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Efeitos do ácido biliar Tauroursodesoxicólico (TUDCA) na homeostase glicêmica: Insulina e Glucagon

Campinas, 2017

Universidade Estadual de Campinas

Instituto de Biologia

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Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Biologia Funcional e Molecular, na Área de Fisiologia

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Campinas, 2017

Ficha catalográfica
Universidade Estadual de Campinas (UNICAMP)
Biblioteca do Instituto de Biologia
Mara Janaina de Oliveira - CRB 8/6972

V643e

Vettorazzi, Jean Franciesco, 1989-
Effects of the bile acid tauroursodeoxycholic (TUDCA) in glycemic homeostasis : insulin and glucagon / Jean Franciesco Vettorazzi. – Campinas, SP : [s.n.], 2017.

Orientador: Everardo Magalhães Carneiro.
Coorientadores: Rosane Aparecida Ribeiro e Ivan Quesada Moll.
Tese (doutorado) – Universidade Estadual de Campinas (UNICAMP), Instituto de Biologia.

1. Ácido tauroursodesoxicólico. 2. Insulina. 3. Glucagon. 4. Obesidade. 5. Diabetes *mellitus* tipo 2. I. Carneiro, Everardo Magalhães, 1955-. II. Ribeiro, Rosane Aparecida. III. Quesada Moll, Ivan. IV. Universidade Estadual de Campinas (UNICAMP). Instituto de Biologia. V. Título.

Informações Complementares

Título em outro idioma: Efeitos do ácido biliar tauroursodesoxicólico (TUDCA) na homeostase glicêmica : insulina e glucagon
Palavras-chave em inglês:
Acid tauroursodeoxycholic
Insulin
Glucagon
Obesity
Diabetes mellitus, Type 2
Área de concentração: Fisiologia
Titulação: Doutor em Biologia Funcional e Molecular
Banca examinadora:
Everardo Magalhães Carneiro [Orientador]
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Licio Augusto Velloso
Bruno Geloneze Neto
Data de defesa: 18-08-2017
Programa de Pós-Graduação: Biologia Funcional e Molecular

‘’Ser de vuelo tan alto,

tan extendido,

que tu carne parece

cielo cernido’’

Miguel Hernandez

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Lista de abreviações

6E-CDCA: 6-Ethyl-chedeoxycholic acid

ADP: Adenosine diphosphate

AKT or PTB: Protein kinase B

ATP: Adenosine triphosphate

AUC: Area under curve

BSA: Bovine serum albumin

cAMP: Cyclic adenosine monophosphate

CREB: cAMP response element-binding protein

DZX: Diazoxide

FXR: Farnesoid X Receptor

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GLP-1: Glucagon-like peptide 1

GLUT-2: Glucose transporter 2

GSIS: Glucose-stimulated insulin secretion

H89: Protein kinase A inhibitor

INT-777: 6-Alpha-ethyl-23(S)-methyl-cholic acid

KATP: ATP-sensitive K+ channel

KLF 11: Kruppel-like factor 11

NAD(P)H: Nicotinamide adenine dinucleotide phosphate

NF449: Gsα-subunit G protein antagonist

OA: Oleanolic acid

OCA: Obeticholic acid

PKA: Protein kinase A

Rp-cAMPS: Competitive inhibitor of the activation of cAMP-dependent protein kinases by cAMP

TCDC: Taurochenodeoxycholic acid

TGR5: G protein-coupled bile acid receptor 1

TβMCA: Tauro β-Muricholic acid

TUDCA: Tauroursodeoxycholic acid

UDCA: Ursodeoxycholic acid

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Resumo

A obesidade e o diabetes mellitus tipo 2 (DM2) vem crescendo consideravelmente em todo mundo, devido principalmente as alterações no estilo de vida e consumo de dietas hipercalóricas. O DM2 caracteriza-se por uma falha na secreção e ação da insulina, além de elevação nos níveis de glucagon, culminando em hiperglicemia e desenvolvimento de complicações cardíacas, renais, visuais, etc. Diversas estratégias que modulam a secreção e ação da insulina/glucagon vem sendo estudadas no tratamento do DM2 e obesidade, dentre os quais destacamos os ácidos biliares. Os ácidos biliares são compostos derivados do colesterol que auxiliam no processo digestivo e, atualmente, são reconhecidos como sinalizadores endócrinos que regulam o metabolismo energético, glicêmico e lipídico. O ácido biliar conjugado a taurina (TUDCA) apresenta atividade de chaperona química, levando a redução do estresse de reticulo em diversos tipos celulares, incluso células beta pancreáticas. Contudo, o papel do TUDCA na manutenção da homeostase glicêmica não foi explorado. Neste trabalho, usando camundongos C57BL6 submetidos ou não a dieta hiperlipidica, buscamos investigar os efeitos do TUDCA na secreção e degradação de insulina, bem como na secreção de glucagon. A exposição de ilhotas pancreáticas ao TUDCA leva a uma potencialização na secreção de insulina estimulada por glicose, efeito não acompanhado de alterações eletrofisiológicas mas dependente do aumento intracelular de AMPc. Em camundongos tratados com dieta hiperlipídica e suplementados com TUDCA durante 15 dias, observamos melhora na tolerância a glicose e sensibilidade a insulina, acompanhado de aumento na expressão hepática da IDE e degradação da insulina. Finalmente, a exposição de ilhotas pancreáticas, assim como linhagem de células alfa pancreáticas α-TC1-9, leva a uma redução na secreção de glucagon frente a glicose, efeito acompanhado de redução nas oscilações intracelulares de cálcio e abertura dos canais de K_{ATP}. Portanto, concluímos que o TUDCA leva ao aumento na secreção de insulina estimulada por glicose, além de regular o clearance de insulina via aumento da expressão hepática da IDE. Além disso, o TUDCA contribui na manutenção da homeostase glicemia via redução na secreção de glucagon estimulada por glicose, demonstrando ser uma possível ferramenta no tratamento da obesidade e DM2.

Abstract

Obesity and type 2 diabetes mellitus (DM2) have been growing considerably worldwide, mainly due to changes in lifestyle and consumption of hypercaloric diets. DM2 is characterized by a failure in insulin secretion and signaling, besides elevation in glucagon levels, culminating in hyperglycemia and the development of cardiac, renal, visual complications, etc. Several strategies that modulate the secretion and action of insulin / glucagon have been studied in the treatment of DM2 and obesity, among which we highlight the bile acids. Bile acids are cholesterol-derived compounds that help in the digestive process and are now recognized as endocrine signals that regulate energy, glycemic and lipid metabolism. Taurine-conjugated bile acid (TUDCA) exhibits chemical chaperone activity, leading to the reduction of reticulum stress in several cell types, including pancreatic beta cells. However, the role of TUDCA in the maintenance of glycemic homeostasis has not been explored. In this study, using C57BL6 mice submitted or not the high fat diet, we sought to investigate the effects of TUDCA on insulin secretion and degradation, as well as on glucagon secretion. Exposure of pancreatic islets to TUDCA potentiates glucose-stimulated insulin secretion, an effect not accompanied by electrophysiological changes but dependent on intracellular increase in cAMP. In mice treated with a high fat diet supplemented with TUDCA for 15 days, we observed an improvement in glucose tolerance and insulin sensitivity, accompanied by increased hepatic expression of IDE and insulin degradation. Finally, exposure of pancreatic islets, as well as the lineag α-TC1-9, reduces glucagon secretion stimulated by glucose, an effect accompanied by reduction in intracellular calcium oscillations and opening of K_{ATP} channels. Therefore, we conclude that TUDCA leads to increased glucose-stimulated insulin secretion and regulates insulin clearance through increased hepatic expression of IDE. In addition, TUDCA contributes to the maintenance of glycemic homeostasis through reduction in glucagon secretion stimulated by glucose, proving to be a possible tool in the treatment of obesity and DM2.

1. Introdução

1.1 Homeostase Glicêmica

A glicose é a principal fonte de energia utilizada pelas células de mamíferos, sendo que o sistema nervoso central, glóbulos brancos e vermelhos, células da córnea e retina utilizam exclusivamente este carboidrato como fonte de energia (*1-4*). Este açúcar apresenta uma fina regulação hormonal pois seu excesso leva a danos celulares e teciduais, e o desenvolvimento de enfermidades como o diabetes mellitus (DM) (*5, 6*). Frente a isso, nosso organismo conta com um conjunto de hormônios que regulam a concentração plasmática de glicose, dentre os quais destacamos a insulina e glucagon, com ações hipoglicemiante e hiperglicemiante, respectivamente (*7, 8*).

A secreção de insulina pelas células beta é controlada continuamente de acordo com a concentração de nutrientes circulantes, em especial, a glicose (*9*). Frente a um aumento na glicemia, ocorre o transporte deste açúcar pelas células beta pancreáticas, através do transportador de glicose (GLUT 2); a glicose então é fosforilada à glicose-6-fosfato pela enzima glicoquinase (GCK) e metabolizada gerando ATP. O resultado é o aumento da relação ATP/ADP, que promove o fechamento dos canais de K^+ sensíveis ao ATP (K_{ATP}), presentes na membrana da célula beta. A redução do efluxo de K^+ resulta em despolarização da membrana e abertura de canais de Ca^{2+} sensíveis à voltagem, resultando no influxo deste íon (*9-12*). O Ca^{2+} é responsável pela extrusão dos grânulos contendo insulina, através da ativação do complexo proteico SNARE (Soluble NSF Attachment Protein Receptor) (*13, 14*). Além da glicose, outros metabólitos como os aminoácidos, ácidos graxos e corpos cetônicos estimulam a secreção de insulina (*15-18*). Ainda, a secreção deste hormônio pode ser modulada por neurotransmissores e hormônios gastrintestinais (*13, 19*). O glucagon e a somatostatina, também secretados pela ilhota pancreática, exercem ação estimulatória e inibitória, respectivamente, sobre a secreção das células beta (*20*).

Uma vez secretada, os efeitos principais da insulina são no fígado, onde inibe a produção hepática de glicose, através das enzimas glicose-6-fosfatase (G6P) e fosfoenolpiruvato carboxilase (PEPCK) (*21*), e no músculo e tecido adiposo, onde estimula a translocação do transportador de glicose do tipo 4 (GLUT-4) para a membrana celular, aumentando assim a captação de glicose (*22-24*). A insulina age

através do receptor de insulina, uma proteína transmembrana com atividade tirosina quinase que, uma vez ativado, sofre autofosforilacao e ativa cascatas de sinalização intracelular. As principais proteínas ativadas pela via da insulina são PI3K e Akt, envolvidas com a translocação do GLUT-4, além de proliferação e sobrevivência celular (*21*). No fígado também ocorre a degradação de insulina, através da enzima IDE (Insulin Degrading Enzyme) (*25*). A IDE pertence à classe de metalopeptidases, e sua ativação depende da presença do íon zinco. Esta proteína é constitutiva, presente em todos tipos celulares, e também no meio extracelular. Cerca de 50% da insulina secretada pelo pâncreas é degradada em sua primeira passagem pelo fígado, além da IDE degradar outros peptídeos como glucagon e amilina. Uma vez ligada ao seu receptor, a insulina é internalizada e sofre degradação em vesículas endocíticas através da IDE (*26*).

Por outro lado, a redução da concentração de glicose no plasma estimula as células α a secretam o glucagon, que promoverá ações para elevação da glicemia (*8*). A secreção de glucagon apresenta um acoplamento estímulo-secreção semelhante ao observado na secreção de insulina. A redução da concentração extracelular de glicose estimula a célula α, que transporta a glicose via transportador de glicose do tipo 1 (GLUT-1), e em sequência metaboliza este açúcar. Ocorre consequente aumento na razão ATP/ADP, o que promove o fechamento dos canais de K_{ATP}. A redução do efluxo de K^+ promove elevação da voltagem da membrana da célula α, resultando na abertura dos canais de Ca^{2+}do tipo T. A entrada de Ca^{2+} por estes canais altera ainda mais o potencial de membrana, e abre os canais de Na^+. O influxo de Na^+ é necessário para abertura dos canais de Ca^{2+} tipo L e N, principais responsáveis pela entrada de Ca^{2+} e a ativação da maquinaria exocitótica dos grânulos contendo glucagon (*27-30*). Além da glicose, a secreção de glucagon pode ser estimulada por adrenalina e alguns aminoácidos como arginina.(*8*)

O glucagon liberado age principalmente no fígado, onde ativa receptores acoplados à proteína G estimulatória (Gs). Algumas das ações do glucagon também podem ocorrer via receptores acoplados à proteína Gq, ativação da fosfolipase C e consequente formação de IP3 e DAG. A ação resultante do hormônio é estimular a glicogenólise por fosforilar e ativar a glicogênio fosforilase e fosforilar e inibir a glicogênio sintase, bem como aumentar a expressão das enzimas chaves da gliconeogênese: glicose-6-fosfatase (G6P) e fosfoenolpiruvato carboxilase (PEPCK)

(*31-33*). Dessa forma, as ações combinadas do glucagon resultam em aumento da produção hepática de glicose. A degradação do glucagon ainda não está esclarecida, mas sabe-se que a IDE participa deste processo (*25*).

1.2 Obesidade e Diabetes Mellitus

A obesidade e as doenças crônicas não transmissíveis têm se tornado uma epidemia em todo mundo, levando os governos a gastarem milhões em saúde pública por ano (*34*). O surgimento destas doenças está relacionado principalmente com alterações no estilo de vida da população, como aumento na ingesta de dietas rica em gorduras, redução ou substituição da atividade física por atividades sedentárias como os equipamentos eletrônicos, uso de tratamentos farmacológicos que levam ao ganho de peso, além do sono inadequado (*35, 36*) . Além disso, o desenvolvimento da obesidade e suas comorbidades pode ter um caráter genético, uma vez que o índice de massa corporal apresenta uma hereditariedade de 40 a 70% (*37*).

A obesidade medeia inúmeras complicações sistêmicas, e é caracterizada por um estado de inflamação crônico. O excesso na ingesta calórica leva a hipertrofia dos adipócitos, disfunção mitocondrial, estresse oxidativo e estresse de retículo, estimulando a liberação de citocinas proinflamatorias, adipocinas e consequente apoptose (*38, 39*). Este ambiente afeta os tecidos periféricos, induzindo resistência periférica a insulina, hiperinsulinemia, dislipidemias, estresse celular, etc (*38, 40*).

O excesso de peso está intimamente relacionado com alterações na homeostase glicêmica (*41*). O ambiente inflamatório induz resistência à insulina no fígado e tecidos periféricos, juntamente com hipersecreção de insulina pelas células beta pancreáticas (*41, 42*). A hiperinsulinemia também leva a disfunção na cascata de sinalização da insulina, uma vez que a incubação de tecidos insulino-responsivos com insulina reduz a atividade tirosina quinase do seu receptor (*43, 44*). A hiperinsulinemia, associada a resistência à insulina e uma moderada hiperglicemia é conhecido como pré-diabetes (*45, 46*). Modelos de pré-diabetes também apresentam redução na degradação da insulina, via redução na expressão proteica da IDE. Este efeito reduz o clearance de insulina e contribui para a hiperinsulinemia (*47, 48*).

A alta demanda na secreção de insulina observada no pré-diabetes induz a apoptose das células beta pancreáticas. Este efeito, associado com a resistência à

insulina, resulta no desenvolvimento do DM2 (*41, 49*). A degradação de insulina também se encontra reduzida no DM2. Pacientes diabéticos apresentam 24% de redução no clearance de insulina (*25, 50, 51*). Além disso, camundongos knockout para a IDE hepática apresentam hiperinsulinemia e posterior desenvolvimento do DM2 (*51*).

O DM2 afeta também a secreção de glucagon pelas células alfa pancreáticas. Pacientes diabéticos apresentam uma falha na supressão da secreção de glucagon estimulada por glicose, uma vez que possuem elevados níveis do hormônio mesmo em hiperglicemia (*27, 41*). As células alfa pancreáticas perdem a capacidade de responder a altas concentrações de glicose, e apresentam resistência à insulina, principal regulador negativo da secreção de glucagon (*29*). A hiperglucagonemia contribui para o aumento na produção e liberação hepática de glicose, elevando a glicemia (*30*).

1.3 Ácidos biliares (BAs) e homeostase glicêmica

Os BAs são compostos sintetizados nos hepatócitos, a partir da molécula de colesterol. Os BAs são primeiramente sintetizados na forma de BAs primários, sendo o ácido cólico (CA) e quenodeoxicólico (CDCA) os principais em humanos, e o ácido muricólico (BMCA) e ácido cólico (CA) em camundongos (*52, 53*). Uma vez sintetizados, os BAs são conjugados ao aminoácido taurina ou glicina através das enzimas BAC (Bile acid:CoA synthase) e BAAT (bile acid:amino acid transferase), efeito que reduz sua toxicidade e aumenta sua solubilidade (*52*) . Apesar de apresentam um caráter anfipático, os BAs podem ser divididos em 2 grupos de acordo com sua afinidade pela água. Os hidrofóbicos compreendem principalmente BAs como CDCA e CA, que possuem facilidade em cruzar a membrana plasmática e são mais tóxicos. Por outro lado, os BAs hidrofílicos, composto pelas formas conjugadas a taurina e/ou glicina, tem maior afinidade por receptores transmembrana uma vez que apresentam dificuldade para cruzar a mesma (*52*).

Uma vez sintetizados, esses compostos são direcionados por ductos hepáticos até a vesícula biliar onde ficam armazenados. A presença de ácidos graxos da dieta no intestino delgado estimula a secreção de colecistocinina, hormônio responsável pelas contrações da vesícula biliar e liberação da bile contendo os BAs (*54-56*). No intestino, os BAs são fundamentais na absorção de lipídios e vitaminas lipossolúveis, uma vez

que agem como detergentes químicos e aumentam a superfície de contato dos lipídios através da formação de micelas (*57*).

Atualmente, os BAs têm se destacado não apenas como moléculas que auxiliam o processo de absorção de gorduras da dieta, mas como sinalizadores endócrinos que regulam o metabolismo glicêmico, lipídico e energético. Este efeito ocorre devido a ativação de receptores intracelulares e transmembrana para BAs localizados em praticamente todos os tipos celulares. Dentre os receptores ativados pelos BAs, destacamos o FXR (Farnesoid X receptor), TGR5 (Takeda G Receptor) e S1PR2 (Sphingosin 1 phosphate receptor 2) (*53*).

O FXR é um receptor com localização citossólica, apresentando maior afinidade por BAs hidrofóbicos (*54*). O FXR regula o metabolismo hepáticos dos BAs, uma vez que a ativação deste receptor nos hepatócitos reduz a expressão da enzima Cyp7a1 (7-α-hidroxilase), inibindo a síntese de BAs (*55, 58*). No metabolismo glicêmico, o FXR regula a secreção de insulina uma vez que a ativação deste receptor em células beta pancreáticas potencializa a secreção de insulina, via fechamento dos canais de K_{ATP} (*59*). A linhagem de células beta βTC6 incubadas por 18 horas com 6E-CDCA, um agonista especifico do FXR, aumenta a transcrição gênica e secreção de insulina, além de aumentar a ativação da Akt e translocação do GLUT-2 para a membrana celular (*60*). Camundongos knockout para o FXR em todos tecidos apresentam aumento nos depósitos hepáticos de lipídios e ácidos graxos livres circulantes, associado com aumento na glicemia, resistência à insulina e intolerância à glicose (*61*).

O TGR5 é um receptor transmembrana com maior afinidade para BAs hidrofílicos (*56, 62*). No fígado, o TGR5 não é expresso em hepatócitos, mas sua ativação protege os coglanciócitos contra apoptose induzida por BAs (*63*). Em células beta pancreáticas, a ativação deste receptor potencializa a secreção de insulina estimulada por glicose (*64, 65*). Já em células alfa pancreáticas, o uso do agonista do TGR5, INT-777, em camundongos diabéticos db/db aumenta a conversão do pró-glucagon em GLP-1, efeito que reduz a concentração plasmática de glucagon (*66*). Este receptor encontra-se expresso também no intestino, onde estimula a secreção de GLP-1 nas células do tipo L (*67*). Já no tecido adiposo marrom, a ativação do TGR5 aumenta o metabolismo energético, através do aumento na atividade da enzima deiodinase-2, que converte T4 em sua forma ativa, T3 (*68*). O tratamento de camundongos diabéticos com

INT-777 melhora a sensibilidade a insulina e tolerância a glicose, além de reduzir lesões renais (*69*).

Finalmente, o receptor S1PR2 também apresenta maior afinidade por BAs hidrofílicos taurina (*70*) . Este receptor apresenta uma localização transmembrana, e sua ativação desencadeia cascatas de sinalização intracelular que ativam, por exemplo, a via da insulina (*70*). No fígado, a ativação do S1PR2 ativa as proteínas Erk e Akt , que regulam genes relacionados com o metabolismo lipídico (*71*). O knockout para o S1PR2 previne parcialmente os efeitos deletérios do tratamento com streptozotocina em camundongos, com redução nos níveis de glicose plasmática e apoptose das células beta pancreáticas (*72*). Em células alfa pancreáticas, o papel do S1PR2 ainda não foi explorado.

A obesidade e DM2 levam a uma alteração no *pool* de BAs, efeito que reflete em prejuízos na sinalização intracelular ativada por seus respectivos receptores (*53, 73*). Pacientes diabéticos apresentam aumento de até duas vezes no *pool* de BAs. Contudo, este *pool* contém maior concentração de BAs hidrofóbicos e não as formas hidrofílicas, que compreendem os conjugados a taurina e glicina (*74*) . Camundongos diabéticos db/db apresentam maior acetilação de histonas no promotor do gene Cyp7a1, enzima chave na regulação da síntese de BAs. Este feito aumenta a expressão da Cyp7a1 e o *pool* de BAs, contudo com maior concentração de formas hidrofóbicas. Apesar de apresentarem aumento no *pool* total de BAs, a obesidade e diabetes alteram a natureza destes compostos.

