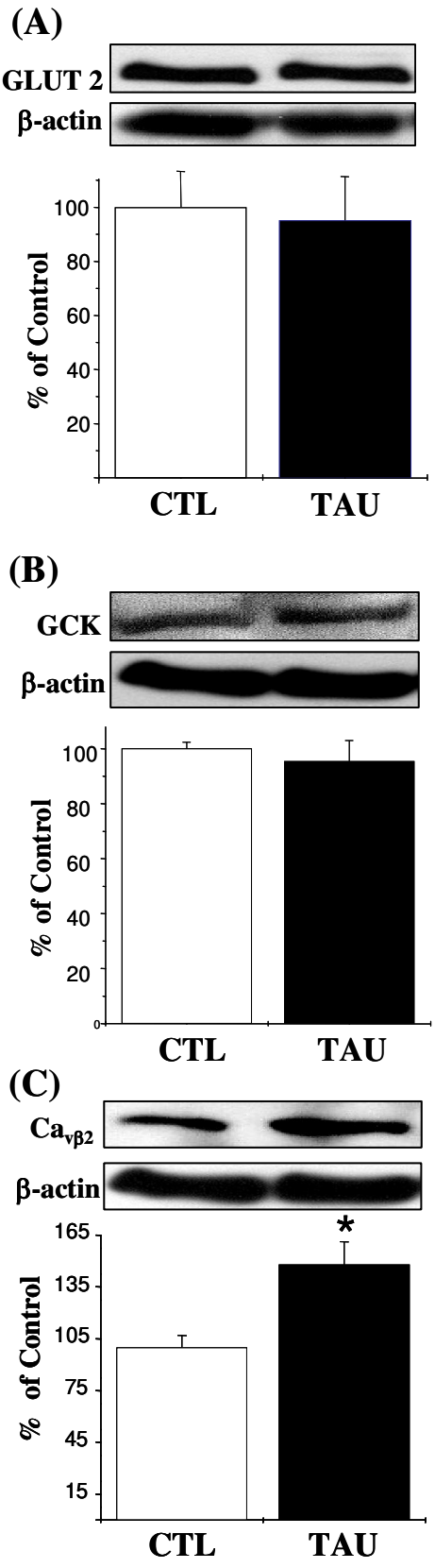
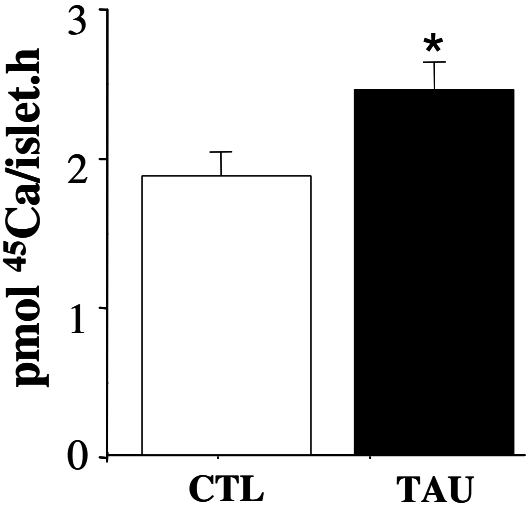

(A)
GLUT 2
β-actin
% of Control
100
80
60
40
20
CTL
TAU
(B)
GCK
β-actin
% of Control
100
80
60
40
20
0
CTL
TAU
(C)
Cavβ2
β-actin
*
% of Control
165
135
105
75
45
15
CTL
TAU

Figure 5



ARTIGO 2

TAURINE SUPPLEMENTATION: INVOLVEMENT OF CHOLINERGIC/PHOSPHOLIPASE C AND PKA PATHWAYS IN POTENTIATION OF INSULIN SECRETION AND Ca^{2+} HANDLING

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Keywords: Ca^{2+} handling; insulin secretion; PKA; PLC; taurine supplementation

Abstract

Taurine (TAU) supplementation increases insulin secretion in response to high glucose concentrations in rodent islets. This effect is probably due to an increase in Ca^{2+} handling by the islet-cells. Here, we investigated the possible involvement of the cholinergic/Phospholipase (PL) C and protein kinase (PK) A pathways in this process. Adult mice were fed with 2% TAU in drinking water for 30 days. After, the mice were sacrificed and pancreatic islets isolated by the collagenase method. TAU-supplemented islets showed higher insulin secretion in the presence of 8.3 mmol/l glucose, 100 μmol/l carbachol (Cch), and 1 mmol/l IBMX. The increase in insulin secretion in response to Cch in TAU-supplemented islets was accompanied by a higher intracellular Ca^{2+} mobilization and $PLC_{\beta 2}$ protein expression. The Ca^{2+} uptake was higher in TAU islets in the presence of 8.3 mmol/l glucose, but similar when the islets were challenged by glucose plus IBMX. TAU islets also showed an increase in the expression of PKAα protein. This protein may play a role in cation accumulation, since the amount of Ca^{2+} in these islets was significantly reduced by the presence of the PKA inhibitor, H89. In conclusion, TAU supplementation increases insulin secretion in response to glucose, favoring both influx and internal mobilization of Ca^{2+}, and these effects seem to involve the activation of both PLC/IP_3 and cAMP/PKA pathways.

Introduction

Insulin secretion from pancreatic β-cells is regulated by several factors including fuels, hormones, and neurotransmitters. These agents modify the intracellular concentrations of several β-cell regulators such as Ca^{2+}, phospholipids, and cyclic nucleotides that influence the amplitude and shape of the insulin secretory response (Jones and Persaud, 1998; Tengholm and Gylfe, 2009).

Ca^{2+} influx contributes to the insulin secretory process, regulating the docking and fusion of the secretory granules with the plasma membrane (Rorsman and Renstrom, 2003), whereas Ca^{2+} mobilized from intracellular stores is essential for the replenishment of the readily releasable pool of secretory granules (Gromada *et al.*, 1999). In addition, Ca^{2+} contributes to the amplification of secretion by activating several enzymes such as the PLC and adenylyl cyclase (AC), which generate the intracellular messengers, inositol-1,4,5-triphosphate (IP_3) plus diacylglycerol (DAG), and cAMP, respectively (Leech *et al.*, 1999; Rhee, 2001; Delmeire *et al.*, 2003; Thore *et al.*, 2005; Thore *et al.*, 2007).

Various hormones and neurotransmitters acting through specific receptors, located at the plasma membrane, potentiate insulin secretion via stimulation of PLC, generating IP_3 and DAG (Jones and Persaud, 1998). IP_3 mediates rapid mobilization of Ca^{2+} from the endoplasmatic reticulum, whereas DAG stimulates PKC (Liu and Gylfe, 1997; Berridge *et al.*, 2003). PLC contributes to the insulin secretion not only when membrane receptors are activated, but also when the intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) increases in response to glucose (Thore *et al.*, 2007).

Evidence points to an interrelationship between cAMP and $[Ca^{2+}]i$. It is known that cAMP/PKA mobilizes Ca^{2+} from intracellular stores and regulates the activity of the L-type Ca^{2+} channels (Ammala *et al.*, 1993; Leiser and Fleischer, 1996; Gao *et al.*, 1997; Dyachok and Gylfe, 2004). cAMP also alters the $[Ca^{2+}]i$, modifying Ca^{2+} oscillations from slow to fast in the presence of stimulatory glucose concentrations (Grapengiesser *et al.*, 1991). In addition, PKA contributes to the first phase of insulin secretion, phosphorylating some proteins involved in the exocytotic process (Seino and Shibasaki, 2005; Hatakeyama *et al.*, 2006).

TAU, a naturally occurring sulfur-containing amino acid, regulates several biological processes, including osmolarity (Schaffer *et al.*, 2000), Ca^{2+} binding and transport (Satoh, 1998; Satoh and Sperelakis, 1998; Palmi *et al.*, 1999), ion channel activity (Lee *et al.*, 2004; Park *et al.*,

2004), insulin secretion, and glucose homeostasis (Loizzo *et al.*, 2007; Carneiro *et al.*, 2008; Ribeiro *et al.*, submitted). Previous data from our laboratory have shown that TAU supplementation improves glucose tolerance and insulin sensitivity in mice and increases nutrient-induced insulin secretion in isolated islets. TAU-supplemented islets show increased Ca^{2+} uptake and higher expression of the L-type β_2 subunit Ca^{2+} channel (Ribeiro *et al.*, 2008). It has also been demonstrated that TAU affects the kinetics of Ca^{2+} movement in different tissues (Satoh, 1998; Satoh and Sperelakis, 1998; Palmi *et al.*, 1999; Lee *et al.*, 2004). Despite reports of a regulatory role of TAU in insulin secretion and Ca^{2+} handling (Carneiro *et al.*, 2008; Ribeiro *et al.*, submitted), little is known about its effects on cholinergic/PLC and PKA pathways in β-cells. These mechanisms regulate and are regulated by $[Ca^{2+}]i$ in β-cells (Ammala *et al.*, 1993; Leiser and Fleischer, 1996; Gao *et al.*, 1997; Dyachok and Gylfe, 2004; Thore *et al.*, 2007; Dyachok *et al.*, 2008).

In this study, we confirm that TAU-supplementation increases insulin secretion in response to glucose in isolated islets. We also show that the secretory capacity and Ca^{2+} handling in these islets were higher in conditions in which IP_3 and cAMP production was increased. Data indicate that TAU modulation of these processes seems to be linked to the activation of cholinergic/PLC and PKA pathways.

Materials and Methods

Materials

$^{45}CaCl_2$ and ^{125}I human insulin were purchased from Amersham International (Little Chalfont, Bucks, UK). Routine reagents, phorbol 12-myristate 13-acetate (PMA), Cch, Forskolin and 3-isobutyl-1-methyl-xanthine (IBMX), *N*-[2-(p-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide (H89) were purchased from Sigma Chemical (St Louis, MO, USA).

Animals

All experiments were approved by the ethics committee at UNICAMP. Three-week-old Swiss mice were obtained from the colony at UNICAMP. The mice were maintained on a 12 hour light/dark cycle (lights on 06:00-18:00 h), controlled temperature (22 ± 1ºC), and allowed free access to water and standard laboratory chow (Rodent chow; Nutrilab, Colombo, Brazil) ad libitum. At 60 days, mice were distributed into two groups: mice that received 2% of TAU in their drinking water for 30 days (TAU group) and those that received only water (CTL group).

Islet isolation and static insulin secretion

Islets were isolated by collagenase digestion of the pancreas. For static incubations, groups of four islets were first incubated for 30 min at 37 ºC in Krebs-Ringer bicarbonate (KRB) buffer with 2.8 mmol/l glucose and 3 g of BSA/l, 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 incubated for 1 h under the following conditions: glucose (8.3 mmol/l) alone or with Cch (100 μmol/l), PMA (100 nmol/l), Forskolin (10 μmol/l) and IBMX (1 mmol/l). At the end of the incubation period, the insulin content of the medium was measured by RIA.

Dynamic insulin secretion

Groups of freshly isolated islets were placed on Millipore SW 1300 filters (8.0 μm pore) and perifused at a flow rate of 1ml/min with KRB buffer gassed with 95% O_2/5% CO_2, pH 7.4 and maintained at 37 ºC. To verify dynamic insulin secretion in response to increased $[Ca^{2+}]i$ induced by Cch, groups of 70 islets were perifused with Ca^{2+}-free KRB buffer containing glucose 8.3 (mmol/l) plus 250 μmol/l of diazoxide and 10 mmol/l EGTA, with or without 100 μmol/l of Cch, as indicated in the figure legends and Results section. In another experimental series, groups of 50 islets were

perifused in a KRB containing 8.3 mmol/l glucose (basal condition) for 30 minutes and, after this period, IBMX (1 mmol/l) or Forskolin (10 μmol/l) was added to the perifusion solution (for more information, see figure legends). Insulin release was measured by RIA.

Uptake of ^{45}Ca

Groups of 150 to 200 islets, derived from the same batch of islets, were preincubated for 30 min at 37 °C in a KRB buffer containing 2.8 mmol/l of glucose, pH 7.4. The islets were then incubated for 30 min in 200 μl of the same medium containing $^{45}CaCl_2$ (60 μCi/ml) and 8.3 mmol/l glucose alone or with IBMX (1 mmol/l) or H89 (10 μmol/l). At the end of the incubation period, 800 μl of ice-cold KRB containing 2 mmol/l of $LaCl_3$ (pH 7.4) was added to stop the reaction. The medium was then removed and an aliquot was saved to determine the amount of ^{45}Ca in the solution. The islets were subsequently washed three times with ice-cold KRB containing La^{3+} and islets were then placed in a Petri dish. Groups of ten islets were transferred to counting vials containing 1 ml of EGTA 50 mmol/l. The uptake of ^{45}Ca was expressed as pmol Ca^{2+} per islet per 30 min of incubation.

Cytoplasmatic Ca^{2+} Oscillations

Fresh pancreatic islets were incubated with fura-2/AM (5 μmol/l) for 1 h at room temperature in KRB buffer containing glucose (5.6 mmol/l) and pH 7.4 and supplemented with BSA. 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 KRB continuously gassed with 95% O_2/5% CO_2, pH 7.4 (37°C) containing glucose (8.3 mmol/l) plus 250 μmol/l of diazoxide and 10 mmol/l EGTA with or without 100 μmol/l of Cch. A ratio image was acquired approximately at 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).

Western blotting

Isolated islets from TAU and CTL groups were solubilized in 100 µl homogenization buffer containing (in mmol/l): 100 Tris pH 7.5, 10 sodium pyrophosphate, 100 sodium fluoride, 10 EDTA, 10 sodium vanadate, 2 PMSF and1% Triton-X 100. The islets were disrupted using a Polytron PT 1200 C homogenizer (Brinkmann Instruments, NY, USA), employing 10s pulses for 3 times. The extracts were then centrifuged at 12,600 g at 4°C for 5 min to remove insoluble material. The protein concentration in the supernatants was assayed using the Bradford dye method and the BioRad reagent. For SDS gel electrophoresis and Western blot analysis, the samples were treated with a Laemmli sample buffer containing dithiothreitol. After heating to 95 ^{o}C for 5 min, the proteins were separated by electrophoresis (55 µg protein/lane, 10% gels). Following electrophoresis, proteins were transferred to nitrocellulose membranes. The nitrocellulose filters were overnight treated 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 rabbit polyclonal antibody to $PLC_{\beta 2}$ (1:500), or PKAα (1:500), or mouse monoclonal antibody to PKCα (1:1,000). All primary antibodies used were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Visualization of specific protein bands was made by incubating the membranes for 2 h with a peroxidase-conjugated secondary antibody (1:10,000; Zymed Laboratories, Inc., San Francisco, CA, USA), followed by detection with enhanced chemiluminescence reagents (Pierce Biotechnology, USA) and exposure to X-ray film (Kodak, AM, Brazil). The band intensities were quantified by

optical densitometry (Scion, Image, Frederick, MD, USA). After assaying the target proteins, Western blotting was repeated using rabbit monoclonal antibody to β-actin (1:10,000, Abcam, Cambridge, MA, USA) as an internal control.

Statistical analysis

Results are presented as means ± SEM for the number of determinations (n) indicated. The statistical analyses were carried out using one way analysis of variance (ANOVA) followed by the Tukey post test for multiple comparisons or Student's t-test for two groups comparisons ($P \leq 0.05$).

Results

Insulin secretion in TAU-supplemented mice islets

Figure 1 shows the insulin secretion induced by sub- and supra-threshold glucose concentrations. At basal glucose, insulin secretion was similar between groups. TAU-supplemented islets released significantly more insulin in the presence of 8.3 and 22.2 mmol/l glucose, compared with the CTL group ($P < 0.03$ and $P < 0.01$, respectively). Since, at a physiological concentration of glucose (8.3 mmol/l), insulin released in TAU-supplemented islets was significantly different from CTL islets this glucose concentration was used in all subsequent insulin release experiments.

Cch and PMA-induced insulin secretion

When the islets were incubated in the presence of 100 μmol/l Cch, insulin secretion was higher in TAU-supplemented than CTL islets (Fig. 2; $P < 0.001$). However, the increment in insulin secretion, induced by 100 nmol/l PMA (that activates PKC), was similar between groups.

Cch-induced intracellular Ca^{2+} mobilization

In the next series of experiments, we analyzed intracellular Ca^{2+} mobilization in TAU and CTL islets. For this purpose, 100 μmol/l Cch was added to a perifusion system with a Ca^{2+}-free medium, containing 8.3 mmol/l glucose, 250 μmol/l diazoxide, and 10 mmol/l EGTA. Figure 3A shows that Cch-induced increase in $[Ca^{2+}]i$ was higher in TAU-supplemented than in CTL islets. The area under the curves (AUC) and the amplitude of $[Ca^{2+}]i$ were significantly higher in TAU-supplemented, compared with CTL islets (1.06 ± 0.1 and 0.19 ± 0.02 vs 0.61 ± 0.08 F340/380.min^{-1} and 0.13 ± 0.01 ΔF340/380, respectively; $P < 0.04$). In accordance, the dynamic insulin release was also higher in the TAU group, compared with the CTL group, when challenged with the same concentrations of Cch (Fig. 3B). The AUC of the total insulin released (min 8 to 20) and peak of secretion (at 9 min) were 24 ± 3 and 4.3 ± 0.5 vs 17 ± 2 ng/70 islets.min^{-1} and 2.5 ± 0.4 ng/50 islets, respectively ($P < 0.05$), for TAU and CTL islets.