1.4 Ácido biliar Tauroursodesoxicólico (TUDCA)

O TUDCA é formado nos hepatócitos a partir da conjugação do ácido biliar ursodesoxicólico (UDCA) com o aminoácido taurina (Tau). O UDCA e seus conjugados perfazem 60% do conteúdo total da bile de ursos, sendo evidenciado que o TUDCA compreende apenas 0,13% do conteúdo total de ácidos biliares no soro de humanos(*75, 76*).

Na farmacologia asiática a primeira descrição do uso do UDCA e TUDCA no tratamento de doenças hepáticas foi relatada no Japão e se tornou comum logo após o início de sua síntese, a partir de ácidos cólicos, em meados dos anos 1950. O UDCA foi inicialmente prescrito para dissolução de cálculos biliares, uma vez que esse ácido biliar

promove solubilização do colesterol e redução de sua saturação na bile. Outra recomendação para uso do UDCA e TUDCA é para tratamento de doença hepática colestática, principalmente cirrose biliar primária. No entanto, seu uso no tratamento de doenças hepáticas cresceu incrivelmente após a descoberta que o tratamento melhora a ação de aminotransferases em pacientes com hepatite crônica (*52, 77*).

O TUDCA apresenta atividade de chaperona química, efeito que melhora o enovelamento proteica e reduz a expressão de marcadores de estresse de retículo em células musculares, cardíacas, auditivas, pancreáticas, pulmonares, etc (*77-81*). Linhagem de células Huh7 derivadas de fígado humano tratadas com tapsigargina, uma droga indutora de estresse de retículo (RE), e incubadas com 100 μM de TUDCA por 18 à 24 h apresentaram redução da expressão de proteínas envolvidas no estresse de retículo endoplasmático (RE) e a fragmentação do DNA via inibição da ativação das proteínas caspase 3 e 7 (*80*). Camundongos ob/ob tratados com 150 mg/Kg de peso corpóreo de TUDCA por 21 dias apresentaram redução do peso corporal, da glicemia, e aumento do gasto energético via aumento da sinalização da leptina no hipotálamo (*79, 82*). O tratamento de camundongos com dieta hiperlipídica associado ao TUDCA reverte alterações no metabolismo glicolipídico hepático (*83*). Além disso, o TUDCA reduziu a expressão dos marcadores de estresse de RE: p-PERK (protein kinase RNA-like endoplasmic reticulum kinase) e ATF-6 e preveniu a redução da expressão do mRNA da pré-pró-insulina de ilhotas isoladas cultivadas por 48h na presença de altas concentrações de glicose (*78*). Além disso, o TUDCA preservou a secreção de insulina e reduziu a apoptose celular de ilhotas isoladas de suínos, tratadas com tapsigargina .

Yang et al (2012) mostraram que o tratamento com TUDCA por 3 semanas (500 mg/Kg de peso corpóreo) via gavagem diminuiu a esteatose hepática em camundongos ob/ob via regulação de vários genes hepáticos envolvidos na regulação da lipogênese. Ainda, camundongos ob/ob tratados com o TUDCA apresentaram menor expressão das proteínas da via de estresse de RE: PERK e JNK (c-Jun amino-terminal kinase) no fígado. Estes camundongos obesos que receberam TUDCA também apresentaram maior sensibilidade periférica à insulina via melhora da ativação da via IRβ/IRS-1 e IRS-2/p-Akt no fígado e tecido adiposo (*79*).

A melhora metabólica evidenciada em modelos experimentais de obesidade que é promovida pelo TUDCA pode estar associada também à ação de hormônios tireoidianos. Sabe-se que nos tecidos periféricos a tiroxina (T4) é convertida a

triidotironina (T3) pela ação da desiodinase 2 (D2). Evidências da literatura demonstraram que o tratamento de 7 à 48hrs com TUDCA induz aumento da atividade da D2 e, portanto do T3 em células MSTO-211H. A cultura primária de células adiposas marrons na presença de TUDCA por 24h apresentou aumento da ação da D2 e do consumo de oxigênio, não tendo efeito sobre células que não expressavam a D2 (*68*).

Os BAs têm se destacado como moléculas sinalizadores com ação endócrina. O tratamento de camundongos obesos e diabéticos com BAs, principalmente as formas conjugadas a taurina, além de alterações no *pool* de BAs relatado em indivíduos obesos e diabéticos, aponta a importância destes compostos na regulação da homeostase glicêmica. O TUDCA age principalmente em vias relacionadas ao estresse de retículo, reduzindo apoptose e aumentando a viabilidade celular. O TUDCA também melhora a homeostase glicêmica em camundongos obesos, efeito associado à redução do estresse de retículo hepático e aumento da sinalização de insulina. Contudo, os efeitos deste composto na secreção e degradação de insulina, dois processos fundamentais na manutenção da normoglicemia, não foi explorado; assim como os efeitos deste composto na secreção de glucagon, principal hormônio hiperglicemiante.

2. Objetivos

1. Investigar os efeitos do TUDCA na secreção de insulina em células beta pancreáticas de camundongos (Artigo 01)
2. Investigar os efeitos do TUDCA na secreção e degradação de insulina em camundongos submetidos à dieta hiperlipídica, assim como em linhagem celular hepática HepG2 (Artigo 02)
3. Investigar os efeitos do TUDCA na secreção de glucagon em células alfa pancreáticas de camundongos e na linhagem celular αTC1-9 (Artigo 03)

3. Artigo 01

THE BILE ACID TUDCA INCREASES GLUCOSE-INDUCED INSULIN SECRETION VIA THE cAMP/PKA PATHWAY IN PANCREATIC BETA CELLS

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3.1 Abstract

Objective: While bile acids are important for the digestion process, they also act as signaling molecules in many tissues, including the endocrine pancreas, which expresses specific bile acid receptors that regulate several cell functions. In this study, we investigated the effects of the conjugated bile acid TUDCA on glucose-stimulated insulin secretion (GSIS) from pancreatic β-cells.

Methods: Pancreatic islets were isolated from 90-day-old male mice. Insulin secretion was measured by radioimmunoassay, protein phosphorylation by western blot, Ca2+ signals by fluorescence microscopy and ATP-dependent K+ (KATP) channels by electrophysiology.

Results: TUDCA dose-dependently increased GSIS in fresh islets at stimulatory glucose concentrations but remained without effect at low glucose levels. This effect was not associated with changes in glucose metabolism, Ca2+ signals or KATP channel activity; however, it was lost in the presence of a cAMP competitor or a PKA inhibitor. Additionally, PKA and CREB phosphorylation were observed after 1-hour incubation with TUDCA. The potentiation of GSIS was blunted by the Gα stimulatory, G protein subunit-specific inhibitor NF449 and mimicked by the specific TGR5 agonist INT-777, pointing to the involvement of the bile acid G protein-coupled receptor TGR5.

Conclusion: Our data indicates that TUDCA potentiates GSIS through the cAMP/PKA pathway.

Keywords: β-cell, bile acids, insulin secretion, TUDCA

3.2 Introduction

Bile acids are molecules derived from cholesterol and synthesized in hepatocytes. They facilitate the digestion and absorption of dietary lipids and fat-soluble vitamins and regulate cholesterol excretion and sterol homeostasis. Before secretion into the gallbladder and duodenum, bile acids undergo a conjugation process with glycine or taurine, which increases their solubility and decreases the toxicity of these compounds [1, 2, 3]. In addition to the digestive function of bile acids, the discovery of bile acid receptors in the last couple of years has emphasized their role as extracellular messengers, which produce both genomic and non-genomic effects through multiple signaling pathways [1, 2, 4, 5]. Many tissues, including the endocrine pancreas, express bile acid receptors [6, 7]. The most important of these receptors are the nuclear receptor Farnesoid X Receptor (FXR) and the G protein-coupled bile acid receptor TGR5 [1, 2, 8].

The activation of FXR can regulate several processes in pancreatic β-cells. In the insulin-producing cell line βTC6, the FXR agonist 6-ethyl-chenodeoxycholic acid (6E-CDCA) increased the expression of insulin and the glucose-regulated transcription factor KLF11. It also induced AKT phosphorylation and GLUT-2 translocation to the plasma membrane, promoting glucose uptake [10]. The activation of FXR by the taurine-conjugated bile acid taurochenodeoxycholic acid (TCDC) increased glucose-stimulated insulin secretion (GSIS) in isolated mouse islets. This effect was associated with the inhibition of ATP-dependent K+ (KATP) channels, changes in β-cell electrical activity, and increased Ca2+ influx [7]. The use of FXR ligands has also been explored in the treatment of glucose homeostasis disorders. The FXR ligand 6-ethyl-chenodeoxycholic acid (6E-CDCA) decreased glucose, triglyceride and cholesterol levels in db/db mice and Zucker fa/fa rats, improving glucose homeostasis in these diabetic models [8]. The FXR agonist obeticholic acid (OCA) ameliorated insulin sensitivity and the metabolic profile in patients with type-2 diabetes [10]. Activation of the G protein-coupled bile acid receptor TGR5 can also regulate pancreatic β-cell function. The TGR5 ligands oleanolic acid (OA) and INT-777 stimulated GSIS in the insulin-producing cells MIN-6 and human islets [6]. This effect depended on the

activation of the Gα stimulatory TGR5 subunit, increasing adenylyl cyclase activity, cAMP levels, and cytosolic Ca2+ concentrations [6]. In rodents, synthetic TGR5 agonists diminished plasma glucose and insulin levels and protected against high-fat diet-induced obesity [11]. TGR5 was also shown to be involved in glucose homeostasis through stimulation of the incretin glucagon-like peptide 1 (GLP-1) secretion [12, 13].

Although bile acids have recently been shown to be signaling messengers that are able to regulate some cellular processes in the endocrine pancreas, there is little information regarding their receptors, their molecular mechanisms and the actions involved. In this study, we analyzed the effects of the taurine-conjugated bile acid tauroursodeoxycholic acid (TUDCA) on the insulin secretory function of pancreatic β-cells. TUDCA and ursodeoxycholic acid (UDCA) are used for the treatment of different liver diseases, such as primary biliary cirrhosis and cholesterol gallstones, but they also seem to have therapeutic potential in non-liver diseases, such as neurological, retinal, metabolic and myocardial disorders [14, 15]. These effects seem to be associated with their anti-apoptotic properties. Additionally, studies in experimental models of obesity have reported that TUDCA can act as a chemical chaperone that ameliorates insulin resistance by reducing endoplasmic reticulum stress and the unfolded protein response [16]. Here, we show that TUDCA potentiates GSIS in pancreatic β-cells, likely through the bile acid receptor TGR5 and activation of the cAMP/PKA pathway.

3.3 Materials and methods

Reagents. TUDCA was purchased from Calbiochem (São Paulo, SP, BRA, cat. 580549), and 125I was purchased from Genesis (São Paulo, SP, BRA). Western Blot reagents were purchased from Bio-Rad (Madrid, Spain), and antibodies were purchased from Cell Signaling (Barcelona, Spain). The remaining reagents were purchased from Sigma Chemical (St. Louis, MO, USA).

Animals. All experiments involving animals were approved by the Animal Care Committee at UNICAMP (License Number: 2234-1) and Miguel Hernández University (ref. UMH.IB.IQM.01.13). Male 90-day-old C57Bl/6 mice were obtained from the breeding colony at UNICAMP and UMH and were maintained at 22 ± 1ºC on a 12-h light–dark cycle with free access to food and water. Mice were euthanized in a CO2

chamber and decapitated for pancreatic islet isolation by collagenase digestion of the pancreas, as previous described [17].

Insulin secretion. For static insulin secretion, pancreatic islets (4 islets per well) were incubated for 30 min with Krebs-Bicarbonate buffer (KBB; (in mM) 115 NaCl, 5 KCl, 2.56 CaCl2, 1 MgCl2, 10 NaHCO3, 15 HEPES), supplemented with 5.6 mM glucose and 0.3 % BSA and equilibrated with a mixture of 95 % O2/5 % CO2 to regulate the pH at 7.4. After 30 min of preincubation time, the medium was removed and immediately replaced with fresh KBB medium containing different glucose and TUDCA concentrations, as well as the different reagents indicated in the experiments. After 1 h of incubation time, the medium was removed and stored at –20°C. For islet insulin content, groups of four islets were collected and transferred to tubes containing 1 mL of deionized water, and the islet cells were homogenized using a sonicator (Brinkmann Instruments, USA). Insulin levels were measured by a radioimmunoassay (RIA). Total islet protein was assayed using the Bradford dye method [18] with BSA as the standard curve.

Cytoplasmic Ca2+ oscillations and NAD(P)H fluorescence. For cytoplasmic Ca2+ oscillations, fresh isolated islets were incubated with fura-2 acetoxymethyl ester (5 μmol/L) for 1 hour at 37ºC in KBB buffer that contained 5.6 mM glucose, 0.3 % BSA and pH 7.4. Islets were then washed with the same medium and placed in a chamber that was thermostatically regulated at 37ºC on the stage of an inverted microscope (Nikon UK, Kingston, UK). Islets were perfused with albumin-free KBB that was continuously gassed with 95 % O2/5 % CO2 (pH 7.4). A ratio image was acquired every 5 s 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), which was equipped with 340 and 380 nm, 10 nm bandpass filters and a range of neutral density filters (Omega opticals, Stanmore, UK). Ca2+-dependent fluorescence in the recordings was displayed as the ratio F340/F380. The analysis was obtained using ImageMaster3 software (Photon Technology International, NJ, USA) [19]. Some data were represented as the area under the curve (AUC) of the last 10 min of the stimuli as a measure of the global Ca2+ entry [20]. NAD(P)H fluorescence was monitored using the same above-mentioned system, but fresh islets were excited with a 365-nm band pass filter, and the emission was filtered at 445 ± 25 nm [21]. An image was acquired every 60 sec.

Western blot analysis. Groups of 250 isolated islets were incubated in KBB medium containing 11.1 mM glucose and 50 μM TUDCA. Islets were then homogenized with 9 μL of Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA) and incubated for 0, 10, 20, 30 and 60 min in the conditions indicated in the figure legends. For SDS gel electrophoresis and western blot analysis, the samples were treated with a Laemmli sample buffer containing dithiothreitol. After heating to 95ºC for 5 min, the proteins were separated by electrophoresis in a 4¬–20% Mini Protean Gel (Bio-Rad, Hercules, CA, USA). Prestained SDS-PAGE standards were included for molecular mass estimation. Transfer to PVDF membranes was performed in a Trans Blot Turbo transfer for 7 min at 25 V with TRIS/glycine buffer (Bio-Rad, Hercules, CA, USA). After the membranes were blocked with 5% non-fat dry milk buffer (5% milk, 10 mM TRIS, 150 mM NaCl and 0.02% Tween 20), they were incubated with a polyclonal antibody against phosphorylated (p)-CREBSer133 (1:1000; Cell Signaling #9198), CREB (1:1000; Cell signaling #4820), pPKA CThr197 (1:1000; Cell Signaling #5661), PKA C-α (1:1000; Cell signaling #4782) or GAPDH (1:1000; Cell Signaling #5174). GAPDH was used as a control for the experiment. The visualization of specific protein bands was performed by incubating the membranes with the appropriate secondary antibodies. Protein bands were revealed by using the Chemi Doc MP System (Bio-Rad, Hercules, CA, USA), which detects the chemiluminescence. The band intensities were quantified with Image Lab Lale 4.1 TM Software (Bio-Rad, Hercules, CA, USA).

Patch-clamp recordings

Islets were dispersed into single cells and cultured as previously described [22]. KATP channel activity was recorded using standard patch-clamp recording procedures. Currents were recorded by using an Axopatch 200B patch-clamp amplifier (Axon Instruments Inc., Union City, CA). Patch pipettes were pulled from borosilicate capillaries (Sutter Instrument Co., Novato, CA) using a flaming/brown micropipette puller P-97 (Sutter Instrument Co.) with resistance between 3 and 5 M when filled with pipette solutions, as specified below. The bath solution contained 5 mM KCl, 135 mM NaCl, 2.5 mM CaCl2, 10 mM HEPES, and 1.1 mM MgCl2 (pH 7.4), and it supplemented with glucose as indicated. The pipette solution contained 140 mM KCl, 1 mM MgCl2, 10 mM HEPES and 1 mM EGTA (pH 7.2). The pipette potential was held at 0 mV throughout the recording process. KATP channel activity was quantified by

digitizing 60 sec sections of the current record filtered at 1 kHz and sampled at 10 kHz by a Digidata 1322A (Axon Instruments Inc., Orleans Drive Sunnyvale, CA, USA) and calculating the mean NPo during the sweep. Channel activity was defined as the product of N, the number of functional channels, and Po, the open state probability. Po was determined by dividing the total time channels spent in the open state by the total sample time. Values of NPo were normalized relative to the channel activity measured in control conditions before the application of different substances. Data sampling was initiated 1 min before (control) and 10–15 min after the application of the test substances. Experiments were carried out at room temperature (20–24ºC). These experiments were performed at 8 mM glucose, since at 11.1 mM glucose concentrations the majority of KATP channels are closed [20, 21, 22].

2.7 Statistical analysis. The results are presented as the mean ± SEM for the number of determinations (n) indicated. Statistical analysis was performed using Student's t test or ANOVA with the appropriate post-test using Graph Pad Prism 5.0 software (La Jolla, CA, USA).

3.4 Results

TUDCA stimulates insulin secretion in isolated islets. Mouse pancreatic islets incubated with TUDCA released more insulin than controls in a glucose-dependent manner. Although this bile acid had no effect at low concentrations, it increased glucose-induced insulin secretion (GSIS) at concentrations higher than 10 μM (Fig. 1). To address the mechanisms involved in the effects of TUDCA on GSIS, we performed the following experiments at a concentration of 50 μM. In agreement with the previous result, figure 2A shows that TUDCA increased insulin release from mouse islets incubated with 11 mM or higher glucose concentrations. The half-maximal effect (EC50) obtained from the dose-response curve (Fig. 2B) was calculated to be 13.78 ± 1.03 mM glucose in islets incubated with TUDCA versus 15.47 ± 0.63 mM in controls. As indicated by the shift to the left of the dose-response curve and the magnitude of the secretory responses, TUDCA increased the β-cell responsiveness to glucose, leading to enhanced GSIS. No differences were observed in the total insulin content between TUDCA-treated and control cells (Fig. 2C), indicating that changes in insulin release were not mediated by TUDCA effects on insulin synthesis.

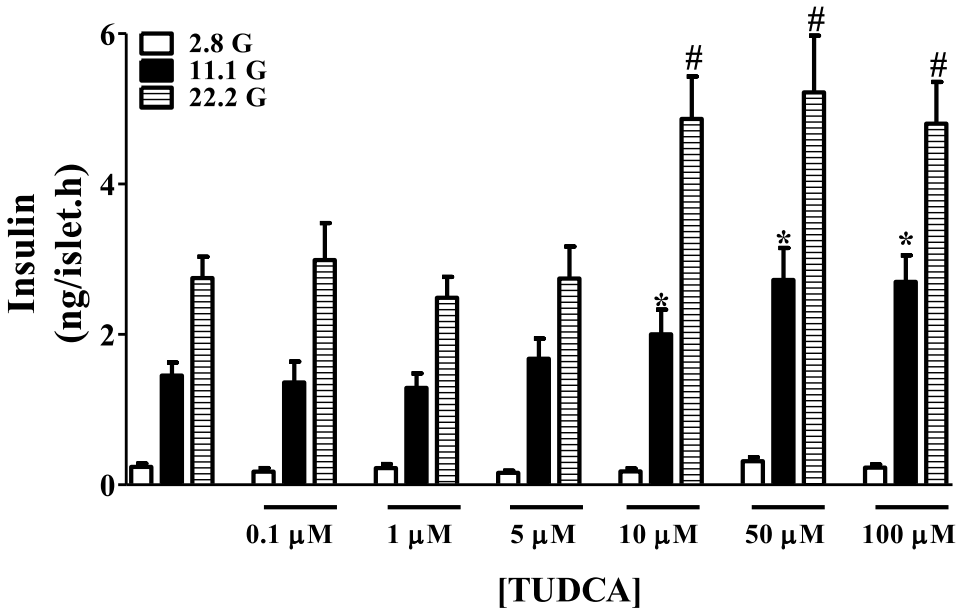


Figure 1: Effects of different TUDCA concentrations on glucose-induced insulin secretion from mouse fresh islets. Groups of 4 islets were incubated for 1 h with 2.8, 11.1, or 22.2 mM glucose (G) in the presence or absence of different TUDCA concentrations. Data are displayed as the mean ± SEM of 10-15 islet groups. In all of the experiments, glucose-induced secretion at 11.1 and 22.2 mM G was found to be significantly higher compared to that of the basal condition (2.8 mM G). * and #, significant differences ($p<0.05$) compared to the control conditions of 11.1 or 22.2 mM G, respectively.