Forskolin and IBMX-induced insulin secretion

Forskolin (10 μmol/l) and IBMX (1mmol/l), which increase cAMP by AC stimulation or by phosphodiesterase inhibition (respectively), significantly stimulated secretion in both types of islets (Fig. 4A). However, while the increase in insulin secretion provoked by IBMX was higher in TAU-supplemented, compared with CTL islets ($P < 0.05$), the increment in the insulin secretion stimulated by forskolin was similar in both groups (Fig. 4A). Using a perifusion system, we confirm that IBMX-potentiated release was significantly higher in TAU-supplemented compared with CTL islets (Fig. 4B). Total insulin release during min 15-50 was 842 ± 152 and 359 ± 139 ng/50 islets.min^{-1}, respectively ($P = 0.05$). Dynamic insulin release induced by Forskolin was also analyzed (Fig. 4C). The amount of insulin secreted during forskolin perifusion time (min 15-50) was similar between TAU and CTL islets (507 ± 60 and 380 ± 27 ng/50 islets.min^{-1}, respectively).

IBMX and H89 effects on ^{45}Ca uptake

As previously observed, TAU-supplemented islets showed increased Ca^{2+} uptake in the presence of high glucose concentrations (Ribeiro, Bonfleur *et al.*, submitted). In order to analyze the involvement of PKA in Ca^{2+} handling by the islets, we measured ^{45}Ca uptake in the presence of 8.3 mmol/l glucose with or without agents that stimulate (IBMX) or inhibit (H89) PKA. At 8.3 mmol/l glucose, the Ca^{2+} uptake was higher in TAU than CTL islets (Table 1; $P < 0.02$). IBMX significantly increased Ca^{2+} uptake in TAU-supplemented and CTL islets by 32% and 58%, compared with the respective controls (glucose alone) (Table 1). At the end of the incubation period, both groups of islets reached similar values of Ca^{2+} uptake. The administration of H89 significantly reduced Ca^{2+} uptake in TAU-supplemented, but only marginally in CTL islets. These results indicated that the participation of the cAMP/PKA pathway in Ca^{2+} influx, in response to glucose, was higher in TAU-supplemented than in CTL islets.

$PLC_{\beta 2}$, PKCα and PKAα protein expression

Western blotting analysis shows that the expressions of $PLC_{\beta 2}$ (Fig. 5A) and PKAα (Fig. 5C) were two-fold higher in TAU-supplemented compared with CTL islets ($P < 0.02$ and $P < 0.002$; respectively), whereas the expression of PKCα was similar between groups (Fig. 5B).

Discussion

Recently, we demonstrated that pancreatic islets from TAU-supplemented mice secreted more insulin in response to glucose and that this effect seems to be linked to a higher Ca^{2+} mobilization. We also showed increased protein expression of the β_2 subunit of the L-type Ca^{2+} channel in these islets (Ribeiro *et al.*, submitted). Here, we investigated the mechanisms involved in the higher insulin release in TAU-supplemented islets; the main findings presented of this study suggest that

TAU supplementation increases the β-cells sensitivity to cholinergic/PLC and cAMP/PKA pathways with a higher Ca^{2+} recruitment from intra- and- extracellular compartments.

In β-cells, Cch provokes an increase in $[Ca^{2+}]i$ in a biphasic manner. The first phase occurs by a rapid mobilization of intracellular Ca^{2+}, induced by IP_3, and the second one depends on Ca^{2+} influx through the store-operated channels located at the cell plasma membrane (Liu and Gylfe, 1997). The intracellular Ca^{2+} mobilization, stimulated by the activation of the PLC and IP_3 production by acetylcholine, was reported to increase insulin granule movement in a PKC-independent manner (Niwa *et al.*, 1998). Here, we observed that insulin secretion in response to PMA, and PKCα protein expression, did not differ between TAU-supplemented and CTL islets. However, increased $PLC_{\beta 2}$ protein levels in TAU islets suggests that augmented activation of PLC followed by IP_3 production may account for increased intracellular Ca^{2+} mobilization in Cch-stimulated TAU-supplemented islets. These data give support to a possible effect of TAU upon IP_3 production, in turn amplifying β-cell response to fuel secretagogues.

It is known that PLC may be activated when the β-cell is depolarized. Since all PLC isoforms require Ca^{2+} (Rhee, 2001), it is possible that, in β-cells, the increase in $[Ca^{2+}]i$ activates this enzyme, leading to an increase in IP_3 and DAG production that may account for an enhanced insulin secretion in response to glucose. Supporting this view, $PLC_{\delta 1}$ activity has been found to increase in the presence of stimulatory glucose concentrations, resulting in a cycle of synthesis and degradation of plasma membrane phosphatidylinositol-4,5-bisphosphate (Thore *et al.*, 2005; Thore *et al.*, 2007).

We show, herein, that TAU-supplemented islets increase insulin secretion in response to IBMX and express more PKAα protein. Thus, in addition to PLC signals, cAMP and PKA may contribute to increasing insulin secretion and islet functionality in the presence of glucose. In support of this assumption, Ca^{2+} uptake, in response to glucose in TAU-supplemented islets, was significantly reduced in the presence of a PKA inhibitor (Fig. 5C). As such, TAU supplementation

seems to alter islet Ca^{2+} handling either by increasing the β_2 subunit Ca^{2+} channels expression (see Ribeiro *et al.*, submitted) or by increasing the expression and/or activity of PKA, since the inhibition of the enzyme provoked only a marginal effect on Ca^{2+} uptake in CTL islets.

Recently, a direct coupling between Ca^{2+} and cAMP has been reported. (Dyachok *et al.*, 2008), monitored the alterations in cAMP levels in β-cells by observing the dissociation of PKA catalytic from the regulatory subunits. These authors showed that glucose induces cAMP level oscillations and that each oscillation was preceded and enhanced by the increase in $[Ca^{2+}]i$. In addition, INS 1 cells, exposed to GLP-1 or IBMX, exhibited cAMP/PKA oscillations synchronized with increases in $[Ca^{2+}]i$ (Dyachok *et al.*, 2006).

In the β-cell, nutrient stimulation leads to membrane depolarization and Ca^{2+} influx. The increase in $[Ca^{2+}]i$ may stimulate or inhibit cAMP formation because β-cells express different AC (Leech *et al.*, 1999). The AC type VIII, present in β-cells, is a Ca^{2+}-sensitive isoform and, in the presence of glucose, may account for the increase in cAMP levels (Delmeire *et al.*, 2003). This increase represents an important signal for $[Ca^{2+}]i$ regulation and insulin exocytosis in β-cells. Moreover, IBMX and forskolin markedly increased the intracellular cAMP levels and, consequently, $[Ca^{2+}]i$ in β-cells (Rajan *et al.*, 1989), probably via phosphorylation of the α1 subunit of the L-type Ca^{2+} channel by PKA (Leiser and Fleischer, 1996). This effect of PKA was also observed in other cell types. PKA phosphorylation of the L-type Ca^{2+} channel (skeletal-muscle, cardiac cells and neurons) increases the open probability of the channel, shifts the voltage-dependence of activation and slows the rate of inactivation, increasing voltage-dependent facilitation of the channel (Mundina-Weilenmann *et al.*, 1991; Bourinet *et al.*, 1994; Gao *et al.*, 1997). PKA also phosphorylates the β_2 subunit of the cardiac Ca^{2+} channel (Brian et al., 1999) and this effect contributes to increase Ca^{2+} channel activity (Kamp and Hell, 2000). In β-cells, PKA acts upon

intracellular Ca^{2+} dynamic via IP_3 receptors, increasing intracellular Ca^{2+} mobilization from the endoplasmatic reticulum (Ammala *et al.*, 1993; Dyachok and Gylfe, 2004).

Therefore, our results suggest that the interrelationship between PKA and Ca^{2+} is enhanced in TAU-supplemented islets and accounts for increased Ca^{2+} handling in the presence of glucose. We do not have a definitive explanation as to why TAU-supplemented islets, when challenged by IBMX, but not forskolin, secreted more insulin than CTL islets, since PKAα expression was increased in the former group of islets. However, we speculate that the maintenance of cAMP levels, rather than the amplification of its production, is more important for the phenomenon. The other possibility, not addressed in this work, is that phosphodiesterase activity may be higher in the TAU group, resulting in a faster degradation of the second messenger.