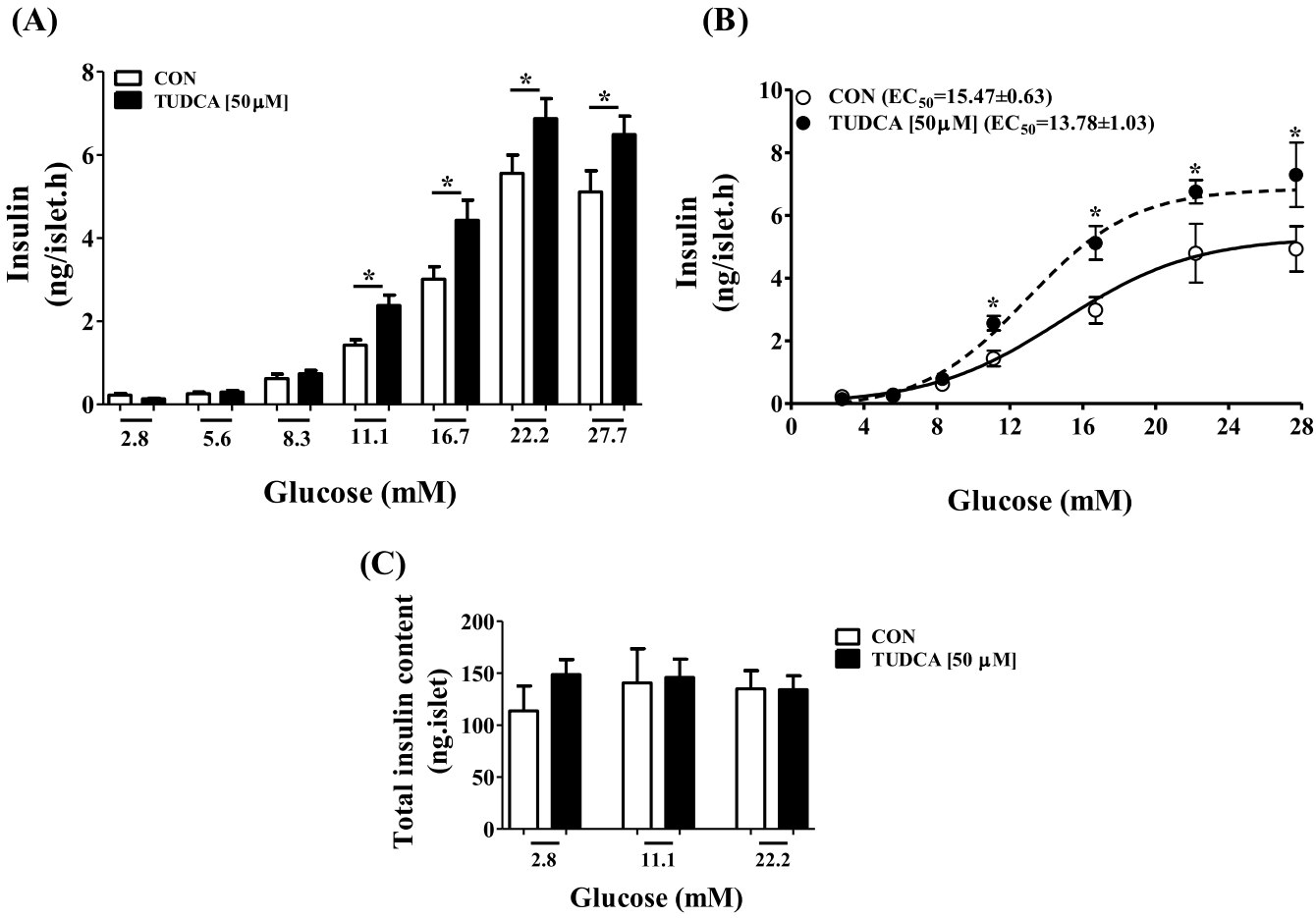


Figure 2: The effect of TUDCA is glucose-dependent. Effects of 50 µM TUDCA on glucose-induced insulin secretion (A, B) and total insulin content (C) from fresh mouse islets. Groups of 4 islets were incubated for 1 h at different glucose concentrations in the presence or absence of 50 µM TUDCA (A). EC50 values are also displayed in (B). Data are displayed as the mean ± SEM and were obtained from 10-15 groups of islets for each glucose concentration. *, significant differences ($p<0.05$) compared to control conditions.

TUDCA did not alter glucose-regulated NAD(P)H levels, electrical activity or Ca2+ signals in isolated islets. Several cell processes are involved in GSIS. When glucose enters β-cells, mitochondrial metabolism increases the cytosolic ATP/ADP ratio, leading to the closure of the KATP channels, which depolarizes the plasma membrane potential. This depolarization activates voltage-dependent Ca2+ channels, triggering a cytosolic Ca2+ rise that stimulates secretion. To study the involvement of

these processes, we first monitored the glucose-induced changes through NAD(P)H levels. These levels increase as a result of glycolysis and Krebs cycle activation by glucose, processes that are coupled to mitochondrial ATP production [23]. When mouse pancreatic islets were perfused in the presence or absence of the bile acid (Fig. 3A, B), no differences in glucose-induced NAD(P)H fluorescence levels were detected between the groups. We also explored the effect of TUDCA on glucose-regulated KATP channel activity because some bile acids, such as TCDC, have been shown to modulate this channel in pancreatic β-cells (Dufer 2012). As shown in Figure 3C and D, TUDCA did not produce any effect on KATP channel activity with 8 mM glucose. These findings also indicate that TUDCA did not affect mitochondrial metabolism (as observed in Figure 3A and B) because the KATP channel is highly sensitive to alterations in mitochondrial function and ATP levels [24]. Diazoxide is a potent KATP channel opener, which hyperpolarizes the plasma membrane, leading to reduced intracellular Ca2+ levels and insulin secretion. As expected, diazoxide decreased insulin secretion induced by 11 mM glucose (Supplementary Fig. 2A). Despite the inhibitory effect of the KATP channel opener, TUDCA was able to increase insulin secretion in the presence of diazoxide, suggesting that TUDCA effects are likely mediated by an alternative pathway that differs from the KATP channel route. Finally, we analyzed the effect of TUDCA on glucose-induced Ca2+ signals. TUDCA did not generate any effect when it was acutely applied to mouse islets in basal conditions (Fig. 4A) or after the generation of a Ca2+ increase with 11 mM glucose (Fig. 4B). No differences were observed in response to 11.1, 16.7 or 22.2 mM glucose in pancreatic islets continuously perfused in the presence of 50 μM TUDCA compared to controls either (Fig. 4C–G and Supplementary. Fig. 1). Thus, it seems that the effect of TUDCA on GSIS is not mediated by KATP channel-dependent mechanisms or Ca2+ signals.

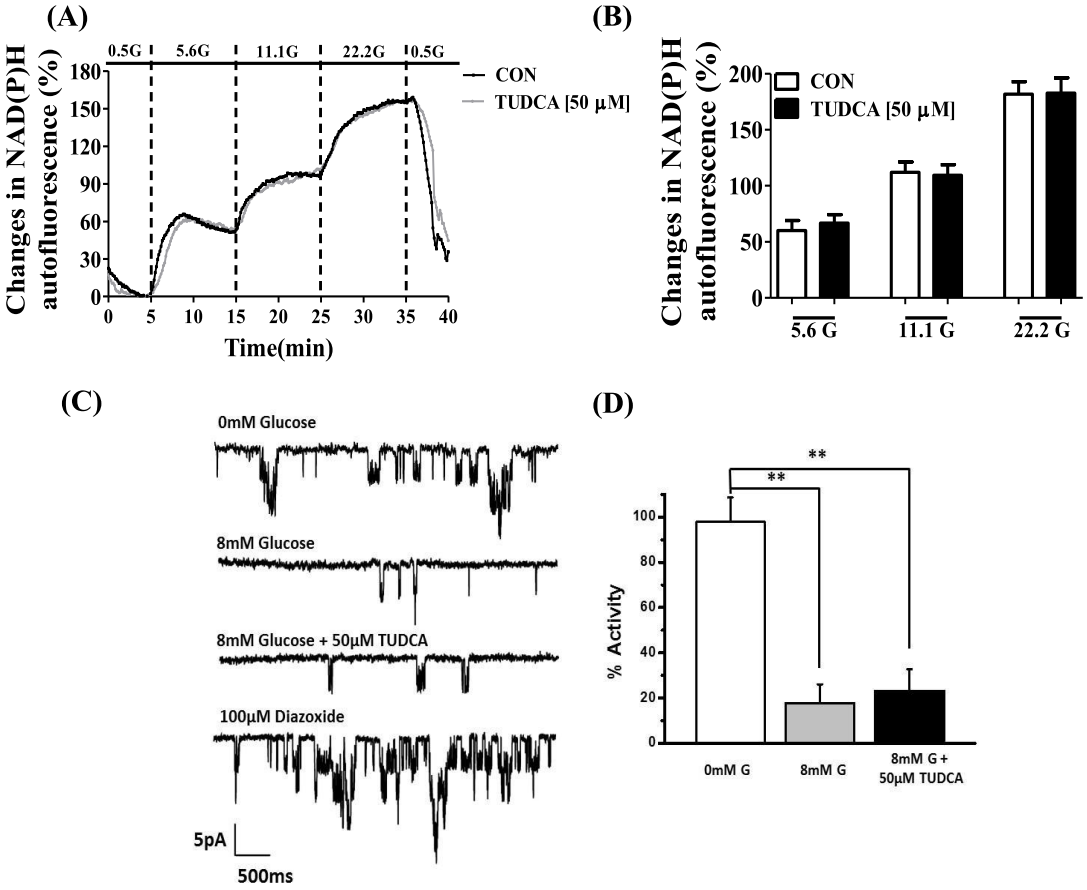


Figure 3: TUDCA effects are not mediated by metabolic changes. (A) Representative records of the changes in NAD(P)H fluorescence (%) in response to 0.5, 5.6, 11.1 or 22.2 mM glucose from fresh mouse islets in the presence or absence of TUDCA. (B) Increment in NAD(P)H fluorescence (%) for each glucose concentration. Data are the mean ± SEM obtained from 4 to 6 independent experiments. (C, D) Regulation of KATP channel activity in pancreatic β-cells of mice by 50 µM TUDCA. TUDCA did not produce any effect on the KATP channel activity at 8 mM glucose. (C) Records of KATP channel activity in the absence of glucose, 10 min after the application of 8 mM glucose, 10 min after the application of 8 mM glucose with 50 µM TUDCA, and 5 min after the application of 100 µM diazoxide. (D) Percentage of the KATP channel activity channel elicited by 0 mM glucose, 8 mM glucose, and 8 mM glucose and 50 µM TUDCA in single β-cells (n=6 cells). **, p□0.01 Student's t-test comparing 8 mM glucose and 8 mM glucose + 50 µM TUDCA with 0 mM glucose.

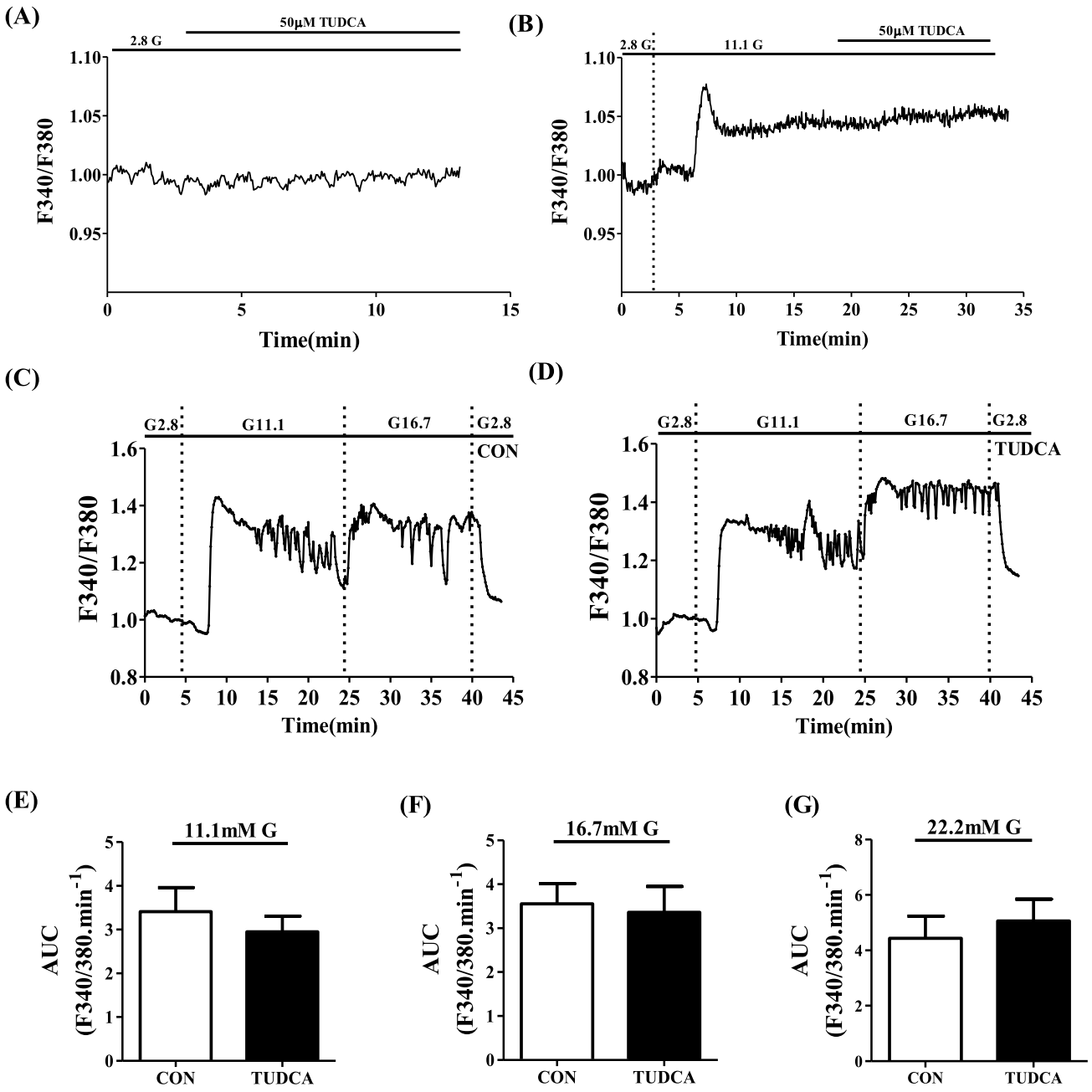
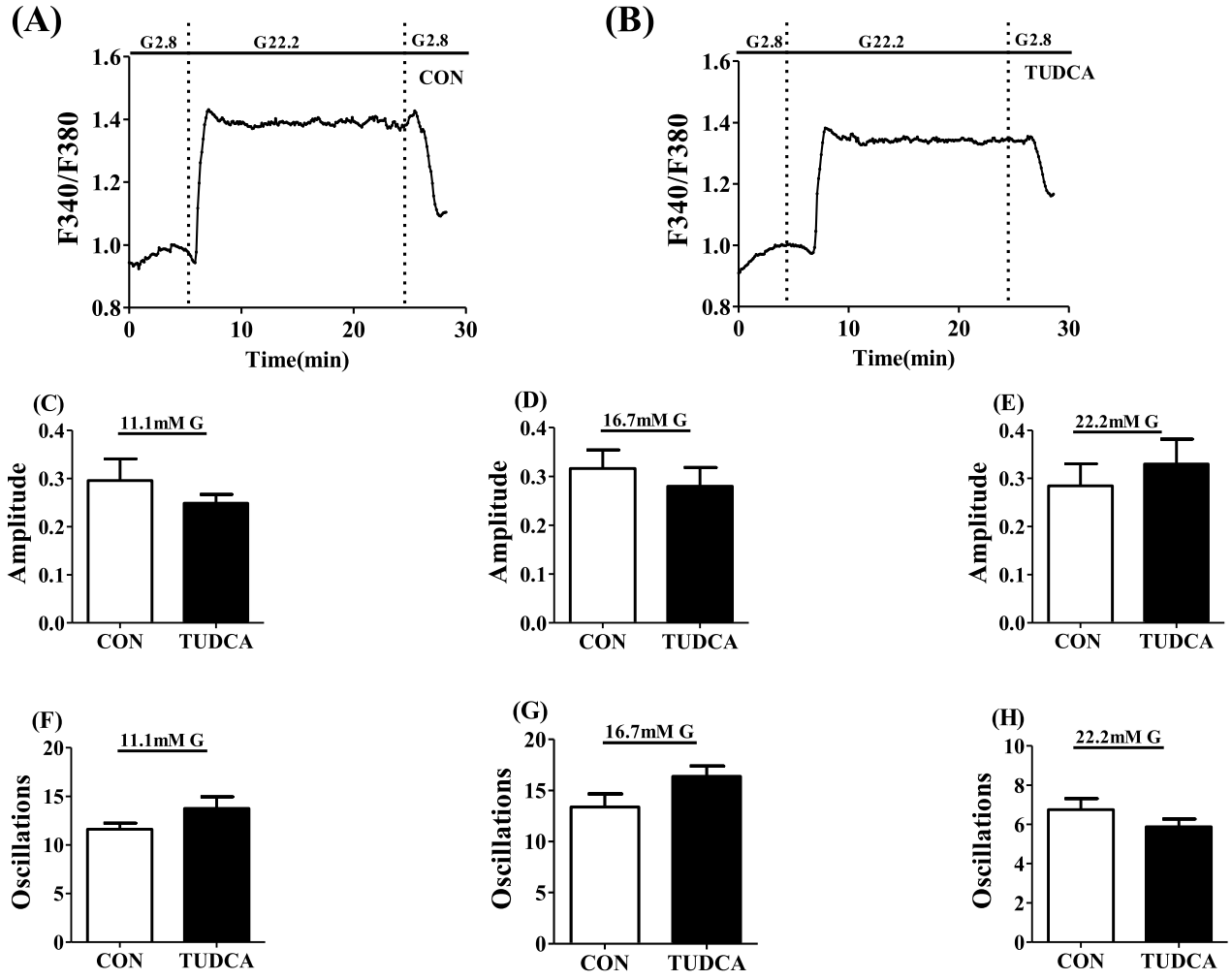


Figure 4: TUDCA does not affect glucose-induced Ca2+ signals. (A, B) Representative Ca2+ recordings from isolated islets showing the lack of TUDCA effects when acutely applied at basal (2.8 mM) and stimulatory (11.1 mM) glucose concentrations. Three independent experiments were performed for each condition. (C, D) Representative Ca2+ recordings in response to 11.1 or 16.7 mM glucose from fresh mouse islets. The experiments were performed in a perfusion system in the continuous

presence or absence of 50 µM TUDCA. The AUC (E, F, G) of Ca2+ is displayed as an indicator of the global Ca2+ entry for the different glucose concentrations. Data are shown as the mean ± SEM and were obtained from 4 to 6 independent experiments.



Supplementary Figure 1: TUDCA does not affect glucose-induced Ca2+ signals. (A, B) Representative Ca2+ recordings from isolated islets showing the effect of TUDCA at basal (2.8 mM) and stimulatory (22.2 mM) glucose concentrations. Three independent experiments were performed in each condition. (C¬¬–H). The amplitude and Ca2+ oscillations from 22.2 and all of the glucose concentrations from the experiments shown in Figure 4. The experiments were performed in a perfusion system in the continuous presence or absence of 50 µM TUDCA. Data are shown as the mean ± SEM and were obtained from 4 to 6 independent experiments.

The effects of TUDCA on GSIS likely depend on the G protein-coupled bile acid receptor TGR5. To further investigate the role of TUDCA on intracellular

pathways, we also explored the types of bile acid receptors that were involved. Given that TUDCA has poor affinity for the nuclear receptor FXR [2, 3], we focused on TGR5, which is a G protein-coupled receptor that couples to the Gα stimulatory subunit, leading to the activation of adenylyl cyclase, the generation of cAMP and, subsequently, the activation of PKA [1]. We used NF449, a specific inhibitor of the Gα stimulatory G protein subunit. This inhibitor did not alter GSIS at 11.1 or 22.2 mM glucose levels (Fig. 5A and B), yet it abolished the stimulatory effects of TUDCA on GSIS at both glucose concentrations. Because there are no commercially available TGR5-selective antagonists [25], we tested the effect of INT-777 (6-alpha-ethyl-23(S)-methyl-cholic acid, 6-EMCA), a potent and selective TGR5 agonist. INT-777 totally mimicked the TUDCA action of 11 mM glucose, whereas it had no effect at basal glucose concentrations (Fig. 5B). These results indicate that a G protein-coupled receptor mediates TUDCA actions, likely via the TGR5 bile acid receptor. We also analyzed the effects of tauro β-muricholic acid (TβMCA), a natural FXR antagonist, to analyze whether this receptor participates in the actions of TUDCA. Incubation with TβMCA did not alter the effect of TUDCA on insulin secretion, indicating that this FXR was not involved (Supplementary Fig. 2B).