In summary, this study confirms and extends previous results showing that TAU supplementation increased insulin secretion and Ca^{2+} handling in the presence of stimulatory concentrations of glucose (Carneiro *et al.*, 2008; Ribeiro *et al.*, submitted). We also provide new evidence that TAU supplementation increased insulin secretion in islets, in response to the cholinergic/PLC and cAMP/PKA pathways and that these effects, at least in part, are probably due to increased protein $PLC_{\beta 2}$ and PKAα expressions and/or by a higher Ca^{2+} mobilization from both external and internal pools.

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Table 1. Islet ^{45}Ca uptake (pmol ^{45}Ca/islet.30min) in the presence of 8.3 mmol/l glucose (G8.3), with or without 1 mmol/l IBMX or 10 μmol/l H89.

	G8.3	**G8.3 + IBMX**	**G8.3 + H89**
CTL	1.05 ± 0.06^{a}	1.66 ± 0.08^{c}	$0.91 \pm 0.05^{a,d}$
TAU	1.30 ± 0.08^{b}	1.70 ± 0.10^{c}	0.76 ± 0.05^{d}

Data are means ± SEM of (14-36) groups of islets. * $P < 0.05$, different words indicate a significant difference.

Figure Legends

Figure 1. Glucose-induced insulin secretion in islets from TAU-supplemented and CTL mice. Groups of 4 islets were incubated for 1 h with different glucose (G) concentrations, as indicated. Each bar represents mean ± SEM of 12-21 groups of islets. * $P < 0.05$ vs CTL.

Figure 2. Insulin secretion, induced by Cch (100 μmol/l) or PMA (100 nmol/l), in islets from TAU and CTL mice. Islets were incubated for 1 h at 8.3 mmol/l glucose (G8.3), with or without Cch and PMA. Each bar represents mean ± SEM of 15 groups of islets. *$P < 0.05$ related to respective CTL.

Figure 3. Cch (100 μmol/l) induced internal Ca^{2+} mobilization (**A**) and insulin secretion (**B**) from TAU-supplemented and CTL islets. The experiments were performed in a perifusium system in a Ca^{2+}-free medium containing: 8.3 mmol/l glucose (G8.3), 250 μmol/l diazoxide, and 10 mmol/l EGTA. For Ca^{2+}, values are the ratio of F340/F380 registered for each group. Data are means ± SEM obtained from 6-7 independent experiments. * $P < 0.05$ indicates significant difference vs CTL.

Figure 4. (**A**) Forskolin (10 μmol/l) and IBMX (1 mmol/l) induced insulin secretion in islets from TAU-supplemented and CTL mice. Islets were incubated for 1 h at 8.3 mmol/l glucose (G8.3), with or without forskolin and IBMX. Dynamic insulin secretion in response to 8.3 mmol/l glucose with or without IBMX (**B**) or Forskolin (**C**) in TAU-supplemented and CTL islets. Data are means ± SEM obtained from 14-15 groups of islets for static incubation and from 4 independent experiments for dynamic measurements. *$P < 0.05$ related to respective CTL.

Figure 5. $PLC_{\beta 2}$, PKCα and PKAα protein expressions in islets from TAU-supplemented and CTL mice. Protein extracts were processed for Western blot detection of $PLC_{\beta 2}$ (**A**), PKCα (**B**), PKAα (**C**) and β-actin (internal control). The bars represent the means ± SEM of the values, determined by optical densitometry. n=3-7; *$P < 0.05$ *vs* CTL.

Figure 1

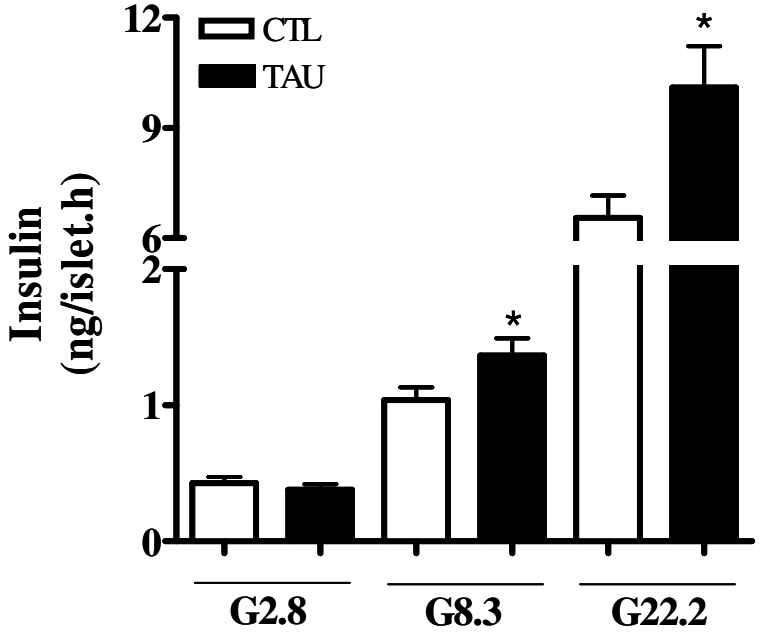


Figure 2

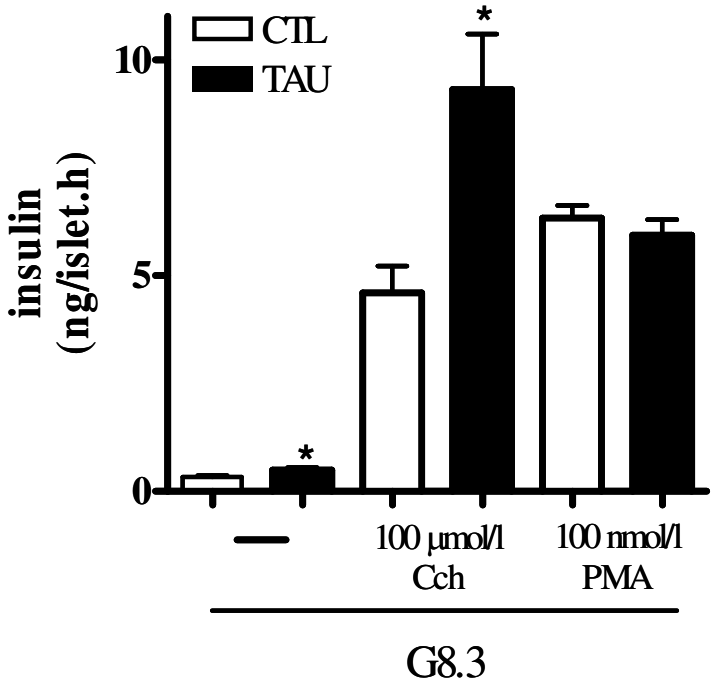

CTL
TAU
10
5
0
insulin
(ng/islet.h)
*
*
—
100 µmol/l
Cch
100 nmol/l
PMA
G8.3

Figure 3

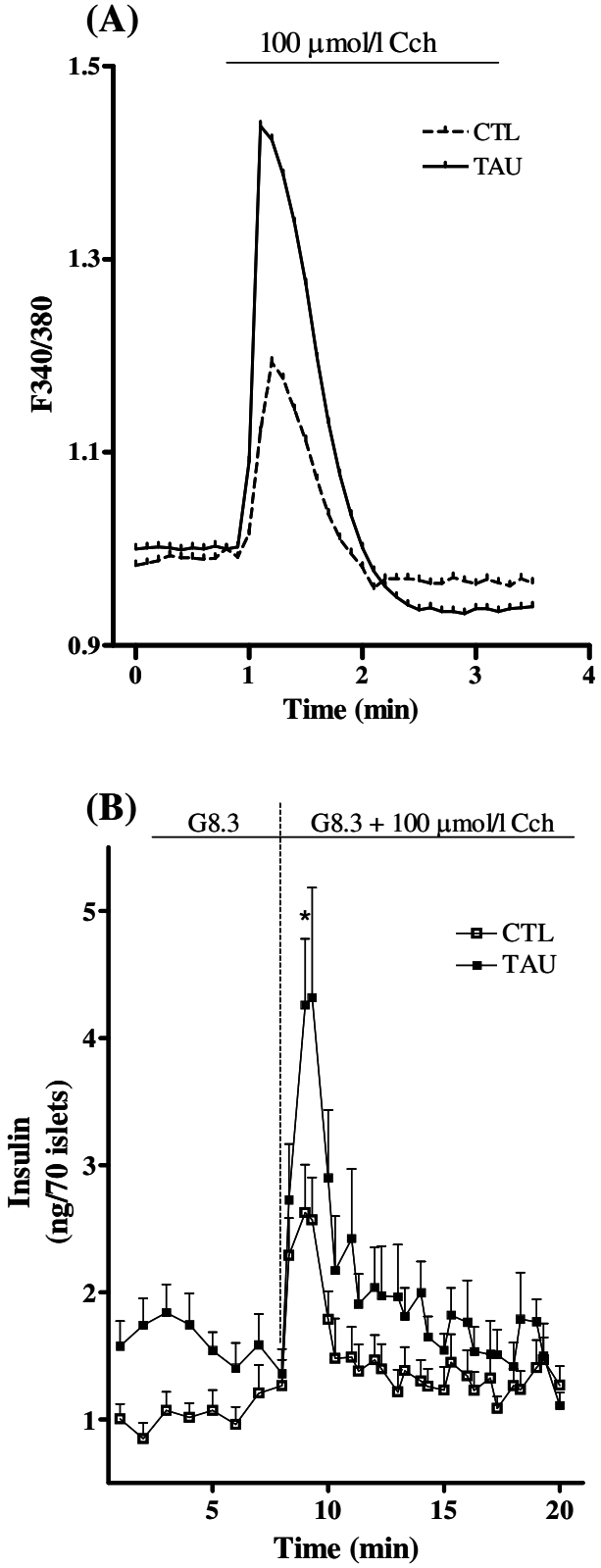

(A)
100 µmol/l Cch
CTL
TAU
F340/380
1.5
1.3
1.1
0.9
0
1
2
3
4
Time (min)
(B)
G8.3
G8.3 + 100 µmol/l Cch
CTL
TAU
*
Insulin
(ng/70 islets)
5
4
3
2
1
5
10
15
20
Time (min)

Figure 4

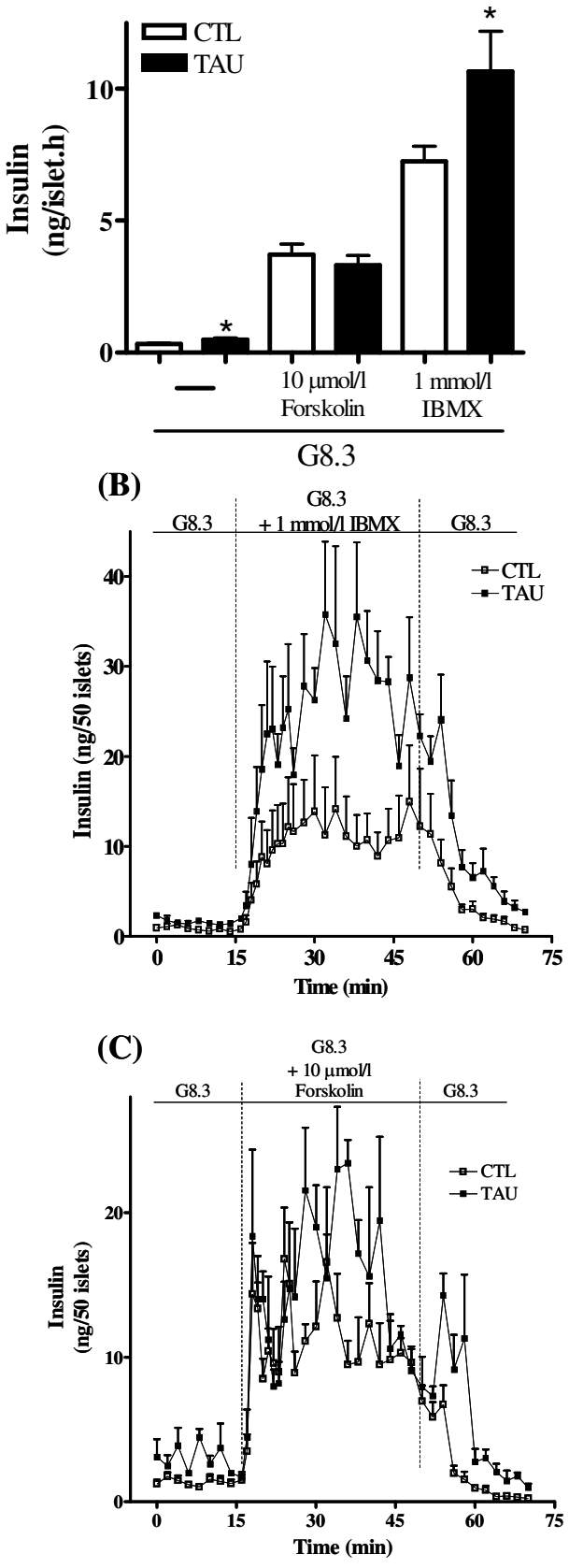
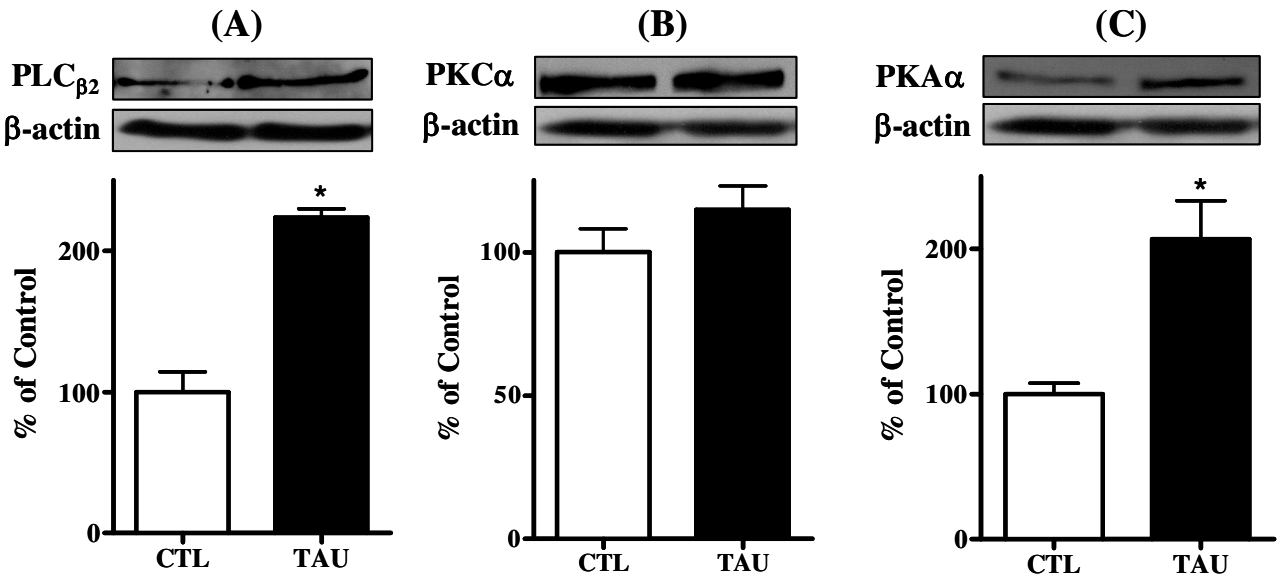

CTL
TAU
Insulin (ng/islet.h)
10 µmol/l Forskolin
1 mmol/l IBMX
G8.3
(B)
G8.3
G8.3 + 1 mmol/l IBMX G8.3
Insulin (ng/50 islets)
Time (min)
(C)
G8.3
+ 10 µmol/l Forskolin
G8.3 Forskolin G8.3
Insulin (ng/50 islets)
Time (min)

Figure 5


(A)
PLCβ2
β-actin
% of Control
200
100
0
*
CTL
TAU
(B)
PKCα
β-actin
% of Control
100
50
0
CTL
TAU
(C)
PKAα
β-actin
% of Control
200
100
0
*
CTL
TAU

DISCUSSÃO

Vários estudos demonstraram uma forte correlação entre a redução da concentração circulante de TAU e o DM em humanos e diferentes modelos experimentais (Franconi *et al.*, 1995; Anuradha e Balakrishnan, 1999; Aerts e Van Assche, 2001; Colivicchi *et al.*, 2004; Tsuboyama-Kasaoka *et al.*, 2006). Além disso, o tratamento com TAU foi benéfico para a prevenção do DM 1 (Arany *et al.*, 2004; Tas *et al.*, 2007), disfunção da célula β (Kaniuk *et al.*, 2007; Oprescu *et al.*, 2007; Xiao *et al.*, 2008), e DM 2 (Nakaya *et al.*, 2000; Nandhini *et al.*, 2005; Tsuboyama-Kasaoka *et al.*, 2006).