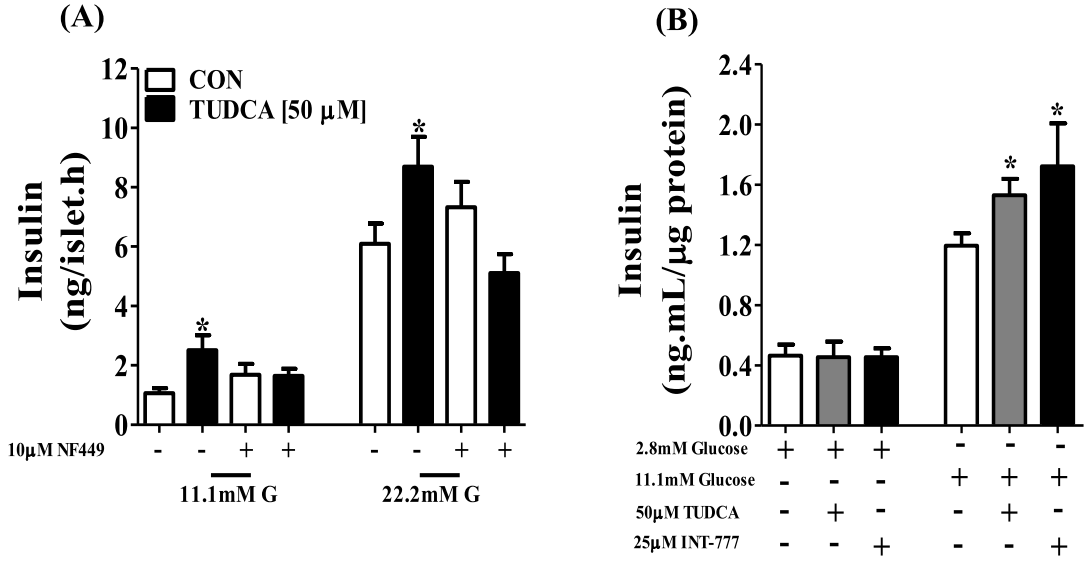
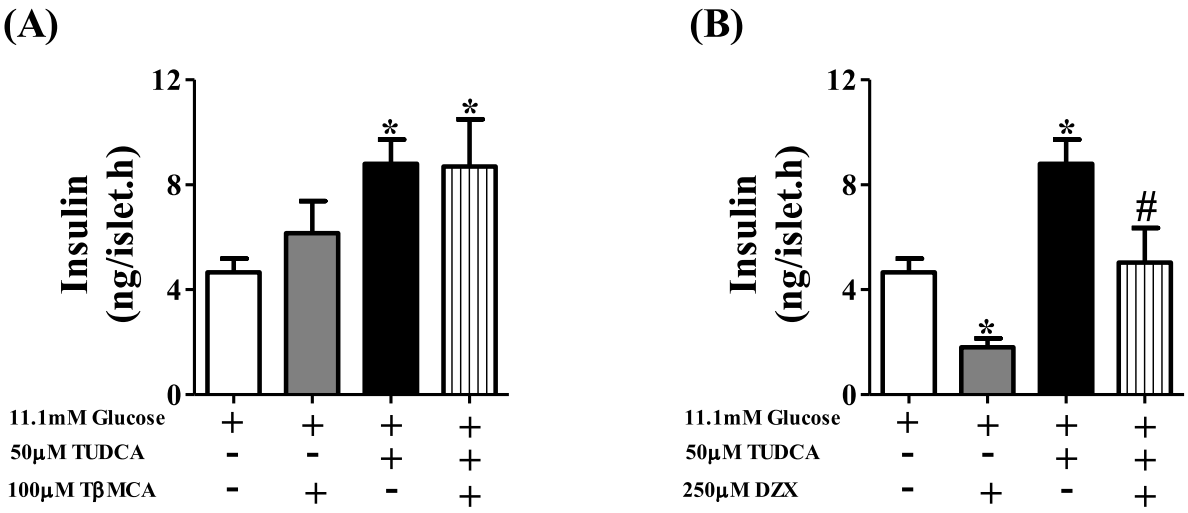


Figure 5: TUDCA effects on GSIS are mediated by a G protein-coupled receptor. (A) TUDCA effects on insulin secretion induced by 11.1 and 22.2 mM glucose from mouse islets were abolished by the Gα stimulatory G protein subunit specific inhibitor NF449. (B) TUDCA effects on insulin secretion induced by 11.1 mM glucose from mouse islets were mimicked by the specific TGR5 agonist INT-777. Groups of 4 islets

were used in each measurement. Data are presented as the mean ± SEM and were obtained from 10 to 12 islets groups. *, significant differences ($p<0.05$) compared to control conditions.



Supplementary Figure 2: TUDCA effects on glucose-stimulated insulin secretion (GSIS) are not mediated by a KATP-dependent mechanism and FXR receptor. (A) TUDCA effects on insulin secretion induced by 11.1 mM glucose from mouse islets were partially abolished by diazoxide. (B) TUDCA effects on insulin secretion induced by 11.1 mM glucose from mouse islets were not abolished by the natural FXR inhibitor TβMCA. Groups of 4 islets were used in each measurement. Data are displayed as the mean ± SEM and were obtained from 6 to 8 islets groups. * and #, significant differences ($p<0.05$) compared to control or control + DZX conditions, respectively.

TUDCA-stimulated insulin secretion is dependent on the cAMP/PKA pathway. To address whether the cAMP/PKA pathway could be modulated by TUDCA, we investigated the effect of the PKA inhibitor H89 and (Rp)-cAMP, a competitive inhibitor of PKA activation by cAMP, on GSIS. In both cases, the inhibition of the PKA pathway completely blunted the TUDCA actions on GSIS from mouse pancreatic islet cells (Fig. 6A and B). In addition, to confirm the activation of this pathway, we analyzed the phosphorylation levels of PKA and its target protein CREB in a time-dependent manner (Fig. 6C and D). TUDCA enhanced PKA and CREB

phosphorylation after being incubated for 20 min. In addition, enhanced pPKA content was also observed after 1 h.

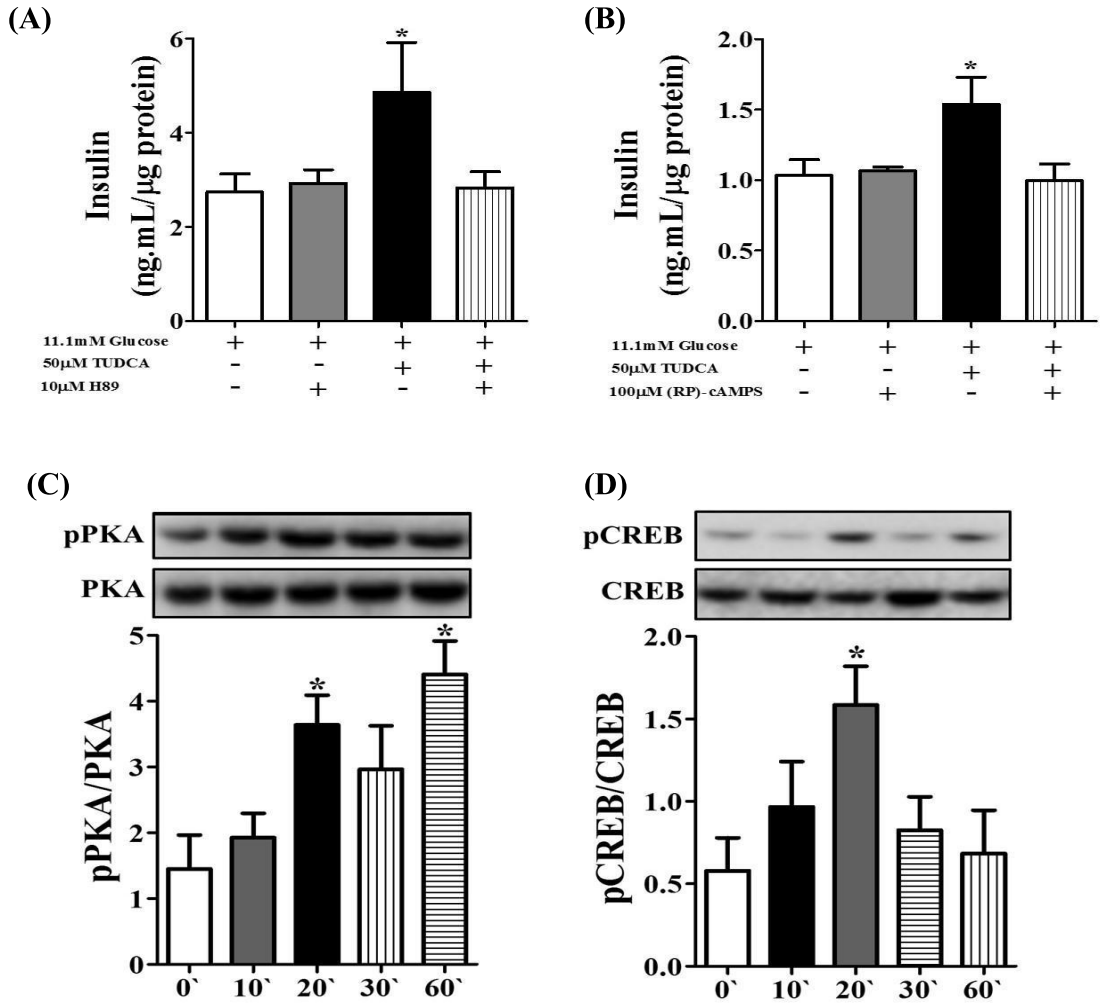


Figure 6: TUDCA actions on GSIS are mediated by the cAMP/PKA pathway. (A, B) Effects of TUDCA on GSIS from mouse islets after 1 h were blunted by the PKA inhibitor H89 (A) or by Rp-cAMP, a competitive inhibitor of PKA activation by cAMP (B). (C, D) TUDCA incubation for 1 h increases the phosphorylation of PKA (C) and CREB (D). Groups of 4 islets were used for insulin secretion measurements, and groups of 250 islets were used in the western blot experiments. Data are shown as the mean ± SEM and were obtained from 10 to 12 groups of islets. *, significant differences ($p<0.05$) compared to control conditions.

3.5 Discussion

The present study shows that the taurine-conjugated bile acid TUDCA has a positive effect on glucose-induced insulin secretion from mouse isolated pancreatic islets, whereas it remains without effect at basal glucose levels. This behavior is similar to that of incretins such as GLP-1. Incretins exhibit an important therapeutic advantage for glycemic control in diabetes because they act on hyperglycemic conditions without favoring hypoglycemic episodes [26]. Thus, glucose-dependent TUDCA action on insulin secretion might be interesting from a therapeutic context. Currently, TUDCA and ursodeoxycholic acid (UDCA) are used for the treatment of several liver diseases [14,15]. In contrast to other bile acids, which are cytotoxic, TUDCA and UDCA exhibit protective properties against apoptosis [27]. Additionally, ongoing research is analyzing the therapeutic potential of TUDCA to alleviate apoptosis in non-liver diseases, such as neurological, retinal, metabolic and myocardial disorders [14, 15]. It has been reported in obese humans and mice that TUDCA ameliorates insulin resistance by reducing endoplasmic reticulum stress [6]. In addition to all of these beneficial properties, here, we show that TUDCA potentiates GSIS via bile acid signaling involving the cAMP/PKA pathway. This effect occurred over a short time period (less than 1 h) and was not mediated by genomic actions because insulin protein synthesis remained unchanged (Fig. 1 and 2). It remains to be explored whether in vitro TUDCA effects on GSIS are also important for in vivo conditions to acutely modulate plasma insulin levels and glucose homeostasis. It would also be interesting to analyze whether in vivo treatment with TUDCA alone or in combination with other therapeutic agents could ameliorate glycemic values in obesity and diabetes animal models.

Although FXR and TGR5 are both expressed in mouse pancreatic islets [6, 7, 9], several findings support that the effects of TUDCA observed in this study were mediated, at least in part, by TGR5. In contrast to the nuclear FXR receptor, TGR5 is a plasma membrane receptor that is coupled to a G protein (Gα stimulatory), which activates adenylate cyclase, increasing cAMP levels. This results in PKA activation, inducing CREB phosphorylation [2, 3, 28]. Our results showed that the effects of TUDCA on GSIS were blocked when we inhibited both a G protein (Gα stimulatory) and PKA (Fig. 5 and 6). Additionally, TUDCA actions were mimicked by a TGR5 selective agonist. We also showed that TUDCA increases PKA and CREB

phosphorylation levels on the same temporal scale as the effects on GSIS. Remarkably, although TUDCA has been reported to activate TGR5 and to induce cAMP production [29, 30], this hydrophilic bile acid and UDCA are not FXR agonists [2, 30] because the latter receptor exhibits more affinity for hydrophobic bile acids. Taurine conjugation of UDCA may also increase its affinity for TGR5 [25, 31]. In contrast to the effects of the FXR agonist TCDC reported in mouse pancreatic islets [7], TUDCA actions on GSIS were independent of KATP channels and changes to cytosolic Ca2+ levels. These findings further support the idea that TUDCA affected secretion in the current study by mechanisms other than FXR activation.

Short-term non-genomic effects on insulin secretion by some bile acids have been previously reported. The conjugated bile acid TCDC induced insulin release at high glucose concentrations via FXR activation in mouse β-cells [7]. In MIN-6 cells and human islets, the TGR5 agonists oleanolic (OA) and lithocholic acid (LCA) stimulated insulin secretion in both basal and stimulatory glucose conditions [6]. TUDCA enhanced insulin secretion in pig pancreatic islets at high glucose concentrations [32]. In the latter study, the bile acid receptor mediating these TUDCA effects was not explored. Our findings are in agreement with these studies, showing that TUDCA stimulates high glucose-induced insulin secretion in the short-term. In βTC6 cells and human islets, the FXR ligand 6E-CDCA [9] was reported to enhance GSIS after an 18 h incubation. However, genomic actions were likely involved at these long periods because this FXR ligand also induced insulin expression.

It has been shown that bile acids can regulate the activity of plasma membrane ion channels and cytosolic Ca2+ signals in different cell types [31]. In mouse isolated islets, the FXR agonist TCDC leads to the blockade of KATP channel currents, stimulating electrical activity and intracellular Ca2+ oscillations [7]. In MIN6 cells, mouse islets and human islets, different TGR5 agonists generate a rise in intracellular Ca2+ [6]. In this latter work, TGR5 activation led to phosphoinositide hydrolysis and Ca2+ release from intracellular stores. In our study, we did not observe any effects of TUDCA on KATP channel activity (Fig. 3), Ca2+ signals or intracellular Ca2+ release (Fig. 4), indicating that these pathways were not involved. It has been shown that the pharmacological activation of PKA can slightly increase glucose-induced intracellular Ca2+ concentrations [33]. Because we did not observe any effect on cytosolic Ca2+ levels, it seems that TUDCA may induce PKA activation to a low extent (at least

compared with a pharmacological agonist) or that PKA-induced activation by TUDCA preferentially affects the secretory process. Indeed, changes in cAMP levels close to the plasma membrane and spatial compartmentalization of several components of the exocytotic process seem to play a major role in GSIS in pancreatic β-cells [34].

TGR5 is a G protein-coupled receptor that leads to adenylate cyclase activation [31]. In the present study, incubation of isolated fresh islets with NF449, a Gαs subunit inhibitor, prevented the effects of TUDCA on GSIS. Likewise, the inhibition of PKA activity with H89 or Rp-cAMPS resulted in the blockade of TUDCA actions. Finally, TUDCA led to PKA phosphorylation and activation of its target CREB in isolated mouse islets in the short-term. All of these findings indicate that the effects of TUDCA on GSIS are cAMP/PKA-dependent. The role of the cAMP/adenylate cyclase pathway in GSIS is well known. Elevation of cAMP concentrations potentiates glucose-dependent insulin secretion through the activation of PKA [33, 35]. PKA phosphorylation affects the regulation of some proteins involved in exocytosis, thus stimulating insulin secretion in pancreatic β-cells [35, 36]. The present results are in agreement with previous studies on enteroendocrine cells showing that TGR5 activation is followed by Gαs release and activation of adenylate cyclase, leading to an increase in cAMP concentration and activation of PKA and CREB [3].

In summary, this study shows an important effect of TUDCA in mouse pancreatic β-cells. This bile acid increases insulin secretion only at high glucose concentrations by a mechanism that is mediated by the cAMP/PKA/CREB pathway. Although our experiments indicate that the TGR5 receptor is likely involved in the effects of TUDCA, we cannot rule out the implication of the FXR receptor and other signaling pathways.

Author contributions

J.F.V., R.A.R., I.Q., E.M.C., A.C.B., and A.N. designed the study, researched data, and wrote the paper. P.C.B., R.C.S.B., B.M., and S.S. researched data. R.A.R., E.M.C., I.Q., and J.F.V. contributed to the discussion and reviewed and edited the manuscript. J.F.V. is the guarantor of this work and with full access to all of the data in the study and takes responsibility for it.

Acknowledgements

We thank M. Carlenossi, M. S. Ramon and M. L. Navarro for their expert technical assistance. This work was supported by grants from Fundacão de Amparo á Pesquisa do Estado de São Paulo (FAPESP 2013/01318-4), Conselho Nacional para o Desenvolvimento Científico e Tecnológico (CNPq 200030/2014-0), Instituto Nacional de Obesidade e Diabetes (CNPq/FAPESP) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). This study was also supported by grants from the Spanish Ministerio de Ciencia e Innovación (BFU2013-42789-P; BFU2011-28358). CIBERDEM is an initiative of the Instituto de Salud Carlos III.

Conflict of interest

All contributing authors report no conflict of interest.

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4. Artigo 02

The bile acid TUDCA improves insulin clearance increasing the insulin-degrading enzyme expression in the liver of obese mice

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4.1 Abstract

Both, insulin secretion and clearance disruption contribute to obesity-induced hyperinsulinemia. However, the insulin clearance reduction seems to be the mainly factor in this context. The liver is the major site for insulin degradation, a process mainly coordinated by the Insulin-degrading enzyme (IDE). The beneficial effects of taurine conjugated bile acid (TUDCA) on insulin secretion as well as insulin sensitivity have been recently described. However, the possible role of TUDCA in insulin clearance had not yet been explored. Here, we demonstrated that treatment for 15 days with TUDCA reestablished plasma insulin to physiological concentrations in high fat diet (HFD) mice, a phenomenon associated with increased insulin clearance and liver IDE expression. TUDCA also increased IDE expression in human hepatic cell line HepG2. This effect was not observed in the presence of an inhibitor of the hepatic membrane bile acid receptor, S1PR2, as well as when its downstream proteins were inhibited, including IR, Pi3K and Akt. These results indicated that treatment with TUDCA may be helpful to counteract obesity-induced hyperinsulinemia, by increasing insulin clearance, an effect that seems to be orchestrated by enhanced liver IDE expression in a mechanism dependent on S1PR2-Insulin pathway activation.

4.2 Introduction

Obesity is the primary cause of hyperinsulinemia, which increases the risk for cancer and cardiovascular disease (1,2), and potentiates insulin resistance, that may trigger type 2 diabetes (T2D) (3). Higher levels of plasma insulin concentration can be attributed to increased insulin secretion and/or decreased insulin clearance (4,5); reduced insulin clearance is likely the primary factor in obesity-induced hyperinsulinemia (6).

Insulin clearance occurs mainly in the liver by the Insulin-degrading Enzyme (IDE), which degrades approximately 50% of insulin in its first passage through the hepatic portal system (7,8). Mice lacking IDE are hyperinsulinemic at 2 months old, showing an improved glucose tolerance. However, they develop glucose intolerance as well as insulin resistance over time (9). This suggests the hyperinsulinemic state, due to reduced insulin clearance, as a trigger for the development of T2D. In the same way, Goto-Kakizaki rats, which have a genetic defect at the IDE gene, as well as some T2D humans, exhibit reduced insulin clearance and augmented plasma insulin concentrations, prior to the onset of T2D (10,11).

Conversely, insulin sensitizer agents such as physical exercise, bariatric surgery and pioglitazone treatment have been found to reduce plasma insulin concentrations in obese rodents, through increased insulin clearance and improved glucose homeostasis (12-14). However, exercise has a low adherence rate (15), bariatric surgery is an invasive procedure (16), and pioglitazone treatment has significant side effects (17).

Thus, the use of endogenous molecules that could increase insulin clearance, without the side effects or adherence concerns, shows potential as a treatment for hyperinsulinemia. In this context, the taurine conjugated bile acid tauroursodeoxycholic (TUDCA) has emerged as a possible candidate due to its beneficial effect upon glucose homeostasis (18-20). In the liver, TUDCA improves insulin sensitivity by reducing endoplasmic reticulum stress (21,22). TUDCA activates liver insulin signaling by the interaction with the sphingosine-1-phosphate receptor 2 (S1PR2), resulting in PI3K/Akt activation (23). However, the effect of TUDCA on insulin clearance as well as upon hepatic IDE expression has not been clarified yet.

Here, using high fat diet (HFD) mice, as an experimental model of hyperinsulinemia, we demonstrated that TUDCA normalizes their plasma insulin concentrations by increasing insulin clearance. This effect is probably due to augmentation of IDE expression in the liver. In in-vitro experiments, using hepatic human HepG2 cell line, we observed that TUDCA also increases IDE expression, by a mechanism dependent on the interaction of TUDCA with the S1PR2 receptor, via the insulin signaling pathway. These findings suggest the treatment with TUDCA as a promising therapeutic intervention for the control of hyperinsulinemia in obese pre-diabetic individuals.

4.3 Materials and methods

Reagents. TUDCA was purchased from Calbiochem (Sao Paulo, Brazil, cat. 580549) and Insulin and C-Peptide Elisa Kits were acquired from Millipore (Darmstadt, Germany, cat. #EZRMI-13K and #EZRMCP2-21K, respectively). Western Blot reagents were purchased from Bio-Rad (Madrid, Spain) and antibodies were acquired from Abcam (Cambridge, UK) and Sigma Aldrich (St Louis, MO, USA). The remaining reagents were purchased from Sigma Aldrich.

Animals. The experiments involving animals were approved by the Animal Care Committee at UNICAMP (license number: 3815-1) and were conducted in accordance to the last revision of the National Institutes of Health (NIH) guide for the care and use of laboratory animal. Male 21-days old C57Bl/6 mice were obtained from the breeding colony at UNICAMP and maintained at 22 ± 1 ºC, on a 12-h light–dark cycle. After 1 month, the mice were fed a standard diet (CON) or a High fat diet with 35% fat (HFD) for 12 weeks. On the last 15 days of these diets, the mice received, i.p., PBS (groups CON and HFD) or 300 mg/kg TUDCA (groups CON+TUDCA and HFD+TUDCA). The mice were killed in a CO2 chamber and decapitated for blood collection and removal of the liver for posterior Western blot analyses.

Glucose and Insulin Tolerance Tests. At the end of treatment with TUDCA, the mice were subjected to 12-h fasting to performe the GTT. The fasting blood glucose level were measured (time 0) by a glucometer. After, the mice received an i.p. glucose load of 2 g/kg body weight and the glycemia was measured at 15, 30, 60 and 120 minutes after the glucose load. For the ITT, the mice were subjected to a 2-h fasting and

the glycemia was measured before (time 0) and 3, 6, 9, 12, 15 and 18 minutes after the i.p. administration of 0.75 U/kg insulin load. The KITT was calculated as previously described 36.

Plasma Insulin and C-peptide measurements. Mouse insulin and C-peptide Elisa Kits were used to measure plasma insulin and C-peptide. The plasma samples was obtained by centrifugation of blood samples at 1100 g, 15 min, 4°C. The assays were performed as indicated on kit protocol. The blood samples for insulin measurements were collected on fed and fasted state, as well as at the GTT times 0, 15 and 60 min. The C-peptide were measured in these same plasma samples of GTT, used for insulin measurements.