Neste sentido, Cherif *et al.* (1998) verificaram que a secreção de insulina de ilhotas de fetos de ratas que foram submetidas à restrição protéica durante a gestação apresenta-se reduzida em resposta a nutrientes. A suplementação com TAU durante a prenhez normaliza a secreção de insulina e a proliferação celular, restaura a síntese de DNA, reduz a apoptose, normaliza a massa de células β, e restaura a expressão de genes envolvidos com o metabolismo e proliferação celular nas ilhotas pancreáticas destes fetos e neonatos desnutridos (Merezak *et al.*, 2001; Boujendar *et al.*, 2002; Merezak *et al.*, 2004; Kalbe *et al.*, 2005; Reusens *et al.*, 2008). Portanto, em modelos de pré-disposição ao DM 2, a suplementação com TAU contribui para a restauração e manutenção da função da ilhota. Contudo, os possíveis mecanismos pelos quais este aminoácido participa da modulação do processo de secreção de insulina não estão estabelecidos.

Em nosso estudo, demonstramos que a suplementação com TAU melhora a homeostase da glicose, e modifica a resposta da célula β a nutrientes e potencializadores da secreção de insulina, e aumenta a expressão da subunidade β_2 do canal de Ca^{2+} do tipo L e de proteínas relacionadas com a potencialização da secreção, regulação da $[Ca^{2+}]i$ e do processo exocitótico dos grânulos de insulina.

Para a melhor compreensão dos parâmetros avaliados em nosso estudo dividimos a discussão em tópicos.

Homeostase Glicêmica

De acordo com o que já foi relatado em outros modelos experimentais (Anuradha e Balakrishnan, 1999; Nakaya *et al.*, 2000; Nandhini e Anuradha, 2002; Nandhini *et al.*, 2005; Tsuboyama-Kasaoka *et al.*, 2006; Loizzo *et al.*, 2007; Carneiro *et al.*, 2008), nosso estudo demonstrou que a suplementação com TAU promove uma melhora no controle da glicemia e ação periférica da insulina, como pode ser observado por meio da menor glicemia pontual e total registradas durante o ipGTT e ipITT (Fig 1; Ribeiro *et al*., artigo 1). Porém, esta modificação no controle glicêmico não é acompanhada por alteração na insulinemia e glicemia de jejum, bem como na concentração plasmática de glicose, insulina e glucagon no estado alimentado (Tab. 1; Ribeiro *et al*., artigo 1).

O efeito da suplementação com TAU sobre a homeostasia glicêmica pode ser decorrente da possível interação do aminoácido com IR. Desta forma, com a elevação da glicemia, no estado alimentado, a concentração plasmática de insulina nos camundongos suplementados não seria alterada, pois o aumento da concentração plasmática de TAU agiria juntamente com a insulina nos tecidos-alvo até a normalização da glicemia. Esta hipótese foi confirmada por um estudo desenvolvido anteriormente pelo nosso grupo de pesquisa, no qual foi observado aumento da fosforilação do IR, em tecido hepático e muscular de camundongos, após a administração ip de TAU, sendo verificado que a fosforilação do IR ocorreu em valores similares aos evidenciados quando insulina foi administrada (Carneiro *et al.*, 2008).

Camundongos suplementados demonstraram um aumento na concentração circulante de glucagon no estado de jejum (Tab. 1; Ribeiro *et al*., artigo1). A elevação da concentração plasmática deste hormônio no jejum está bem documentada. Glucagon e insulina agem de formas antagônicas buscando a normalização da glicemia e fornecimento de glicose aos tecidos. No jejum, a redução da concentração circulante deste açúcar estimula a secreção de glucagon, o qual atua sobre estoques de

glicose existentes no fígado (Gromada *et al.*, 2007). Portanto, a elevação plasmática de glucagon sugere que a TAU modifica os processos de controle da glicemia, tanto em situações onde a concentração plasmática deste açúcar apresenta-se elevada, quanto em condições que a mobilização da glicose é necessária, como no estado de jejum para evitar hipoglicemia.

Secreção de Insulina

Ao estudarmos a secreção de insulina em ilhotas isoladas, foi evidenciado que ilhotas pancreáticas do grupo suplementado com TAU apresentam uma maior elevação e sustentação da secreção dinâmica de insulina em resposta ao aumento da glicose (Fig. 2; Ribeiro *et al*., artigo 1). Este efeito não é devido ao aumento do metabolismo ou captação deste açúcar, já que a oxidação da glicose e expressão da GCK e GLUT 2 não foram alterados pela suplementação com TAU. Porém, ilhotas TAU apresentaram maior captação de íons Ca^{2+} na presença de glicose, e expressão protéica da subunidade β_2 do canal de Ca_v (Fig. 4 e 5; Ribeiro *et al*., artigo 1).

Uma vez que o influxo de Ca^{2+} através dos canais de Ca_v em resposta a vários estímulos nas células β resulta na secreção de insulina, é provável que a regulação da expressão e da atividade dos canais de Ca_v seja um fator determinante para a secreção do hormônio. Em diferentes tipos celulares foi demonstrado que a subunidade β participa no processo de translocação dos canais de Ca^{2+} para a membrana plasmática, aumenta a amplitude da corrente do Ca^{2+} e a cinética de ativação do canal (Chien *et al.*, 1995; Josephson e Varadi, 1996; Dolphin, 2003; Yang e Berggren, 2006). Além disso, foi sugerido que a subunidade β também regule a expressão gênica (Hibino *et al.*, 2003). Apesar de em células β a função desta subunidade não estar totalmente estabelecida, Ihara *et al.* (1995), ao transfectarem em células ovarianas uma subunidade α1 que é expressa em células RINm5F e em ilhotas de ratos, verificaram que o canal de Ca_v só apresenta-se ativo quando co-expresso a

subunidade β_2, o que sugere que a atividade destes canais pode estar sob o controle desta subunidade em células β.

Além da subunidade β_2, a subunidade β_3 é expressa em células RINm5F, ilhotas de ratos e camundongos (Safayhi *et al.*, 1997; Iwashima *et al.*, 2001; Berggren *et al.*, 2004), e parece ser importante no controle da $[Ca^{2+}]i$ e na geração de mensageiros intracelulares na célula β, uma vez que a sua deleção eleva a secreção de insulina em ilhotas de camundongos por aumento das oscilações da $[Ca^{2+}]i$ em resposta à glicose, devido à uma maior produção de IP_3 e da mobilização de Ca^{2+} de estoques intracelulares (Berggren *et al.*, 2004).

Além disso, os canais do tipo L interagem fisicamente com as proteínas SNARE: sintaxina 1, SNAP25 (*synaptosomal-associated protein*) e sinaptotagmina formando um complexo denominado "excitossoma" (Wiser *et al.*, 1999), o qual é essencial para expor o pool de grânulos à alta $[Ca^{2+}]i$ que ocorre na vizinhança dos canais de Ca^{2+} do tipo L e permitir a rápida exocitose dos grânulos de insulina que contribuem para a primeira fase da secreção (Wiser *et al.*, 1999; Mears, 2004; Trus *et al.*, 2007). Portanto, as evidências de que as subunidades β apresentam um importante papel na regulação da atividade dos canais de Ca_v e da $[Ca^{2+}]i$, juntamente com nossos resultados que demonstraram que a suplementação com TAU eleva o conteúdo total de insulina armazenado, sugerem que ilhotas de animais tratados com TAU apresentam maior resposta secretória de insulina na presença de glicose provavelmente pelo aumento do influxo de Ca^{2+}, e da interação deste cátion com o aparato exocitótico, beneficiando o acoplamento estímulo/secreção de insulina.

Ilhotas pancreáticas do grupo TAU apresentaram aumento da secreção de insulina na presença de L-leucina (Fig. 2 e 3; Ribeiro *et al*., artigo 1). Esta modificação da resposta secretória à L-leucina no grupo TAU é devido ao maior catabolismo da L-leucina, já que a oxidação deste aminoácido é maior em ilhotas TAU. A secreção de insulina em resposta ao BCH (um análogo não metabolizável da L-leucina; (Liu *et al.*, 2003) e ao BCH/L-glutamina, que são estímulos que priorizam a atividade

da GDH; bem como na presença de combinações de estímulos que reduzem ou inibem o metabolismo da L-leucina como: L-leucina/L-glutamina, BCH/L-glutamina/L-leucina, ou BCH/L-glutamina/L-leucina e o inibidor de transaminase AOA (ácido aminooxiacético) (Malaisse *et al.*, 1982; Gao *et al.*, 2003; MacDonald *et al.*, 2008), apresenta-se semelhante entre ilhotas TAU e CTL. Desta forma, nossos dados sugerem que o tratamento *in vivo* com TAU modifica a secreção de insulina na presença de L-leucina primariamente por alteração na transaminação deste aminoácido ao KIC, elevando assim o aporte de acetil-CoA para o ciclo de Krebs, consequentemente geração de ATP, despolarização da membrana da célula β, e possivelmente maior elevação da $[Ca^{2+}]i$ em ilhotas TAU.

Cabe novamente salientar que a célula β pancreática é equipada de vários tipos de canais de Ca_v, e que no processo exocitótico da insulina os íons Ca^{2+} e os canais de Ca_v estão diretamente relacionados com a adequada secreção do hormônio frente à elevação da concentração de glicose (Yang e Berggren, 2006). Além da contribuição da subunidade β do canal sobre o controle da atividade dos canais de Ca_v e da mobilização do cátion de estoques intracelulares (Ihara *et al.*, 1995; Berggren *et al.*, 2004), quinases como a PKA e PKC, e mensageiros intracelulares como IP_3, gerado pela ativação da PLC, contribuem para a regulação da $[Ca^{2+}]i$ por meio de ações sobre os canais de Ca_v e/ou da mobilização intracelular de Ca^{2+} (Liu e Gylfe, 1997; Niwa *et al.*, 1998; Kamp e Hell, 2000; Kang *et al.*, 2005). Assim, a secreção de insulina, bem como o movimento e captação de íons Ca^{2+} foram avaliados em resposta a estímulos que ativam estas quinases.

Em nosso estudo, a secreção de insulina na presença do Cch foi maior no grupo TAU comparado ao CTL (Fig. 2; Ribeiro *et al*., artigo 2). Este aumento não foi dependente da PKC, uma vez que a secreção em resposta ao PMA, ativador desta enzima, não difere entre os grupos estudados. A maior secreção de insulina frente ao Cch pode ser devido à maior produção de IP_3, uma vez que ilhotas TAU apresentaram aumento da expressão protéica da $PLC_{\beta 2}$, e maior

mobilização de Ca^{2+} de estoques intracelulares em resposta ao Cch (Fig. 5 e 3, respectivamente; Ribeiro *et al*., artigo 2). Portanto, novamente verificamos que as possíveis contribuições sobre o processo de secreção de insulina moduladas pela TAU ocorreram por regulação da $[Ca^{2+}]i$.

O aumento na resposta secretória ao Cch e da expressão da $PLC_{\beta 2}$ em ilhotas TAU ressalta uma das vias pela qual este aminoácido pode contribuir para a preservação da função da célula β e homeostase da glicose. Estudos demonstraram que a deleção do receptor muscarínico 3 (M3) em células β reduz a tolerância à glicose e a secreção de insulina em camundongos. Contudo, camundongos com superexpressão do M3 somente nas células β pancreáticas tornaram–se resistentes ao desenvolvimento de hiperglicemia e de intolerância à glicose induzida por dieta (Gautam *et al.*, 2007).

Dentre as várias quinases que regulam a atividade dos canais de Ca_v está a PKA (Kamp e Hell, 2000). Em miócitos a PKA fosforila os canais de Ca^{2+} do tipo L resultando no aumento do número de canais que estão prontos para serem abertos durante a despolarização, e é sugerido que a fosforilação do canal pela PKA seja necessária para que o canal se torne ativo (Kamp e Hell, 2000). A expressão da subunidade β_2 é maior em ilhotas TAU, e pelo menos com relação à subunidade β_{2a} é relatado que existam três resíduos serina ($Ser^{459/478/479}$) que são fosforilados pela PKA, e esta fosforilação aumenta a atividade do canal (Gao *et al.*, 1997; Gerhardstein *et al.*, 1999). Desta forma, sugerimos que o maior influxo de Ca^{2+} em ilhotas do grupo suplementado pode estar sendo regulado por esta quinase, uma vez que evidências demonstraram uma interrelação entre a $[Ca^{2+}]i$ e a via cAMP/PKA em células β. Sendo relatado nestas células que há modificação do padrão das oscilações da $[Ca^{2+}]i$ na presença de AMPc (Grapengiesser *et al.*, 1991), e que a concentração deste mensageiro frente à glicose oscila assim como a $[Ca^{2+}]i$ (Dyachok *et al.*, 2008). A PKA também age sobre o retículo endoplasmático da célula β contribuindo para a mobilização de Ca^{2+} de estoques

intracelulares (Kang *et al.*, 2005), além de atuar no controle da $[Ca^{2+}]i$ na célula β, esta quinase também está envolvida no processo de exocitose dos grânulos de insulina (Seino e Shibasaki, 2005).

Ao estudarmos a secreção de insulina em resposta a agentes que elevam a concentração de AMPc na célula β, foi observado que ilhotas TAU secretam mais insulina na presença do inibidor de fosfodiesterase IBMX. Porém, a liberação do hormônio na presença de forskolin, um ativador da AC, foi similar entre os grupos estudados, apesar da expressão protéica da PKAα em ilhotas TAU ter sido significativamente maior em relação à ilhotas CTL (Fig. 4 e 5; Ribeiro *et al*., artigo 2). Ainda, a PKA parece modular a captação de íons Ca^{2+} em ilhotas do grupo TAU na presença de glicose, pois o acúmulo deste cátion foi reduzido neste grupo quando a atividade da PKA foi inibida pelo H89 (Tab. 1; Ribeiro *et al*., artigo 2).

Os resultados obtidos em nosso estudo demonstram que o efeito global da TAU em aumentar a resposta secretória da ilhota deve ocorrer por regulação da $[Ca^{2+}]i$. Estes efeitos podem ser derivados tanto do aumento da expressão de subunidades que modulam a função do canal de Ca_v, bem como da expressão de proteínas e/ou da produção de mensageiros intracelulares que contribuem para o influxo e mobilização intracelular de Ca^{2+} em ilhotas do grupo suplementado.

Suplementação com TAU e Função Secretória da Ilhota

Observamos que a suplementação com TAU alterou a sensibilidade secretória da ilhota aos nutrientes, pois além da modificação do padrão secretório de insulina, ilhotas do grupo TAU apresentaram aumento na secreção de glucagon na presença de baixa concentração de glicose (Ribeiro *et al*., artigo 1).

O processo de secreção de glucagon é regulado por nutrientes, hormônios, e pelo sistema nervoso autonômico. O estímulo para a secreção deste hormônio é a redução da concentração

circulante de glicose que é rapidamente detectada pelas células α pancreáticas. A glicose é captada por essas células através do transportador GLUT1 e, uma vez metabolizada, aumenta a relação ATP/ADP, promovendo o fechamento de um canal de K_{ATP} e elevação do potencial de membrana em repouso, que ativa canais de Na^+ sensíveis à voltagem e canais de Ca^{2+} do tipo *N* que são responsáveis pelo desenvolvimento do potencial de ação e pela exocitose dos grânulos que contém glucagon (Gromada *et al.*, 2007).

Além de agir sobre estoques de glicose, sugere-se que glucagon exerça efeito parácrino sobre a célula β, estimulando a secreção de insulina (Kawai *et al.*, 1995; Cabrera *et al.*, 2006). Várias evidências contribuem com essa idéia, pois as células β apresentam receptor para o hormônio glucagon (Moens *et al.*, 1996; Moens *et al.*, 1998), e a utilização de um antagonista para glucagon reduz a secreção de insulina em ilhotas de humanos (Huypens *et al.*, 2000). Desta forma, sugerimos que além da suplementação com TAU alterar a resposta secretória dos hormônios da ilhota, TAU possa modificar o controle parácrino dos hormônios do pâncreas endócrino, pois ilhotas do grupo TAU apresentam maior potencialização da secreção de insulina quando glucagon é adicionado ao meio de incubação.

Considerações Finais

É importante ressaltar que as modificações observadas quanto ao manejo diferenciado de íons Ca^{2+} em ilhotas do grupo suplementado demonstra que a TAU pode exercer papel relevante na preservação da função das células β em situações que resultarão no DM, já que em diferentes modelos experimentais desta doença a expressão gênica e a atividade dos canais Ca_v apresentam-se alterados (Wang *et al.*, 1996; Wang *et al.*, 1999; Iwashima *et al.*, 2001; Yang e Berggren, 2006).

Foi observado em linhagem de células secretoras de insulina (GH3) cultivadas na presença de soro de pacientes com DM 1 aumento da atividade dos canais de Ca_v, elevação da $[Ca^{2+}]i$ basal, fragmentação do DNA e destruição destas células (Juntti-Berggren *et al.*, 1993). O mesmo foi verificado em células β de camundongos NOD, as quais apresentaram a $[Ca^{2+}]i$ basal três vezes maior devido a expressão de canais Ca_v3. Estes canais apresentam baixo limiar para ativação, e não são normalmente encontrados em células β. Esta alteração parece ser decorrente do ataque de citocinas que ocorre nas células insulinares de animais NOD, já que células β de camundongos *Swiss* e a linhagem de células TC3 tratadas com interleucina 1β e interferon γ, demonstraram a expressão deste mesmo canal com aumento da $[Ca^{2+}]i$ basal e da apoptose (Wang *et al.*, 1996; Wang *et al.*, 1999). Além disso, ilhotas pancreáticas de ratos OLETF (*Otsuka Long-Evans Tokushima Fatty*) com DM 2 apresentam redução da secreção de insulina e do influxo de Ca^{2+}, e a expressão gênica das subunidades do canal de Cav: $\alpha 1.3$, β_2 e β_3 apresentam-se reduzidas (Iwashima *et al.*, 2001).