Cell culture and treatment. HepG2 liver cell line were cultured in DMEN (Vitrocell, Campinas, SP, Brazil), enriched with 10% (vol./vol.) fetal bovine serum (FBS) for 3 days, under a humidified condition with 5% CO2 at 37ºC. After that, the cells were incubated in the presence, or not, of different TUDCA concentrations (T50, T100 and T200 μM) during 24-h. The concentration of 100 μM was adopted for the following experiments. The inhibitors of insulin pathway and bile acid receptors were added when necessary, as describe on figure legends. In the experiments with S961, MK2206 or Wortmannin, the cells were submitted to a 6-h serum starved before treatment.

Western blot analysis. Liver samples were collected and homogenized with 500 μL of Cell Lysis Buffer. For HepG2 Western blot, after treatment, the cells were collected in trypsin/EDTA, washed with PBS, and homogenized in urea anti-protease/anti-phosphatase buffer. For SDS (sodium dodecyl sulfate) polyacrylamide gel electrophoresis, all samples were treated with a Laemmli buffer containing dithiothreitol. After heating to 95ºC for 5 min, proteins were separated by electrophoresis in a 10% polyacrylamide gel. The transfer to nitrocellulose membranes was performed in a Trans Blot transfer for 2-h in 100V, with Tris/Glycine buffer. After, the membranes were blocked with 5% non-fat dry milk buffer (5% milk, 10 mM TRIS, 150 mM NaCl and Tween 20 0.02%) during 1-h, and then, they were incubated with a polyclonal antibody against IDE (Abcam, cat. ab32216). Tubulin (Sigma Aldrich, cat. 6074) was used as control of the experiment. Visualization of specific protein bands was performed by incubating the membranes with appropriate secondary antibodies. Protein bands were visualized using the Amersham Imager 600 (GE Healthcare Life

Sciences, Buckinghamshire, UK) which detects the chemiluminescence. The band intensities were quantified with the Image J software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. The data were presented as means ± standard errors media (SEM) for 4-8 animals, or obtained for 3 different cells experiments, each one in triplicate. The comparisons between all groups were performed by one-way Anova analysis followed by Newman-Keuls test. When the comparisons were determined between two groups Student´s t-test was adopted. The difference between the groups were considered statistically significant if $P \leq 0.05$.

4.4 Results

TUDCA reduced body weight, fat pads weight and blood glucose in HFD mice. As expected, body weight was significantly higher in HFD, compared with CON mice (Table 1). This was accompanied by higher perigonadal and retroperitoneal fat pad weight, as well as higher fed/fasted blood glucose concentrations. TUDCA treatment reduced body and fat pad weight in the HFD+TUDCA mice (Table 1). Moreover, TUDCA treatment returned fed/fasted glucose concentrations to levels similar to the control mice (Table 1). TUDCA treatment failed to alter all these parameters in CON+TUDCA mice.

Table 1. Final characterization of CON, CON+TUDCA, HFD and HFD+TUDCA mice.

	CON	CON+TUDCA	HFD	HFD+TUDCA
Body Weight (g)	30.33±1.21^{a}	29.68±1.48^{a}	41.22±1.18^{b}	33.88±1.27^{ab}
Perigonadal fat pad weight (g)	0.314±0.03^{a}	0.276±0.02^{a}	1.309±0.10^{b}	0.8746±0.06^{c}
Retroperitoneal fat pad weight (g)	0.154±0.01^{a}	0.133±0.01^{a}	0.630±0.06^{b}	0.336±0.03^{c}
Fasted Glycemia (mg/dL)	90±4.32^{a}	91.5±10.0^{a}	111±5.42^{b}	89.38±5.56^{a}
Fed Glycemia (mg/dL)	137±7.78^{a}	121±6.02^{a}	161±2.00^{b}	138±6.48^{a}

Different letters indicate statistically significant differences (One-way ANOVA followed by Newmans-Keuls posttest, $P < 0.05$). Data are mean ± SEM (n = 4-8).

Table 1. Final characterization of CON, CON+TUDCA, HFD and HFD+TUDCA mice. Different letters indicate statistically significant differences (One-way ANOVA followed by Newman-Keuls posttest, $P \leq 0.05$). Data are mean ± SEM (n = 4-8).

TUDCA improved glucose tolerance and insulin sensitivity in HFD mice. To investigate the effects of TUDCA on glucose homeostasis, we performed glucose and insulin tolerance tests (GTT and ITT). After glucose load, during GTT, all groups had a maximal glucose peak at 15-30 minutes (Figure 1A). However, HFD mice presented higher glucose concentrations indicating an impairment of glucose tolerance, as judged by the higher AUC of blood glucose, compared with the other groups (Figure 1B). Interestingly, HFD+TUDCA mice presented improved glucose tolerance (Figure 1A), as observed by the lower AUC of blood glucose, during GTT (Figure 1B). HFD group displayed higher blood glucose during the ITT, compared with CON mice (Figure 1C). Insulin sensitivity was lower in HFD mice, as judged by the glucose disappearance rate (KITT) (Figure 1D). The treatment with TUDCA restored insulin sensitivity in HFD+TUDCA mice (Figure 1D), improving the KITT values (Figure 1D). Finally, we assessed the plasma insulin levels in the fed and fasted state. The HFD increased plasma insulin concentrations in both states (Figure 1E and F) and the treatment with TUDCA restored them in HFD+TUDCA to levels similar to those found in CON mice (Figure 1E and F).

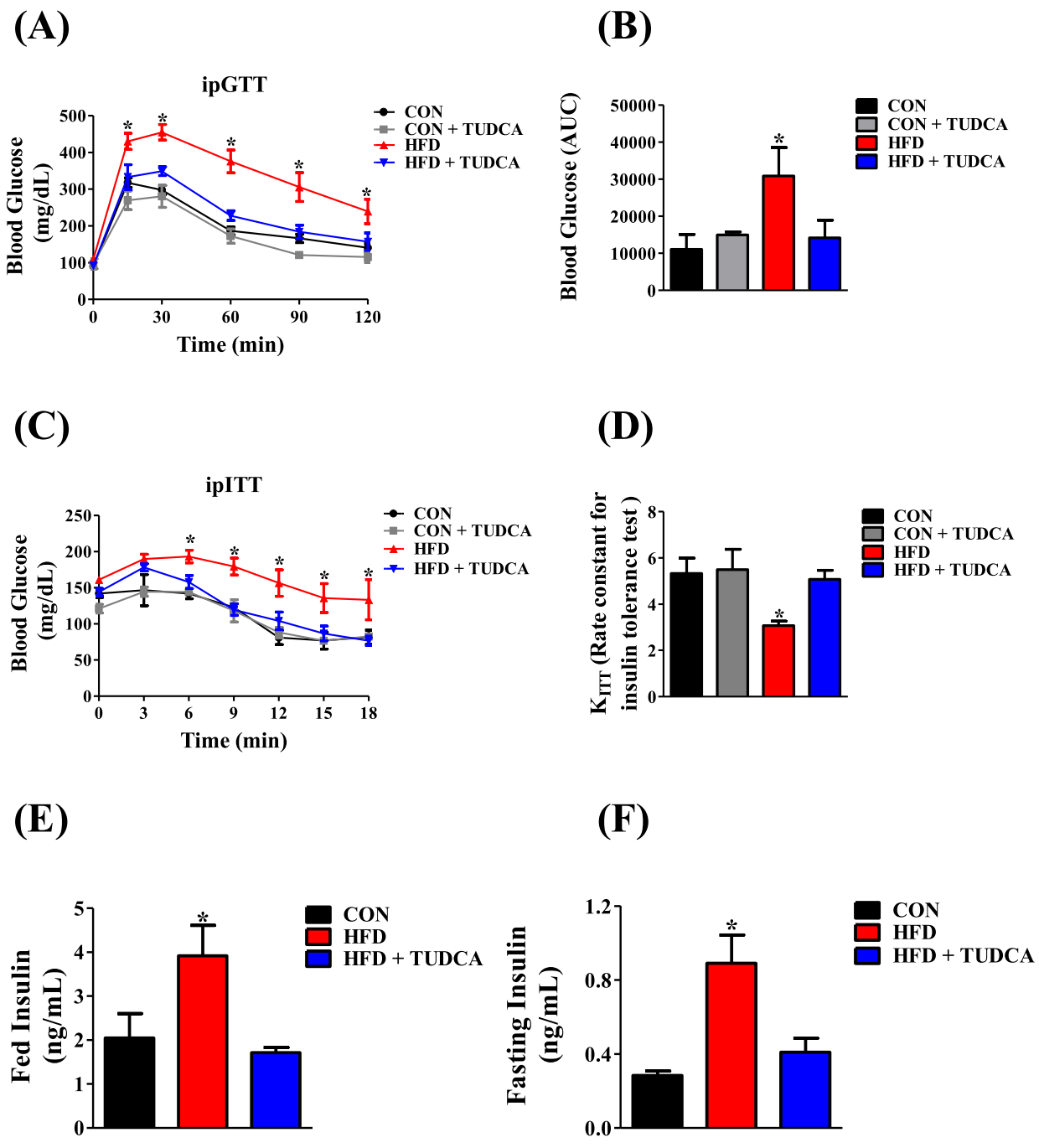

Figure 1. TUDCA treatment improves glucose tolerance, insulin resistance and insulinemia in HFD mice. Blood glucose during GTT (A) and ITT (C). Area under the curve (AUC) of total blood glucose concentration during GTT (B) and glucose disappearance rate during ITT (KITT) (D). Plasma insulin in fed (E) and fasting (F) state. Mice were fed a control diet (CON and CON+TUDCA) or high fat diet (HFD and

HFD+TUDCA) for 12 weeks, and received or not i.p. 300 mg/kg TUDCA during 15 days, as indicated. Data are mean ± SEM (n=4-8). * $P \leq 0.05$ vs CON.

TUDCA increased insulin clearance and IDE expression in the liver of HFD mice. Plasma insulin concentration is controlled by insulin secretion and insulin clearance. To measure insulin secretion we assessed the plasma concentration of C-peptide during GTT. After glucose administration, the HFD group presented increased C-peptide levels during the test (Figure 2C), as we observed in the AUC graphic (Figure 2D), indicating higher insulin secretion in this group, compared with CON group. Plasma insulin concentration also was increased in the HFD group (Figure 2A and B), reducing the C-peptide/insulin ratio. Insulin and C-peptide are co-secreted by the pancreatic β cells (ratio 1:1); however C-peptide has a longer half-time than insulin, thus reduction in the C-peptide/insulin ratio indicates a reduced insulin clearance, as we observed in the HFD mice (Figure 2E and F). The TUDCA treatment, in HFD mice, did not alter the higher insulin secretion as we observed by the elevated plasma C-peptide concentration during GTT (Figure 2C and D); however it reduced the plasma insulin concentration (Figure 2A and B), restoring the C-peptide/insulin ratio to similar levels of CON (Figure 2E and F), indicating a reestablishment of insulin clearance in these HFD+TUDCA mice. To elucidate the mechanism whereby TUDCA restored insulin clearance in HFD mice, we also investigated the IDE expression in the liver of these mice. As expected, the HFD reduces IDE expression (Figure 3) supporting the lower insulin clearance in these mice. Interestingly, TUDCA treatment restored the IDE protein expression, in the HFD+TUDCA mice, to similar levels of CON mice (Figure 3) explaining, at least in part, the reestablishment of insulin clearance in these mice.

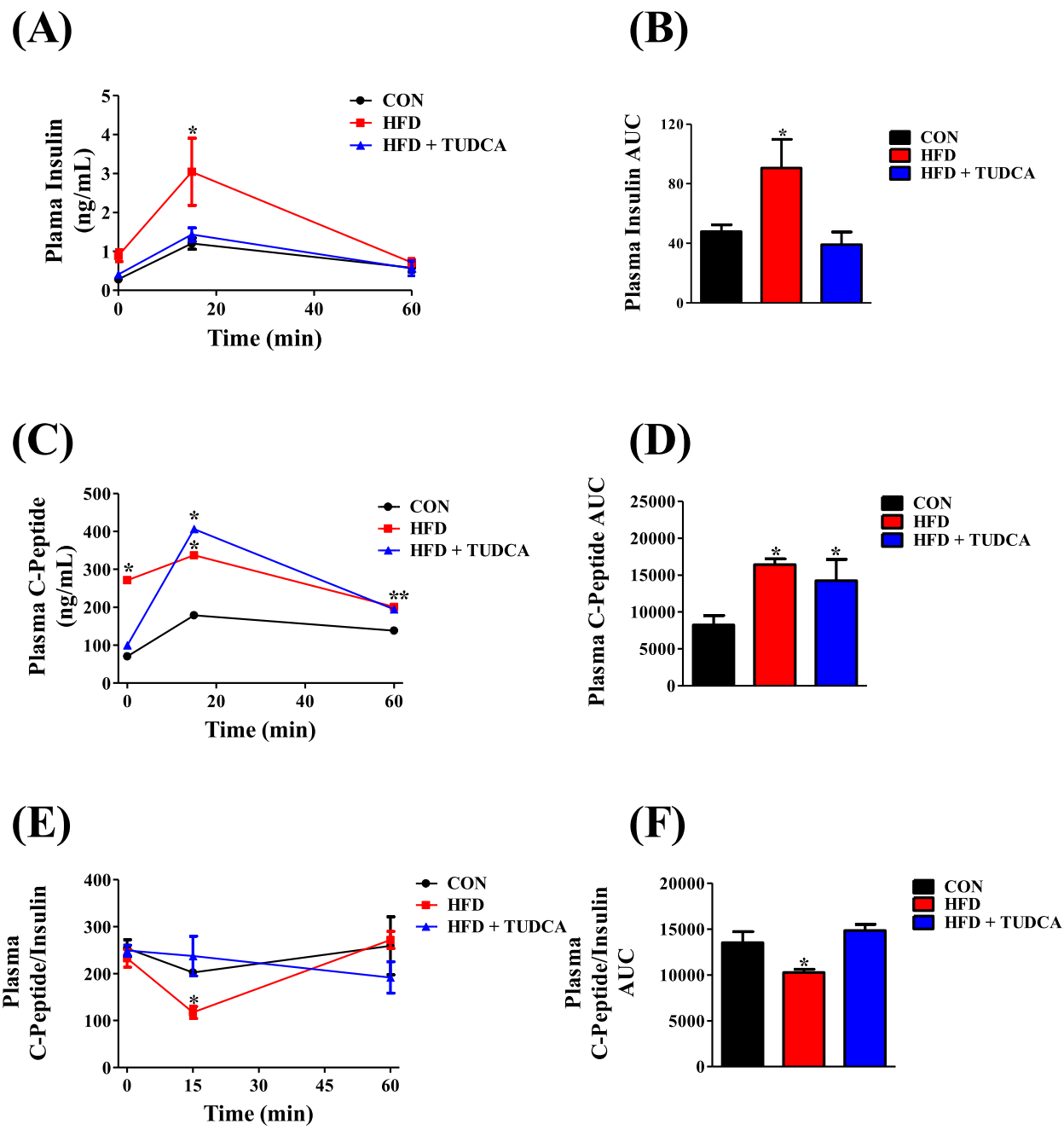

Figure 2. TUDCA treatment increases insulin clearance in HFD mice. Plasma levels of insulin (A), C-peptide (C) and the C-peptide/Insulin ratio (E). AUC of plasma insulin concentration (B), C-Peptide (D) and C-Peptide/Insulin ratio (F). Mice were fed a control diet (CON) or high fat diet (HFD and HFD+TUDCA) for 12 weeks, and received or not i.p. 300 mg/kg TUDCA during 15 days, as indicated. Data are mean ± SEM (n=4-8). * $P \leq 0.05$ vs CON.

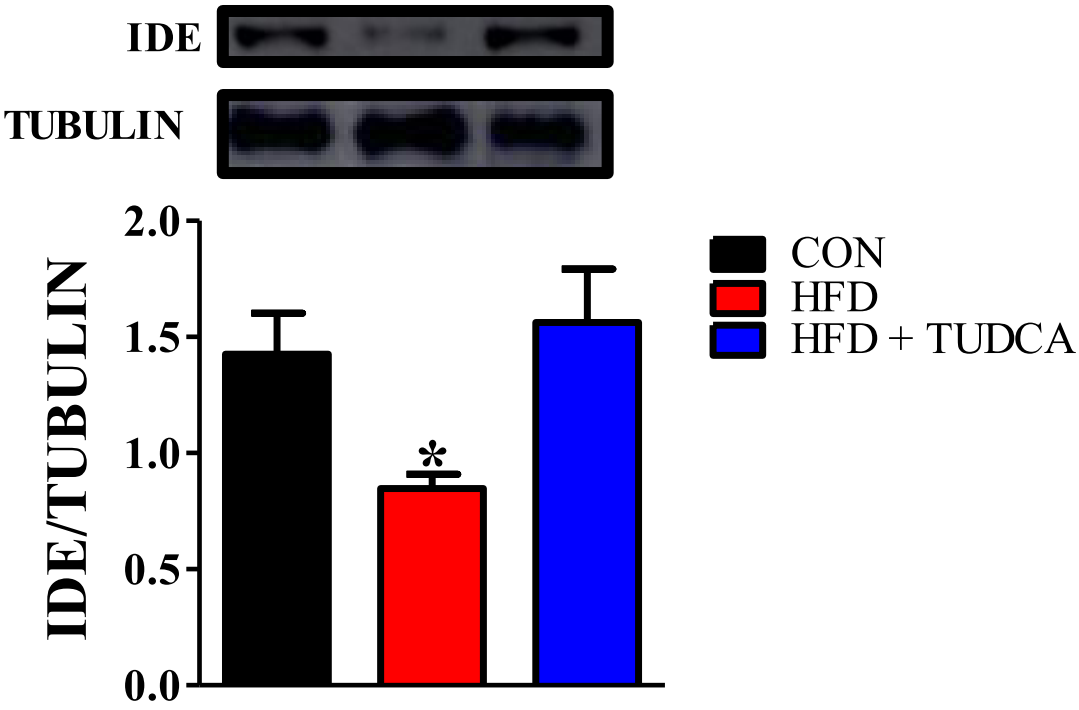

Figure 3. TUDCA treatment increases IDE expression in HFD mice. Protein expression of IDE in the liver and its representative immunoblottings images. Mice were fed a control diet (CON) or high fat diet (HFD and HFD+TUDCA) for 12 weeks, and received or not i.p. 300 mg/kg TUDCA during 15 days, as indicated. Data are mean ± SEM (n=4-8). Data are mean ± SEM (n=4-8). * $P \leq 0.05$ vs CON.

TUDCA increased IDE expression in HepG2 cell line by an S1PR2–IR pathway dependent mechanism. To assess the direct effect of TUDCA on IDE expression, we performed in vitro experiments using a human liver cell HepG2. First, we performed a dose-response curve to different concentrations of TUDCA, for 24-h. We observed that TUDCA increased IDE expression at 50, 100 and 200 µM (Figure 4A). Thus, 100 µM TUDCA was used in the subsequent experiments. It is known that the activation of the insulin pathway increases IDE expression in neurons 24 and the bile acid TUDCA activates insulin pathway mainly by S1PR2 receptor in the liver 23. The Figure 4B shows that in the presence of the S1PR2 inhibitor JTE-013, TUDCA failed to increase the expression of IDE. TUDCA also failed to increase IDE expression in cells incubated with S196, (an IR inhibitor) 25 MK-2206 (an Akt inhibitor) or Wortmannin (a PI3K inhibitor) 26 (Figure 4C and D, respectively).

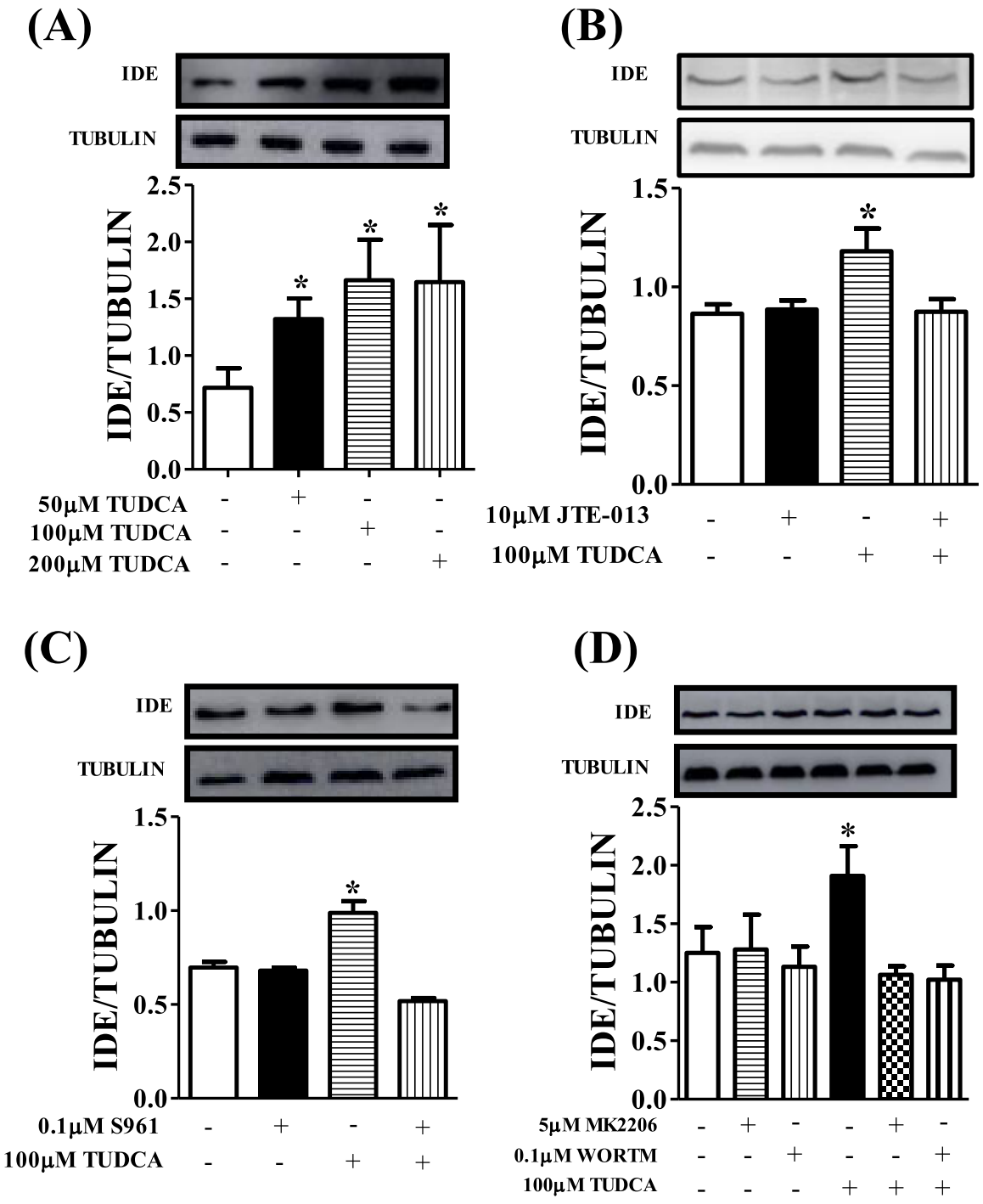

Figure 4. TUDCA modulates IDE expression in HepG2 cells by a S1PR2 -IR receptor pathway. Protein IDE expression in HepG2 cells treated or not with different concentrations of TUDCA for 24-h (A). Effect of TUDCA on IDE expression in the presence of 10 μM sphingosine-1-phosphate receptor 2 inhibitor (JTE-013) (B), 0.1 μM insulin receptor inhibitor (S961) (C), 5μM of Akt inhibitor (MK2206) (D) or 0.1 μM of

PI3k inhibitor (Wortmannin) (D). Data are mean ± SEM (n=4-6). *P ≤ 0.05 vs control conditions.

4.5 Discussion

The plasma insulin concentration is controlled by the interaction between insulin secretion and insulin clearance (7,8). Prolonged hyperinsulinemia is associated with reduced IR tyrosine kinase activity, and the normalization of plasma insulin concentrations recovers insulin signaling (3). In addition, non-obese mice with over-expression of the insulin gene present high insulin levels, associated with impaired insulin sensitivity, and consequently T2D (27). Thus, therapeutic interventions toward insulin clearance may be relevant. Here, we demonstrate that TUDCA treatment (15 days) ameliorates insulin clearance in HFD mice, probably by increasing IDE expression in the liver. In addition, we demonstrate that TUDCA increases IDE expression in hepatic human cell-line HepG2, through a mechanism dependent of the S1PR2/Insulin pathway.

As already shown (28), we observed that TUDCA treatment decreased body weight due to a reduction in fat pad deposits associated with improved glucose tolerance as well as insulin sensitivity. However, the possible role of TUDCA treatment upon insulin clearance had not been explored yet.

In both the HFD and HFD+TUDCA mice groups, plasma C-peptide concentrations were increased. In HFD mice, this increase indirectly reflects an augmented insulin secretion, probably, as a consequence of the insulin resistance (29). In HFD+TUDCA, which had lower insulin resistance than HFD mice, the increased insulin secretion may be a direct effect of TUDCA on pancreatic islets (30). Despite the HFD+TUDCA mice showing increased plasma C-peptide, their plasma insulin concentration was lower, resulting in a higher plasma C-peptide/insulin ratio. This suggests TUDCA treatment as an important insulin clearance booster. Our results indicate that the increased insulin clearance in the HFD+TUDCA mice was probably due to augmented expression of liver IDE, the most important organ responsible for insulin clearance(7).

In an attempt to explore the direct effect of TUDCA upon IDE, we incubated the human hepatic cell line HepG2, with or without TUDCA, and we confirmed that TUDCA per se increase IDE expression in these cells. These data reinforce our premise

that bile acid may increase insulin clearance by increasing IDE expression in the liver, contributing to the normalization of insulin levels in hyperinsulinemic pre-diabetic mice.

In addition to the well known effects of bile acids as regulators of lipid digestion and absorption in the small intestine (31), it was suggested that they also act as hormones, dependent on the bile acid type binding with specific receptors (31-33). In the hepatic cell TUDCA is a ligand of a G-protein coupled protein receptor called S1PR2 (23). The biding of TUDCA and other conjugated bile acids to S1PR2 activates the insulin pathway, at the IR-PI3K-Akt level. In addition, TUDCA also improves liver insulin signaling by reducing endoplasmic reticulum stress (22). Here, we show that TUDCA increases insulin clearance in HFD and that this effect seems to be dependent on S1PR2-IR-PI3K-Akt pathway.

Previous studies have demonstrated that different interventions, which improve insulin signaling, are associated with increased insulin clearance and IDE expression in obese mice (13, 13). In fact, neural cell exposure to insulin increased IDE expression, whereas insulin pathway inhibition, at PI3K level, abolished its effect (24). This evidence support our findings, about the possible role of insulin pathway over IDE expression.

Although this evidence indicated that increased insulin-signaling pathway might induce IDE expression, others studies have proposed a different conception of this issue, suggesting that IDE expression could also affect the insulin pathway (34). Likewise, human subjects, with a genetic defect at the IDE gene, develop insulin resistance and type 2 diabetes (35). In the same way, the Goto-Kakizaki rats, with a polymorphism at IDE gene, develop hyperinsulinemia, insulin resistance, and ultimately T2D (10). IDE knockout mice are hyperinsulinemic, which contributes to the maintenance of a chronic insulin signaling, and as a consequence the insulin negative feedback, resulting in insulin resistance by reduced IR expression in skeletal muscle, adipose tissue and liver (9).

Here, we provide evidence that TUDCA may be a therapeutic strategy to counteract obesity-induced hyperinsulinemia. We found that TUDCA increased insulin clearance in HFD mice, probably through increased IDE expression in the liver, reestablishing their plasma insulin levels. Our results also indicated that TUDCA-induced IDE expression seems to be mediated by S1PR2-Insulin signaling pathway.

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Acknowledgements

We thank Marise M. C. Brunelli, Jheynifer C. Souza and Bil for technical assistance, and Bridgett A. Bollin for English editing.

This study was supported by the Fundação de Amparo e Pesquisa do Estado de São Paulo (FAPESP, grant numbers 2013/01318-4, 2014/01717-9 and 2015/12611-0) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Grant number 449794/2014-8).

Author contributions

JFV, JMCJ and MAK contributed to research design and JFV, MAK, GMS, PCB, SMF, RCSB and LSLM conducted the experiments and acquired data. ACB and EMC provided all reagents. JFV contributed to data analysis and JFV, MAK and JMCJ to data interpretation. JFV, JMCJ and MAK wrote the manuscript. ACB, JMCJ and EMC revised the manuscript. All authors reviewed and approved the final version of the manuscript.

Additional information

Competing financial interests: The authors have declared that there is no competing interests.

5. Artigo 03

TUDCA reduces glucagon secretion via S1PR2-Insulin pathway in pancreatic alpha cells

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5.1 Abstract

Type 2 diabetes (T2D) is an epidemic that affects 9% of total world population, and is related to the onset of cardiac disease, renal failure and blindness. T2D is characterized by a decrease in insulin secretion by pancreatic β cells, as well as insulin resistance on peripheral tissues. Despite insulin is the main hormone involved in T2D dysfunction, glucagon is also impaired presenting elevated plasmatic levels, which contributes to hyperglycemia. Molecules which could interact with glucagon secretion also are important to T2D treatment. Bile acids have emerged as new endocrine signaling molecules that regulate glucose, lipid, and energetic metabolism. The taurine conjugated bile acid TUDCA regulates insulin secretion and signaling, however the effect of this compound on glucagon secretion is unknow. Here, using isolated pancreatic islets from C57Bl6 mice and the α cell line αTC1-9, we clarify the effects of TUDCA on glucagon secretion. The exposure of pancreatic islets and αTC1-9 cell line to 50μM TUDCA reduces glucose induced glucagon secretion. This effect is dependent of activation of the S1PR2/Insulin pathway, since the inhibition of S1PR2, PI3K and Akt blunted the TUDCA effect on glucagon secretion. Moreover, TUDCA increases the activity of ATP-sensitive potassium channel (KATP) in a population of alpha cells and reduces calcium oscillations in islets α cells. So, we conclude that the taurine conjugated bile acid TUDCA reduces glucagon secretion by altering electrical activity and calcium influx, due to the activation of the S1PR2/Akt pathway.

5.2 Introduction

Type 2 diabetes (T2D) affects 400 million people around the world, and is the main cause of cardiac disease, renal failure and blindness [1]. At onset of T2D, there is an increase in insulin resistance coupled with increased pancreatic islet β-cell insulin secretion, leading to a normoglycemic/hyperinsulinemic state. Frequently, the islet β-cell undergoes a progressive loss of function, which culminates in apoptosis and reduction in insulin plasma levels [2, 3]. Moreover, a defect in islet α-cell in the suppression of glucagon secretion upon hyperglycemic conditions contributes to the hyperglycemia observed in T2D [4, 5].

Glucagon secretion counteracts the effect of insulin and reverses hypoglycemia enhancing hepatic glucose output and gluconeogenesis. This hormone is secreted by pancreatic α cell in response to low glucose concentration, intestinal incretins as GLP-1/2, aminoacids and the sympathetic hormone adrenaline. As the pancreatic β cells, α cells express an ATP-dependent potassium channels (KATP) that couples the glucose metabolism to electrical activity. The pancreatic α-cell has a glucose transporter, SLC2A1, and at low glucose concentrations the KATP channel activity leads the membrane potential to a level at which Ca2+ and Na+ channels are activated. This activation leads to an increased Ca2+ influx, which culminates in glucagon secretion [6-8]. Insulin has a paracrine effect upon α-cell activating the Akt/PI3K signaling pathway, opening KATP channels, which results in membrane hyperpolarization and subsequent inhibition of calcium influx, reducing glucagon secretion [9, 10]. Insulin resistance could affect pancreatic α-cell, preventing the insulin effect on the inhibition of glucagon secretion which contributes to the hyperglucagonemia observed in type 1 and 2 diabetes.

The main target of glucagon is the liver, where activates a G-protein Coupled Receptor that enhances cAMP production and subsequently PKA and CREB phosphorylation resulting in glycogenolysis and gluconeogenesis activation and glucose release to the blood [11, 12]. Glucagon secretion is altered in all forms of diabetes, leading to enhanced hepatic glucose output, contributing to hyperglycemia. The treatment with glucagon receptor antagonists, as well as the knockout of glucagon receptor (Gcgr), improves glucose homeostasis. However, the Gcgr mice presents elevated glucagon levels and pancreatic α cell hyperplasia [11, 13-15]. Molecules which

reduce glucagon secretion could be effective on hyperglucagonaemia treatment, contributing to reduced liver glucose release and glycaemia.

The taurine conjugated bile acid Tauroursodeoxycholic (TUDCA) has emerged as a potential therapeutic tool in diabetes and obesity treatment. TUDCA protects pancreatic beta cells from Endoplasmic Reticulum Stress, reducing apoptosis and increasing viability [16]. The administration of TUDCA in leptin receptor deficient ob/ob mice, as well as high-fat diet (HFD) models, improves glucose homeostasis by enhanced insulin signaling in liver [17-19]. TUDCA also increases glucose induced insulin secretion in pancreatic β cells, and increases energetic metabolism in brown adipose tissue by the activation of the Deiodinasa 2 enzyme [20, 21]. However, the effect of this compound on glucagon secretion and pancreatic α cell is unknown.

Here, we demonstrated that acute exposure to TUDCA inhibits glucose -induced glucagon secretion in mouse isolated islets and α-TC1-9 cell lines. This effect is probably associated with increased KATP activity and reduced α cell calcium oscillations, as well as is linked to the activation of the Sphingosine-1-Phosphate Receptor 2 (S1PR2) and insulin pathway in pancreatic α cells.

5.3 Material and methods

Reagents: TUDCA was purchased from Calbiochem (São Paulo, SP, Brazil, cat. 580549), Wortmannin and JTE-013 from TOCRIS (Minneapolis, MN, USA, cat. 1232 and 2392, respectively), MK2206 from ChemCruz (Santa Cruz Biotechnology, Texas, USA, cat. sc-364537) and glucagon elisa kit was purchased from Mercodia (Uppsala, Sweden, cat. 10-1271-01). The remaining reagents were purchased from Sigma Chemical (St. Louis, MO, USA).

Animals: All experiments involving animals were approved by the Animal Care Committee at Miguel Hernández University (Ref. UMH.IB.IQM.01.13). Male 90-day-old C57Bl/6 mice were obtained from the breeding colony at UMH and were maintained at 22 ± 1 °C on a 12-h light–dark cycle with free access to food and water. Mice were euthanized in a CO2 chamber and decapitated for pancreatic islet isolation by collagenase digestion of the pancreas, as previous described.

Cell culture: αTC1-9 cell line was adopted as a model of pancreatic alpha cell, and purchased from ATCC (Barcelona, Spain, cat. CRL2350). αTC1-9 cell line was

cultured in a humidified atmosphere containing 5% CO2 and maintained in DMEM 5030 medium, supplemented with 11.1 mM glucose, 15mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2mM L-glutamine, 0.1mM non-essential aminiacids, 1% penicillin/streptomycin and 10% Fetal Bovine Serum (FBS) until 60–80% confluence. After this period, the cells were submitted to glucagon secretion.

Glucagon secretion: For static glucagon secretion, pancreatic islets (15 islets per well) or αTC1-9 cell line were recovered for 2 hours in isolation medium containing 11.5mM NaCl, 10mM NaHCO3, 5mM KCl, 1.1mM MgCl26H2O, 25mM Hepes and 1.2mM Na2HPO4 supplemented with 5.6mM glucose and 0.25% Bovine serum albumin (BSA) equilibrated with a mixture of 95% O2/5% CO2 to regulate the pH at 7.4. After that, the islets/cells were pre-incubated for 1hr with Krebs-bicarbonate buffer (KBB) containing 11.5mM NaCl, 5mM KCl, 2.56mM CaCl2, 1mM MgCl2, 10mM NaHCO3, 15mM HEPES, supplemented with 5.6 mmol/L glucose and 0.3% BSA and equilibrated with a mixture of 95% O2/5% CO2 to regulate the pH at 7.4. After 1hr of preincubation time, the medium was removed and immediately replaced with fresh KBB medium containing different glucose concentration and 50μM TUDCA, as well as the different reagents indicated in the experiments. After 1 h of incubation time, the medium was removed and stored at − 80 °C with 15uL Aprotinin. For islet glucagon and protein content, groups of 15 islets were collected and transferred to tubes containing 20uL buffer lysis (alcohol-acid), and the islet cells were homogenized using a sonicator (Brinkmann Instruments). Glucagon levels were measured by Elisa Kit. Total islet protein was assayed using the Bradford dye method [17] with BSA as the standard curve.

Calcium oscillations :For cytoplasmic alpha cell Ca2+ oscillations, fresh isolated islets were recovered for 2hrs in isolation medium. After that, islets were incubated with 4 μmol/L Fluo-4AM probe (Thermo Fisher, Waltham, Massachusetts, USA, cat. F14201) for 1 hour at 37 °C in KBB buffer that contained 5.6 mmol/L glucose, 0.3% BSA and pH 7.4. Islets were then washed with the same medium and placed in a chamber treated with Poli-Lysine, that was thermostatically regulated at 37 °C on the stage of a confocal microscope Zeiss LSM 510 (Carl Zeiss, Germany). Islets were perfused with albumin-free KBB that was continuously gassed with 95% O2/5% CO2 (pH 7.4), containing 0.5mM glucose in the presence or absence of 50μM TUDCA and 5μM Adrenaline. The confocal microscope allows the acquisition of sections from 0.5

and 12.4µm. Fluorescence changes were monitored with a 40X immersion microscope objective, by exciting the calcium probe at 488nm. An image was acquired every 2 seconds. The analysis was obtained using the software from confocal LSM Pascal 5 (Carl Zeiss, Germany).

Patch-Clamp recordings: Islets were dispersed into single cells and cultured as previously described [21]. KATP channel activity was recorded using standard patch-clamp recording procedures. Currents were recorded by using an Axopatch 200B patch-clamp amplifier (Axon Instruments, Union City, CA). Patch pipettes were pulled from borosilicate capillaries (Sutter Instrument, Novato, CA) using a flaming/brown micropipette puller P-97 (Sutter Instrument) with resistance between 3 and 5 MΩ when filled with pipette solutions, as specified below. The bath solution contained 5 mmol/L KCl, 135 mmol/L NaCl, 2.5 mmol/L CaCl2, 10 mmol/L HEPES, and 1.1 mmol/L MgCl2 (pH 7.4), and it supplemented with glucose as indicated. The pipette solution contained 140 mmol/L KCl, 1 mmol/L MgCl2, 10 mmol/L HEPES and 1 mmol/L EGTA (pH 7.2). The pipette potential was held at 0 mV throughout the recording process. KATP channel activity was quantified by digitizing 60 s sections of the current record filtered at 1 kHz and sampled at 10 kHz by a Digidata 1322A (Axon Instruments, Orleans Drive Sunnyvale, CA) and calculating the mean NPo during the sweep. Channel activity was defined as the product of N, the number of functional channels, and Po, the open state probability. Po was determined by dividing the total time channels spent in the open state by the total sample time. Values of NPo were normalized relative to the channel activity measured in control conditions before the application of different substances. Data sampling was initiated 1 min before (control) and 10–15 min after the application of the test substances. Experiments were carried out at room temperature (20–24 °C). These experiments were performed at 0.5mM glucose, a stimulatory concentration for pancreatic alpha cell.

Statistical Analysis: The results are presented as the mean ± SEM for the number of determinations (n) indicated. Statistical analysis was performed using Student's t test or ANOVA with the appropriate post-test using Graph Pad Prism 5.0 software (La Jolla, CA).

5.4 Results

TUDCA reduces glucose induced glucagon secretion in pancreatic α cell and αTC1-9 cell line. To determine the effects of TUDCA on glucagon secretion, pancreatic islets and αTC1-9 cell line were exposed to 50μM TUDCA, concentration already published for experiments with pancreatic β cells and insulin secretion. In the presence of low glucose concentration (0.5mM), which stimulates pancreatic α cells, TUDCA reduces glucose induced glucagon secretion in pancreatic islets (Fig. 1A, B) and αTC1-9 cell line (Fig. 1C). Glucagon secretion was inhibited at high glucose concentrations (11 mM). At these glucose level, TUDCA did not produce any effect.

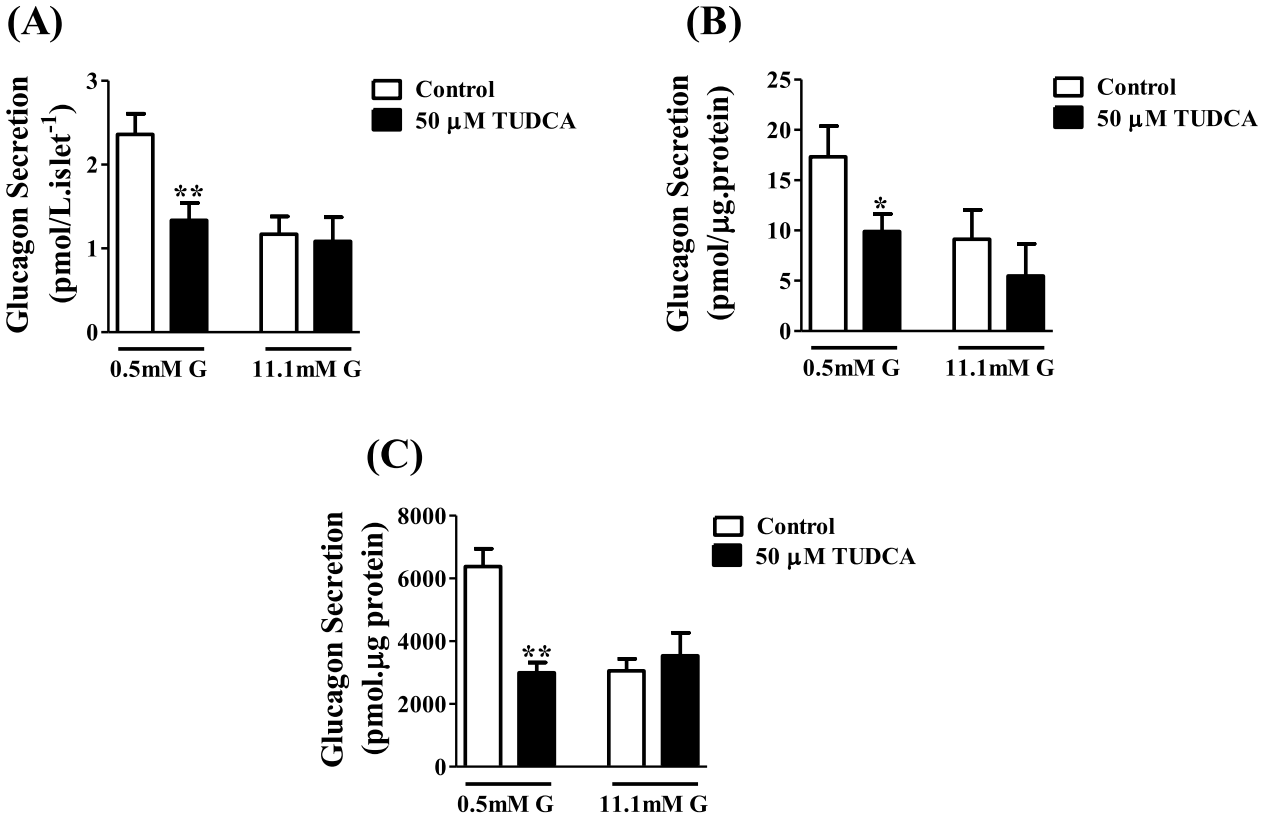

Figure 1. The bile acid TUDCA reduces glucose induced glucagon secretion. Pancreatic islets (A, B) and pancreatic alpha cell line αTC1-9 were exposed for 1hr to 0.5 or 11.1mM glucose, in the presence or not of 50μM TUDCA, as indicated in the graph. Glucagon secretion was measured using an Elisa kit, and normalized by islet number (A) or protein content (B, C). Data are expressed as mean ± SEM from 12

wells/condition, obtained from islets of 8 mice. * and ** indicates statistically significant difference from control condition, $p<0.05$.