Portanto, conforme nossos dados e evidências da literatura que demonstram que a TAU participa do controle da atividade de canais iônicos (Satoh, 1996; Tricarico *et al.*, 2000; Park *et al.*, 2004) e regula a $[Ca^{2+}]i$ em diferentes tecidos (Satoh e Sperelakis, 1998; Palmi *et al.*, 1999; Chen *et al.*, 2001). Além disso, que a suplementação com TAU retarda o aparecimento do DM, eleva a taxa de proliferação e reduz a apoptose nas células β de camundongos NOD (Arany *et al.*, 2004), e no DM 2 o tratamento com TAU em ratos OLETF aumenta a tolerância à glicose e sensibilidade à insulina (Nakaya *et al.*, 2000). Sugerimos que nestes modelos a TAU participe no controle da homeostase da glicose, da manutenção da massa insular, e da integridade das estruturas celulares do pâncreas endócrino, por modulação da $[Ca^{2+}]i$, via regulação da expressão do canal de Ca_v, ou da modulação de proteínas que contribuirão para a regulação da concentração deste cátion na célula β. E como o Ca^{2+}, além de ser importante para o acoplamento estímulo/secreção, é também

fundamental para o desenvolvimento, sobrevivência, crescimento e morte das células β. A utilização de biomoléculas que contribuem para a regulação da $[Ca^{2+}]i$ no DM é essencial para a manutenção da função do pâncreas endócrino.

CONCLUSÕES

Os resultados obtidos neste estudo permitem as seguintes conclusões:

- Camundongos suplementados com TAU por 30 dias demonstram aumento da tolerância à glicose e da sensibilidade periférica à insulina;
- A suplementação com TAU aumenta a secreção de insulina, de ilhotas isoladas, em resposta a nutrientes e agentes potencializadores da secreção;
- O aumento da secreção de insulina na presença de glicose é acompanhado por uma maior captação de íons Ca^{2+}, sem alteração para o metabolismo do açúcar em ilhotas do grupo TAU;
- Aumento da captação de íons Ca^{2+} pode ser decorrente da maior expressão protéica da subunidade β_2 do canal de Ca_v;
- O conteúdo total de insulina é maior em ilhotas do grupo TAU;
- A TAU eleva a secreção de insulina em resposta à L-leucina por aumentar o seu catabolismo;
- O aumento na secreção de insulina em resposta ao Cch é possivelmente decorrente da maior expressão protéica da $PLC_{\beta 2}$ e da liberação de Ca^{2+} de estoques intracelulares em ilhotas do grupo TAU;
- Ilhotas TAU apresentam maior expressão protéica da PKAα, e aumento na secreção de insulina em resposta ao IBMX;
- A PKA deve estar envolvida com o maior influxo de Ca^{2+} em ilhotas do grupo TAU, pois a captação do cátion na presença de glicose é minimizada, nestas ilhotas, quando a atividade da PKA é inibida.

- Ilhotas TAU, apresentam aumento na secreção de glucagon na presença de baixa concentração de glicose, o que sugere que a função secretória como um todo da ilhota é modificada pela suplementação com TAU.

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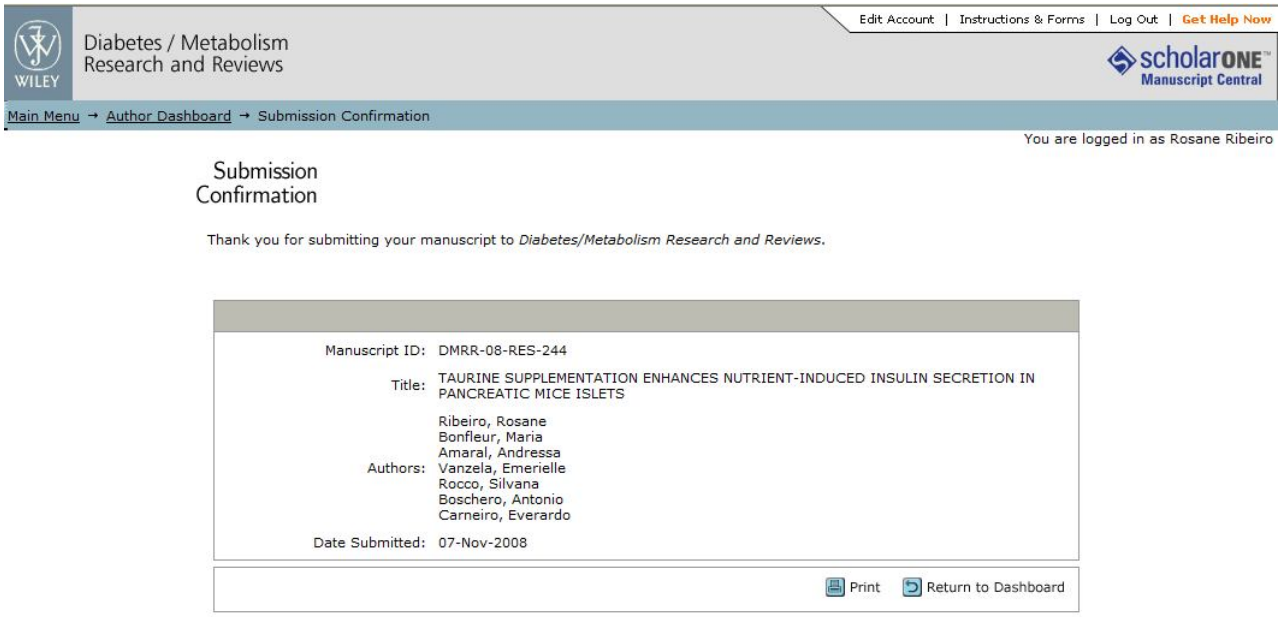
ANEXOS

Publicação do Artigo 1



02-Mar-2009

Dear Dr Ribeiro

I am pleased to inform you that your revised manuscript "TAURINE SUPPLEMENTATION ENHANCES NUTRIENT-INDUCED INSULIN SECRETION IN PANCREATIC MICE ISLETS" is accepted for publication in Diabetes/Metabolism Research and Reviews. The manuscript files you have submitted will be electronically transferred to the Production Department at John Wiley & Sons, Ltd. You will receive proofs of your article in due course via email.

Thank you for submitting your paper to Diabetes/Metabolism Research and Reviews and we will look forward to receiving additional manuscripts from you in the future.

Sincerely,

Prof. Juleen Zierath
Diabetes/Metabolism Research and Reviews

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha tese de Doutorado intitulada REGULAÇÃO DA SECREÇÃO DE INSULINA EM ILHOTAS PANCREÁTICAS DE CAMUNDONGOS SUPLEMENTADOS COM TAURINA:

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

( ) está inserido no **Projeto CIBio/IB/UNICAMP** (Protocolo n°_____), intitulado _____;

(X) tem autorização da **Comissão de Ética em Experimentação Animal/IB/UNICAMP** (Protocolo n° 1201-1);

( ) tem autorização do **Comitê de Ética para Pesquisa com Seres Humanos/FCM/UNICAMP** (Protocolo n°_____);

( ) tem autorização de comissão de bioética ou biossegurança externa à UNICAMP.
Especificar: _____

Rosane Ap. Ribeiro
Aluno: ROSANE APARECIDA RIBEIRO

_____
Orientador: EVERARDO MAGALHÃES CARNEIRO

Para uso da Comissão ou Comitê pertinente:
(X) Deferido ( ) Indeferido

Ana Maria Aparecida Guaraldo
Nome:
Função:

Profa. Dra. ANA MARIA A. GUARALDO
Presidente
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Comissão de Ética na Experimentação Animal
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CERTIFICADO

Certificamos que o Protocolo nº **1201-1**, sobre **"Secreção e sinalização insulínica e movimento do cálcio em ilhotas pancreáticas de camundongos suplementados com Taurina"**, sob a responsabilidade de **Prof. Dr. Everardo Magalhães Carneiro / Rosane Aparecida Ribeiro**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal (CEEA)-IB-UNICAMP em 27 de fevereiro de 2007.

CERTIFICATE

We certify that the protocol nº **1201-1**, entitled **"Insulin signaling and secretion, and calcium oscillations in Langerhans islets from Taurine treated mice"**, 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 February 27, 2007.

Campinas, 27 de fevereiro de 2007.

Profa. Dra. Ana Maria A. Guaraldo
Presidente

Fátima Alonso
Secretária Executiva

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