TUDCA modulates electrical activity and calcium signals in pancreatic α cell. Glucagon secretion in pancreatic α cells is stimulated by low glucose concentration. These cells maintain a membrane potential of -60mV in low glucose concentration, opening T-type Ca2+ channels, depolarizing the membrane enough to activates Na and N-type Ca2+ channels and leading to action potentials. The Ca2+ influx by N-type Ca2+ channels triggers glucagon secretion [6, 7]. We started to determine if TUDCA could interact with electrical activity in α cells, resulting in reduced glucagon secretion. The KATP channel experiments shows that in 0.5mM glucose, pancreatic α cells have a low KATP activity (Fig. 2A, B). However, in the presence of TUDCA, we observed an increased KATP channel activity. Moreover, TUDCA reduces the appearance of action currents (Fig. 2A, B), which are indicative of the formation of action potentials. These effects suggest that TUDCA reduces the firing of action potentials by increasing KATP channel activity. However, we observed both effects only in 2 cells out of 5, and thus, the global TUDCA effect on KATP channel activity is not statistically significant (Fig. 2C). Since these are preliminary results, we will continue performing more experiments to fully determine these effects. Concomitantly, TUDCA reduces calcium oscillations in pancreatic alpha cells, effect that is likely attributed to reduced potential action firing (Fig. 3A).

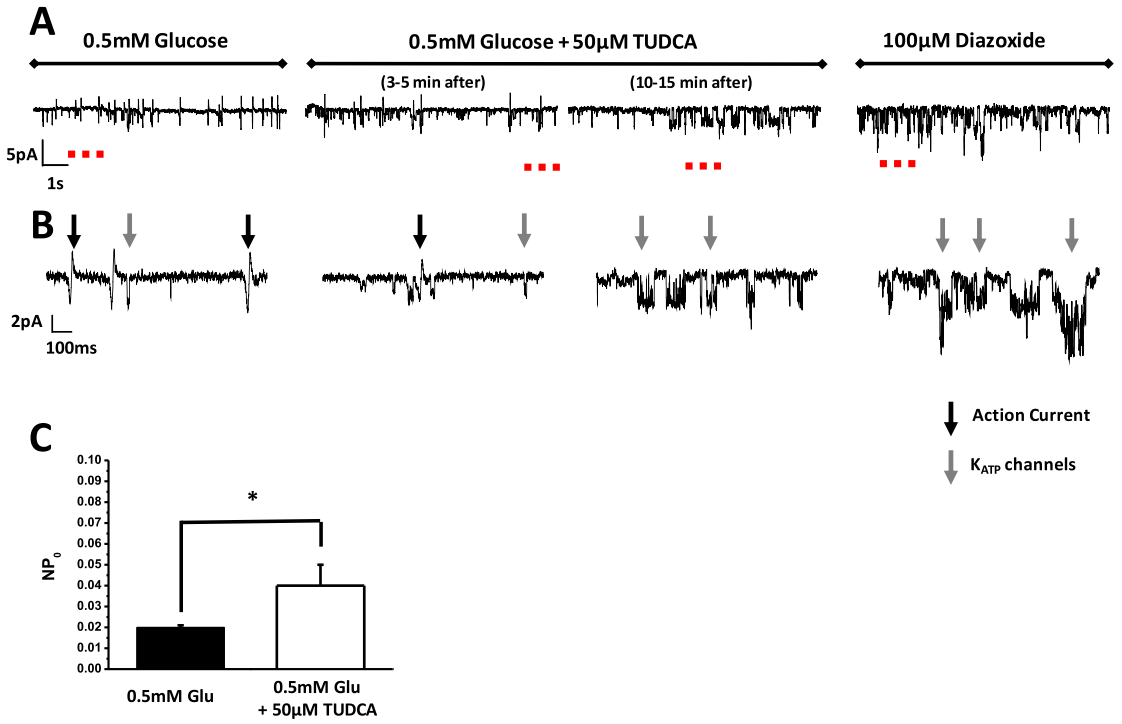

Figure 2. TUDCA effect on KATP channel activity in pancreatic α cells. A. Records show KATP channel activity in the presence of 0.5mM G, concentration at which action currents were generated. 10-15min after application of 50µM TUDCA no action currents were observed in 2 of 5 recordings. KATP channels were activated in this period. B. Expanded traces showed in A (red dotted line) C. KATP channel activity channel elicited by 0.5mM G and 10-15min after application of 50µM TUDCA (n=5 cells). Ns, non-significant.

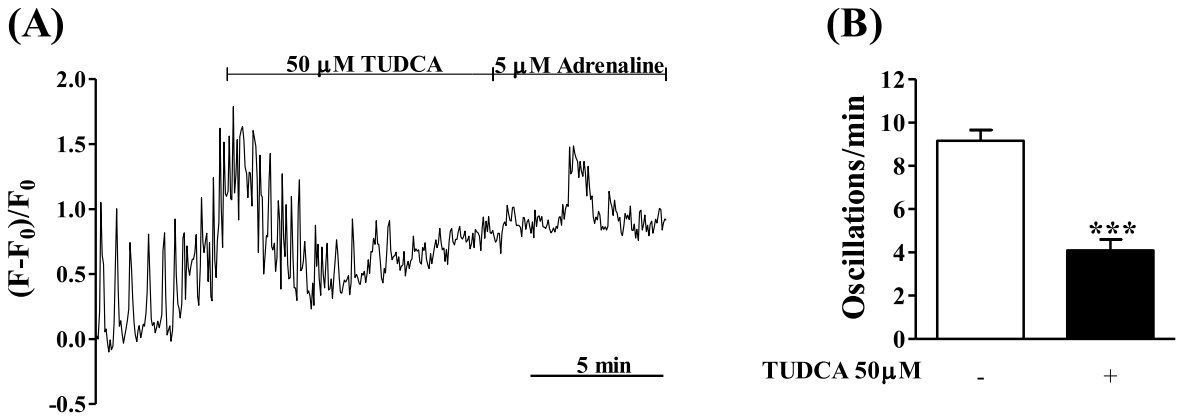

Figure 3. TUDCA inhibits Calcium oscillations induced by low glucose concentration in pancreatic islet α cells. Intact mouse islet was loaded with the

calcium probe Fluo-4 for 1hr, before exposure to 0.5mM glucose, 50μM TUDCA or 5μM adrenaline, as indicated in the graph. The calcium pattern (A) and oscillations (B) were acquired by confocal microscopy. Data are expressed as mean ± SEM, from 53 pancreatic alpha cells, obtained from 10 islets of 4 mice. individual experiments. *** indicates statistically significant difference from control condition, $p<0.05$.

The effect of TUDCA on glucagon secretion is dependent of the activation of the S1PR2, and not by TGR5 receptor. To determine the receptors involved in TUDCA action on pancreatic alpha cell, we decided to investigate the main bile acid receptors activated by TUDCA, S1PR2 and TGR5. In the liver, TUDCA activates S1PR2 which in turn is associated with the activation of the insulin pathway. However, in pancreatic β cells and brown adipose tissue, TUDCA activates the TGR5 receptor. In the presence of the S1PR2 inhibitor JTE-013, TUDCA is unable to reduce glucose-regulated glucagon secretion (Fig. 4A, B), showing that S1PR2 is essential for TUDCA action. The TGR5 did not have a pharmacological inhibitor, so we tested the effect of an agonist. Surprisingly, the incubation with INT-777, an agonist of TGR5, increases glucose-regulated glucagon secretion (Fig. 4C, D), effect opposite to that of TUDCA. So, these data point to the involvement of S1PR2, and not TGR5, on TUDCA effects. Moreover, the expression of S1PR2 is showed in Figure 5.

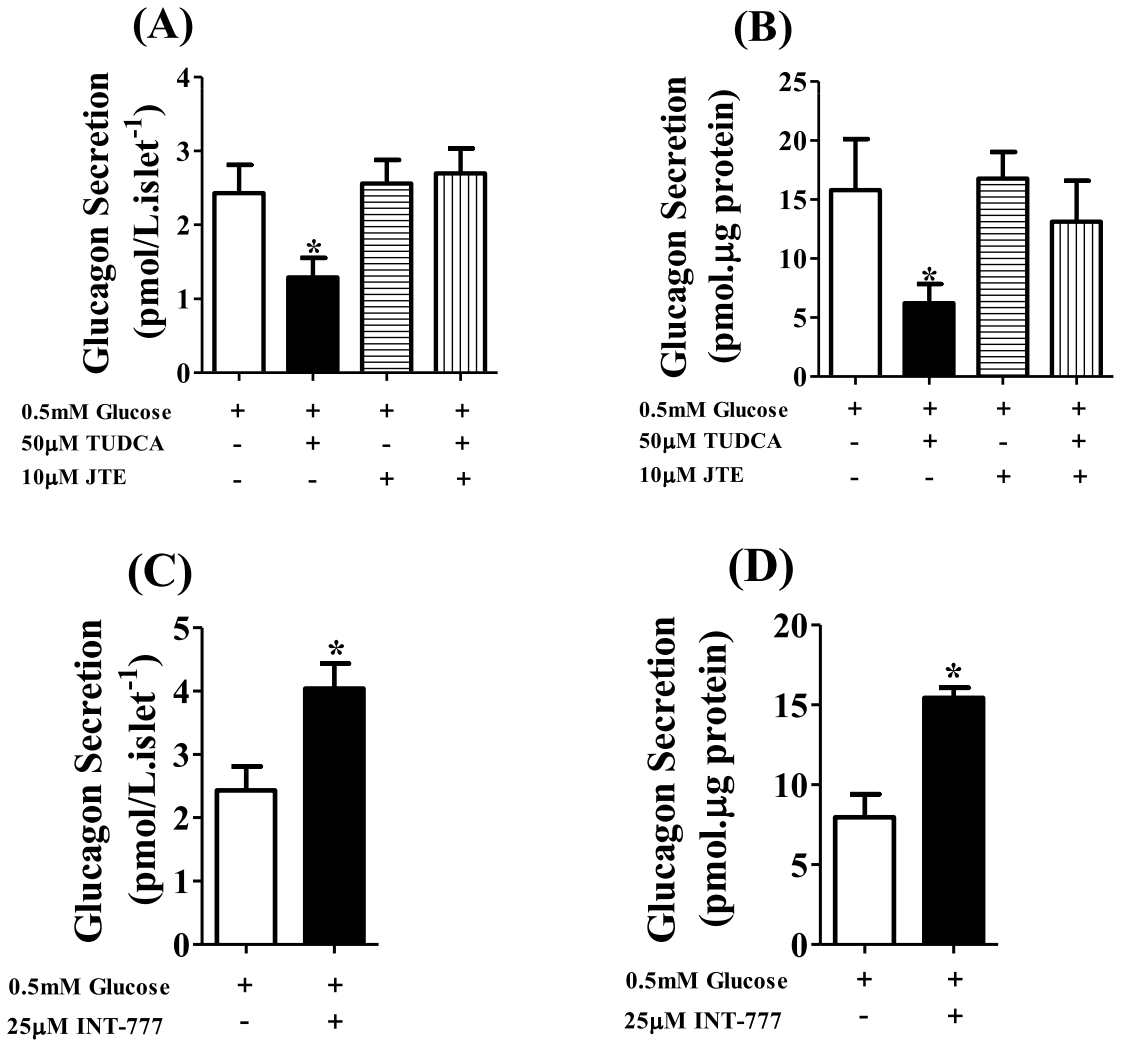

Figure 4. TUDCA effect on pancreatic alpha cell is dependent of the activation of Sphingosine-1-Phosphate Receptor 2 (S1PR2), and not TGR5. Mouse pancreatic islets were pre-incubated 1hr with 10µM JTE-013, inhibitor of the S1PR2, before exposition to 0.5mM glucose in the presence or absence of 50µM TUDCA, as indicated on the graph (A, B). Moreover, pancreatic islets were incubated 1hr with the TGR5 agonist INT-777 (C, D). The glucagon secretion was measured by Elisa kit, and normalized by islet number (A, C) or protein content (B, D). Data are expressed as mean ± SEM from 6 wells/condition, obtained from islets of 8 mice. * indicates statistically significant difference from control condition, $p<0.05$.

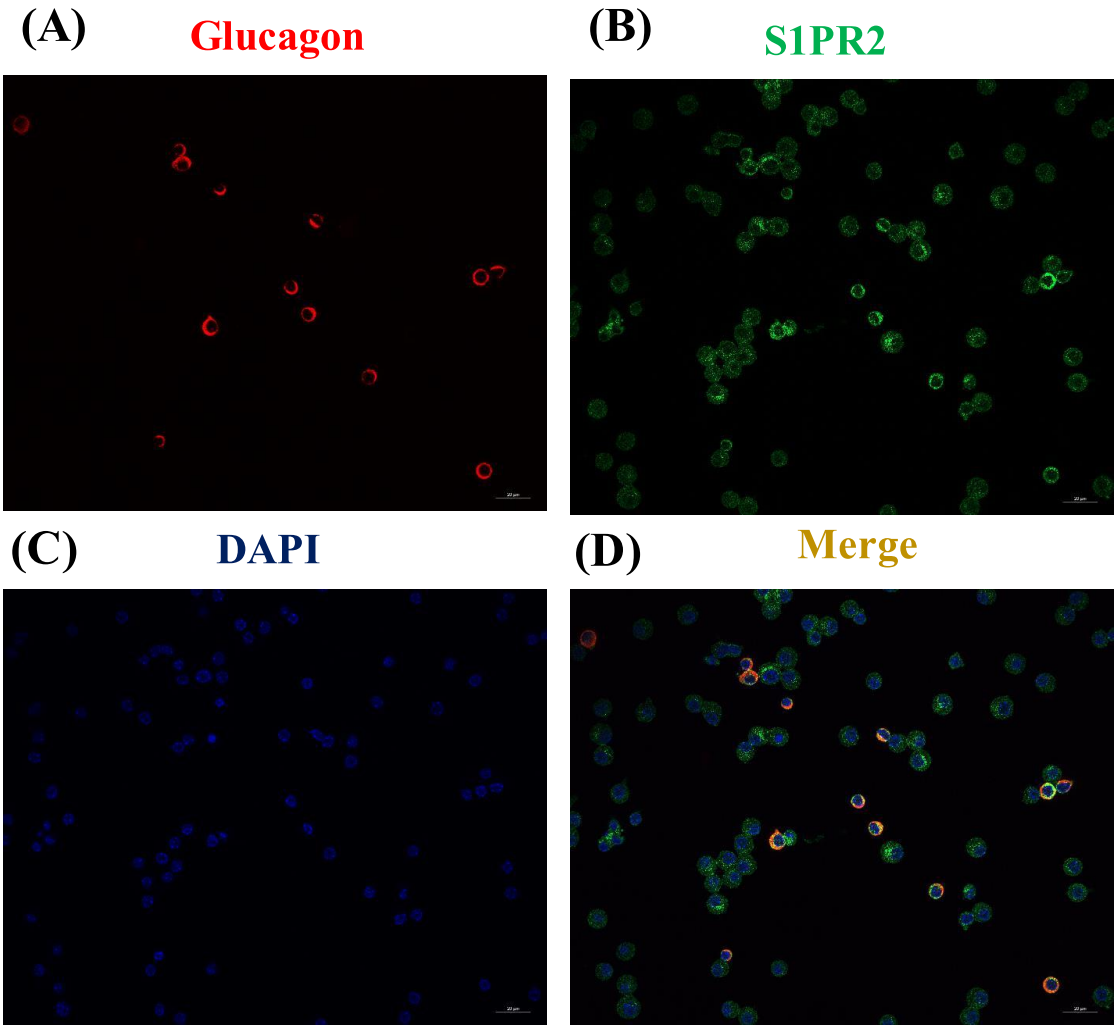

Figure 5. S1PR2 expression in pancreatic alpha cell from mouse islets. Immunofluorescence to Glucagon (A), S1PR2 (B), nucleus staining DAPI (C) and the merge (D). Fresh isolated islets were submitted to dispersion, and the glucagon and S1PR2 positive cells obtained by Immunofluorescence.

Insulin pathway is also involved in TUDCA action on pancreatic α cells. Since the activation of S1PR2 in the liver triggers the insulin pathway, we decided to investigate if the insulin route is also involved in the effects of TUDCA. Pancreatic islets were preincubated with the PI3K inhibitor Wortmannin, and with the Akt inhibitor MK2206. Both compounds blunted the effects of TUDCA on glucose-regulated glucagon secretion (Fig. 5A, B), pointing to the involvement of the S1PR2/Insulin pathway activation by TUDCA in pancreatic α cell.

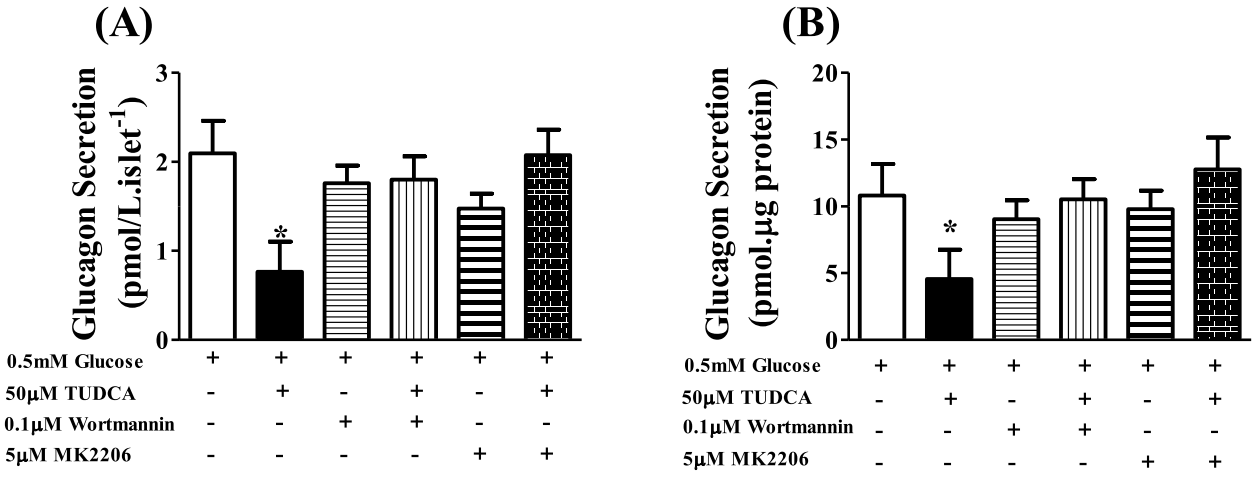

Figure 6. The insulin pathway is also involved in the TUDCA action on pancreatic alpha cells. Mouse pancreatic islets were pre-incubated 1hr with 0.1µM Wortmannin, a PI3K inhibitor, or 5 µM MK2206, an Akt inhibitor, before exposure to 0.5mM glucose in the presence or absence of 50µM TUDCA, as indicated on the graph (A, B). The glucagon secretion was measured by Elisa kit, and normalized by islet number (A) or protein content (B). Data are expressed as mean ± SEM from 6 wells/condition, obtained from islets of 6 mice. * indicates statistically significant difference from control condition, $p<0.05$.

5.5 Discussion

T2D is characterized by hyperglycemia due to impaired insulin secretion and signaling, as well as increased glucagon levels (*42, 84*). In this context, molecules which interacts with pancreatic beta and alpha cell has potential therapeutic interest. TUDCA increases glucose induced insulin secretion as well as insulin signaling (*65, 79*), and here we demonstrate that this bile acid reduces glucose induced glucagon secretion, effect associated with S1PR2/Insulin pathway activation, increased K_{ATP} channel activity and reduced calcium oscillations. Besides we did not use diabetic models to investigate TUDCA's effect on this context, the study of TUDCA *per se* on glucagon secretion allows the research about molecules that could modulates alpha cell function and helpful on hyperglucagonemia observed in T1D and T2D.

The bile acids have highlight on metabolic diseases treatment, once the activation of bile acid receptors FXR, TGR5 and S1PR2, mainly, modulates lipidic, glycemic and energetic metabolism (*53*). TUDCA and others taurine conjugated bile acids have a hydrophilic profile, which give them grater affinity by membrane receptors as TGR5 and S1PR2 (*52, 71*). TUDCA could activates both receptors, however seems that S1PR2 is responsible by the effects observed, once S1PR2 inhibition blunted the effects of TUDCA on glucagon secretion. No TGR5 inhibitor were used, once no pharmacological inhibition is commercially available. However, the stimulation of TGR5 by INT-777 for 1hr, same time for TUDCA incubation, increases glucose induced glucagon secretion, effect opposite to TUDCA. TGR5 activation leads to increased AMPc production, which activates a series of intracellular pathways that acutely, in pancreatic alpha cells, increases glucose induced glucagon secretion (*66*).

S1PR2 is a G-protein coupled receptors activated by sphingosine 1-phosphate-(S1P), a signaling sphingolipid released from ceramides that regulates cell proliferation, survival, and transcriptional activation (*72*). However, in the last years the discovery of S1PR2 as bile acid receptor has change the overview about this receptor. S1PR2 is activated by bile acids as TUDCA and TCA, and its activation on liver stimulates insulin receptor autophosphorylation, resulting in increased PI3K and Akt activation, regulating gluconeogenic genes, glycogen and fatty acid synthesis, fatty acid oxidation and bile acid synthesis (*71*). On pancreatic alpha cell, insulin receptor activation inhibits glucose induced glucagon secretion. This effect is due to Akt activation that

phosphorylates GABA receptors, translocate this receptors to plasma membrane and change the membrane potential by increasing K_{ATP} channel activity, reducing calcium oscillations and consequently glucagon secretion (*28, 30, 85*). Once TUDCA activates S1PR2 on liver and improves insulin signaling, we supposed that this receptor could be activated by TUDCA on pancreatic alpha cells, resulting on insulin signaling activation and reduced glucagon secretion.

The identification of S1PR2 expression on pancreatic alpha cells confirm the importance of this receptor on glucagon secretion, due to the high fluorescence emitted by S1PR2 antibody incubation. The use of S1PR2 inhibitor, JTE-013, as well as insulin pathway inhibitors like Wortmannin and MK2206, confirm that TUDCA use this pathway to inhibits glucagon secretion, once these inhibitors isolated blunted the effects of TUDCA. We also confirm that TUDCA could be acting by S1PR2/Insulin dependent mechanism, once the effects of TUDCA on electrical activity in pancreatic alpha cells is similar to that observed with insulin.

Electrical activity in alpha cells orchestrates the calcium influx, indispensable on glucagon granule exocytosis. The K_{ATP} channels connects cellular metabolism with electrical activity, effect that occurs in a small-time scale (*30, 86*). Patch-Clamp records allows the fine data acquisition of changes on electrical activity, and TUDCA increases K_{ATP} channel activity in low glucose concentrations. TUDCA also reduce potential action firing, which reflects on reduced calcium oscillations. On pancreatic beta cells, TUDCA did not modulates K_{ATP} channel activity, and these effect is associated with the activation of another receptor, TGR5. However, on alpha cells TUDCA seems to activate S1PR2, and not TGR5, which increases K_{ATP} channel activity and results in membrane hyperpolarization.

TUDCA have demonstrated a series of effects on pathologies. This bile acid is largely used to treat ocular diseases, hepatic alterations and recently has been used on glycemic disorders (*68, 78, 79, 83, 87*). TUDCA treatment in high fat diet model's improvers hepatic glycolipid disorders. On pancreatic beta cells, TUDCA reduces apoptosis and increases glucose induced insulin secretion. Here, we demonstrate that this taurine conjugated bile acid also regulates alpha cell function, reducing glucose-induced glucagon secretion by a S1PR2/Insulin pathway dependent mechanism. TUDCA shows potent therapeutic importance and could be used as a treatment to

hyperglucagonemia on T1D and T2D, contributing to reduced glucagon secretion and normoglycemic maintenance.

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Acknowledgements

We thank M. Carlenossi, M. S. Ramon and M. L. Navarro for their expert technical assistance. This work was supported by grants from Fundacão de Amparo á Pesquisa do Estado de São Paulo (FAPESP 2013/01318-4), Conselho Nacional para o Desenvolvimento Científico e Tecnológico (CNPq 200030/2014-0), Instituto Nacional de Obesidade e Diabetes (CNPq/FAPESP) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). This study was also supported by grants from the Spanish Ministerio de Ciencia e Innovación (BFU2013-42789-P; BFU2011-28358). CIBERDEM is an initiative of the Instituto de Salud Carlos III.

Conflict of interest

All contributing authors report no conflict of interest.

6. Discussão

O TUDCA modula a homeostase glicêmica, agindo em órgãos como fígado e pâncreas endócrino. Com este trabalho nós demonstramos que este BA potencializa a secreção de insulina estimulada por glicose em células beta pancreáticas, dependente da via do AMPc/PKA. Na degradação de insulina, o TUDCA aumenta a expressão da IDE em células HepG2 e fígado de camundongos obesos através do receptor S1PR2, refletindo em melhora na homeostase glicêmica. Em células alfa pancreática, o TUDCA reduz a secreção de glucagon estimulada por glicose, também através do receptor S1PR2 e mediadas por alterações eletrofisiológicas envolvidas com os canais de Ca^{2+} e K_{ATP}.

Os BAs têm se destacado no tratamento de diversas patologias, assim como obesidade e DM2. Estes compostos levam a redução do peso corpóreo e depósitos de gordura (*68, 83*), aumento na β-oxidação e redução na esteatose hepática(*83*), melhora na tolerância a glicose e sensibilidade a insulina (*79, 81, 83, 88*), modulação na secreção de insulina e glucagon (*59, 66*), além de serem utilizados no tratamento do Alzheimer (*89*), doenças cárdicas (*88*), renais (*69*), visuais (*76*) e pulmonares (*90*) em modelos animais. Estes efeitos são decorrentes do uso de BAs conjugados a taurina, como o TUDCA.

O TUDCA foi inicialmente utilizado no tratamento de doenças hepáticas por muitos anos (*55, 91*). Recentemente, este BA tem sido aplicado no tratamento de alterações na homeostase glicêmica, uma vez que o TUDCA reduz o estresse de reticulo hepático melhorando a sinalização de insulina, além de proteger as células beta pancreáticas contra apoptose. Nos demonstramos aqui que, além dos efeitos supracitados, este BA age diretamente na célula beta, potencializando a secreção de insulina, supostamente pela ativação do receptor TGR5. As células beta pancreáticas expressam o receptor para BAs TGR5, e sua ativação potencializa a secreção de insulina através do aumento intracelular de AMPc e fosforilação de PKA (*64*). O efeito do TUDCA em potencializar a secreção de insulina é dependente desta mesma via, pois a inibição do AMPc ou PKA impede sua ação na ilhota pancreática.

A ativação da PKA em ilhotas pancreáticas está relacionada com aumento na secreção dos grânulos contendo insulina. Este efeito foi observado através do uso de GLP-1, que também aumenta a produção intracelular de AMPc e consequente secreção

de insulina (*19*). O aumento no AMPc induzido pelo GLP-1 ativa a PKA, que fosforilada estimula a snapina, uma proteína moduladora do processo de exocitose inicialmente descrita em neurônios (*92*). A ativação da snapina é necessária para a interação entre as proteínas relacionadas a maquinaria exocitotica, e sua superexpressao mimetiza os efeitos do GLP-1 em potencializar a secreção de insulina (*93*). Além disso, sabe-se que a ação da PKA na secreção de insulina se dá principalmente no recrutamento e iniciação da exocitose, efeito que ocorre após o influxo de cálcio (*94, 95*) Estes dados corroboram com os efeitos do TUDCA, aumentando a secreção de insulina dependente de AMPc/PKA sem alteração no influxo de cálcio. O TUDCA também mimetiza os efeitos do GLP-1 uma vez que ambos compostos só atuam na secreção de insulina em altas concentrações de glicose (*96*), não oferecendo riscos de hipoglicemia.

Na manutenção da glicemia, a insulina é finamente regulada por 3 processos: secreção, sinalização e degradação. Os BAs como TUDCA regulam a secreção e sinalização de insulina. Aqui demonstramos pela primeira vez que estes compostos também regulam o processo de degradação de insulina hepática, via aumento na expressão da IDE, o que reflete em alterações na insulinemia. O papel da IDE na homeostase glicêmica e DM2 ainda não está esclarecido. Contudo, alterações na expressão desta enzima, assim como na degradação da insulina, são relatados na obesidade, pré-diabetes e DM2.

O tratamento de camundongos obesos com pioglatizona ou exercício físico, ambos sensibilizadores da via da insulina, aumenta a expressão hepática da IDE e degradação de insulina, efeito que reflete em redução de 50% na insulina plasmática e 20% na glicemia (*48, 97*). O uso do TUDCA em camundongos obesos levou ao mesmo efeito observado com pioglatizona ou exercício, com aumento da expressão hepática da IDE, redução da insulina plasmática e redução na glicemia de jejum e alimentado. Existe uma associação entre a via da insulina e a expressão da IDE que parece regular esta enzima e a concentração de insulina. O mecanismo de ação do TUDCA na modulação da expressão hepática da IDE foi analisado em linhagem de hepatócitos HepG2, efeito associado a ativação do receptor S1PR2 e da via da insulina, uma vez que a inibição desta via impede o aumento na expressão da IDE estimulado pelo TUDCA.

Em neurônios, a redução na sinalização da insulina reflete em redução na expressão da IDE. Este efeito está associado ao aumento nos depósitos do composto β-

amilóide, um dos alvos da IDE, culminando no desenvolvimento do Alzheimer (*89, 98*). O uso do TUDCA no tratamento do Alzheimer foi explorado em camundongos que apresentam aumento nos depósitos β-amilóide. O TUDCA reduziu os depósitos deste composto e melhorou a capacidade cognitiva e memória dos camundongos com Alzheimer. Apesar do mecanismo de ação do TUDCA não ser explorado neste contexto, acreditamos que também seja via aumento na expressão da IDE. Uma vez aumentada, a IDE consegue degradar o composto β-amilóide e reverter os danos ocasionados pelo Alzheimer.

O TUDCA parece modular também a degradação de insulina após procedimentos de cirurgia bariátrica. A realização de cirurgias que excluem a porção inicial do intestino tem sido muito utilizada no tratamento da obesidade e DM2 (*99, 100*). Após a realização do procedimento, os pacientes apresentam principalmente redução no peso corpóreo e glicemia. A glicemia sofre redução já nas primeiras semanas após o procedimento, antes mesmo da perda de peso. Este efeito está associado à melhora na sensibilidade hepática a insulina e consequente aumento na expressão da IDE e degradação da insulina (*101*). Curiosamente, o TUDCA apresenta aumento de até 36 vezes após estes tipos de procedimentos e pode estar implicado no aumento na expressão da IDE (*102*).

Os efeitos atribuídos ao TUDCA na homeostase glicêmica derivam de uma gama de fatores. Este BA regula a secreção (*65*), sinalização (*82*) e degradação da insulina. Contudo, sabemos que o DM2 é uma desordem bi-hormonal, e que o glucagon também está implicado no desenvolvimento e manutenção da hiperglicemia nesta doença (*103*). Frente a isto, nós analisamos também pela primeira vez se a incubação aguda á BAs altera a secreção de glucagon estimulada por glicose.

O glucagon, principal hormônio hiperglicemiante, apresenta elevados níveis em indivíduos obesos e diabéticos. Este efeito é decorrente, principalmente, de uma falha na secreção de glucagon que perde sua regulação frente a glicose e insulina. A falta da ação inibitória da glicose e insulina na secreção de glucagon induz a hiperglucagonemia, que aumenta a liberação hepática de glicose e contribui para a hiperglicemia (*8, 27, 30, 104*). Uma vez que a insulina é o principal agente inibitório da secreção de glucagon e que o TUDCA estimula a via da insulina, buscamos investigar se este BA também regula a secreção de glucagon em células alfa pancreáticas.

A incubação de ilhotas pancreáticas, assim como células αTC1-9, com TUDCA reduziu a secreção de glucagon. Este efeito foi associado a ativação do receptor S1PR2 que ativa a via de sinalização da insulina na célula alfa. A ativação desta via aumenta a atividade dos canais de K_{ATP}, o que resulta em hiperpolarização da membrana, redução na geração de potenciais de ação e consequente redução no influxo de cálcio (*20, 105*). O TUDCA também ativa a sinalização da via da insulina na célula alfa, uma vez que o uso de inibidores da PI3K e Akt bloquearam seu efeito. Além disso, as alterações observadas na presença do TUDCA são semelhantes as desencadeadas pela insulina, indicando que este composto também age na função da célula alfa pancreática.

Portanto, o TUDCA modula a homeostase glicêmica através da insulina e glucagon, efeito decorrente da ativação dos receptores TGR5 em células beta pancreáticas, ou S1PR2 no fígado e células alfa pancreáticas. Este efeito reflete na regulação na concentração destes hormônios, principais reguladores da homeostase glicêmica.

7. Conclusão

1. O TUDCA potencializa a secreção de insulina em ilhotas isoladas de camundongos C57Bl6. Este efeito não está relacionado a alterações eletrofisiológicas, como influxo de cálcio ou atividade dos canais de K_{ATP}, mas depende da via do AMPc/PKA.
2. O TUDCA aumenta a degradação de insulina em células HepG2 e camundongos obesos através do aumento na expressão da IDE. O efeito do TUDCA em células hepáticas é dependente da ativação do receptor S1PR2, e consequente via da insulina.
3. O TUDCA reduz a secreção de glucagon estimulada por glicose em células alfa pancreáticas, efeito associado a ativação do receptor S1PR2, que aumenta a atividade dos canais de K_{ATP} e reduz as oscilações de cálcio.

Neste contexto, o TUDCA parece um candidato promissor para o tratamento de alterações na homeostase glicêmica, como o DM2. Através do aumento na secreção, sinalização e degradação de insulina, juntamente com a redução na secreção de glucagon, este BA contribui para a manutenção da normoglicemia. Além disso, devido a sua baixa toxicidade, e por ser um composto fisiologicamente funcional, este BA pode ser administrado na tentativa de prevenir ou reverter o DM2, além de outras patologias como o Alzheimer.

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9. Anexos

✓ **Publicação do Artigo 01**

METABOLISM CLINICAL AND EXPERIMENTAL 65 (2016) 54–63

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The bile acid TUDCA increases glucose-induced insulin secretion via the cAMP/PKA pathway in pancreatic beta cells

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✓ **Submissão e resposta do Artigo 02**

Manuscript #	SREP-17-15526
Current Revision #	0
Submission Date	7th April 17
Current Stage	Waiting for Revision
Title	The bile acid TUDCA improves insulin clearance increasing the insulin-degrading enzyme expression in the liver of obese mice
Manuscript Type	Original Research
Corresponding Author	Dr. Everardo Carneiro (emc@unicamp.br) (University of Campinas)
Contributing Authors	Dr. Jean Vettorazzi , Dr. Mirian Kurauti , Miss Gabriela Moreira Soares , Miss Patrícia Borck , Dr. Sandra Mara Ferreira , Mrs. Renato Branco , Miss Luciana de Souza Lima Michelone , Dr. Antonio Boschero , Dr. José Maria Costa-Júnior
Authorship	Yes
Abstract	Both, insulin secretion and clearance disruption contribute to obesity-induced hyperinsulinemia. However, the insulin clearance reduction seems to be the mainly factor in this context. The liver is the major site for insulin degradation, a process mainly coordinated by the Insulin-degrading enzyme (IDE). The beneficial effects of taurine conjugated bile acid (TUDCA) on insulin secretion as well as insulin sensitivity have been recently described. However, the possible role of TUDCA in insulin clearance had not yet been explored. Here, we demonstrated that treatment for 15 days with TUDCA reestablished plasma insulin to physiological concentrations in high fat diet (HFD) mice, a phenomenon associated with increased insulin clearance and liver IDE expression. TUDCA also increased IDE expression in human hepatic cell line HepG2. This effect was not observed in the presence of an inhibitor of the hepatic membrane bile acid receptor, S1PR2, as well as when its downstream proteins were inhibited, including IR, Pi3K and Akt. These results indicated that treatment with TUDCA may be helpful to counteract obesity-induced hyperinsulinemia, by increasing insulin clearance, an effect that seems to be orchestrated by enhanced liver IDE expression in a mechanism dependent on S1PR2-Insulin pathway activation.
Techniques	Life sciences techniques, Protein techniques [Protein expression];
Subject Terms	Biological sciences/Physiology/Metabolism Health sciences/Endocrinology/Endocrine system and metabolic diseases/Obesity
Competing Financial Interest	There is **NO** Competing Interest.
Applicable Funding Source	Fundação de Amparo a Pesquisa do Estado de São Paulo 2014/01717-9 [Carneiro] Fundação de Amparo a Pesquisa do Estado de São Paulo 2013/01318-4 [Vettorazzi] Conselho Nacional de Desenvolvimento Científico e Tecnológico 449794/2014-8 [Michelone] Fundação de Amparo a Pesquisa do Estado de São Paulo 2015/12611-0 [Boschero]

Dear Dr Carneiro,
Your manuscript entitled "The bile acid TUDCA improves insulin clearance increasing the insulin-degrading enzyme expression in the liver of obese mice" has now been reviewed and the reviewer comments are appended below. You will see that, while they find your work of interest, they have raised points that need to be addressed by a major revision.
We therefore invite you to revise and resubmit your manuscript, taking into account the points raised. At the same time, we ask that you ensure your manuscript complies with our format requirements explained in full at:
https://www.nature.com/srep/publish/guidelines
We hope to receive your revised paper within four weeks. If you cannot send it within this time, please let us know so that we can close your file. In this event, we will still be happy to reconsider your paper at a later date as long as you haven't submitted similar or related work elsewhere in the meantime.
We look forward to hearing from you soon. Best regards,

Maria Garcia .Editorial Board Member

Scientific Reports

- ✓ **Atividades cientificas e publicações desenvolvidas durante a tese**

  - ✓ **Estágio no exterior**

    1. Nome: Jean Franciesco Vettorazzi

       Processo: 200030/2014-0 – Cnpq

       Duração: Abril/2014 á Marco/2015

       Modalidade: Doutorado Sanduíche no Exterior - SWE

       Projeto: Regulação das células Alfa-pancreáticas e da secreção do glucagon em modelo de obesidade genética: Camundongos ob/ob

       Local: Universidad Miguél Hernández de Elche, Elche, Espanha

    2. Nome: Jean Franciesco Vettorazzi

       Processo: 2017/07127-4 - FAPESP

       Modalidade: Bolsa Estágio de Pesquisa no Exterior – BEPE

       Duração: Julho/2016 á Janeiro/2017

       Projeto: O ácido biliar TUDCA modula a secreção de glucagon em células alfa pancreáticas

       Local: Universidad Miguél Hernández de Elche, Elche, Espanha

  - ✓ **Cursos**

    1. Título: Cromatografia Líquida (HPLC ou CLAE): Fundamentos, Instrumentação e Aplicações

       Realização: INSTITUTO INTERNACIONAL DE CROMATOGRAFIA (IIC)

       Período: 02, 03 e 04 de dezembro de 2015

  - ✓ **Resumos apresentados em congresso**

1. Título do trabalho: Taurine supplementation improves glucose homeostasis and prevents pancreatic alterations in leptin deficient ob/ob mice.

   Autores: Santos-Silva, JC. **Vettorazzi, JF**; Irles, E; Rickli, S; Nadal, A; Boschero, AC; Ribeiro, RA; Carneiro, EM.

   Evento: II Workshop pós BFM Unicamp – Campinas/Brasil

   Duração: 13/14 de agosto de 2014

2. Título: The bile acid TUDCA increases pancreatic betta-cell responsiveness to glucose via the cAMP/PKA pathway.

   Autores: **Vettorazzi JF**, Ribeiro RA, Borck PC, Branco RC, Soriano S, Merino B, Boschero AC, Nadal A, Quesada I, Carneiro EM.

   Evento: XXX Reunião Anual da Federação da Sociedade brasileira de Biologial Experimental –FESBE – São Paulo/Brasil

   Duração: 09 a 12 de setembro/2015

3. Título: TUDCA protects pancreatic α-cell against endoplasmatic reticulum (er) stress;

   Autores: **Vettorazzi, J.F**., Ribeiro, R.A., Borck, P.C., Branco, R.C.S., Merino, B.. Bru, E., Quesada, I. M. , Carneiro, E.M.

   Evento: V Encontro sobre Síndrome Metabólica – Cuiabá, Brasil

   Duração: 29 e 30 de outubro/2015

4. Título: TUDCA modulates alpha cell function and glucagon secretion

   Autores: **Vettorazzi, J.F**., Ribeiro, R.A., Borck, P.C., Branco, R.C.S., Merino, B.. Bru, E., Quesada, I. M. , Carneiro, E.M.

   Evento: The Islet Study group meeting - ISG.

   Duração: 16 a 18 de setembro/2016 – Garmisch/Alemanha

5. Título: The bile acid TUDCA modulates ER stress in beta and alfa cell exposed to different stressor

Autores: **Vettorazzi, J.F**., Ribeiro, R.A., Borck, P.C., Branco, R.C.S., Merino, B.. Bru, E., Quesada, I. M. , Carneiro, E.M.

Evento: Diabetes, hypertension, metabolic syndrome and pregnancy – DIP 2017 – Barcelona, Espanha

Duração: 8 a 12 de marco/2017

- ✓ **Premiações**

1. Menção Honrosa na XXX Reunião Anual da Federação da Sociedade brasileira de Biologial Experimental -FESBE- 09 a 12 de setembro/2015. The bile acid TUDCA increases pancreatic betta-cell responsiveness to glucose via the cAMP/PKA pathway. Vettorazzi JF, Ribeiro RA, Borck PC, Branco RC, Soriano S, Merino B, Boschero AC, Nadal A, Quesada I, Carneiro EM.
2. Artigo científico indicado para concorrer ao prêmio Metabolism Young Investigator Award, realizado anualmente pela revista Metabolism.

- ✓ **Extensão e outros**

1. Monitor da oficina Master chefe nutricional (Centro de pesquisa, Inovação e Difusão – CEPID – FAPESP)

   Faculdade de Ciências Médicas da Unicamp

   Período: 11/11/205

2. Apresentação no Workshop New Horizons in Diabetes Therapy

   Título da palestra: TUDCA and the treatment of glucose homeostasis disorders

   Universidade Estadual de Campinas – Unicamp

   Período: 18/05/2016

3. Apresentação de Seminário para o curso de pós graduação em Biociências da Universidade de Alicante, Espanha

   Título: The effects of the bile acid TUDCA on glucose homeostasis

Universidad de Alicant – Alicante, Espanha

Período: 11/11/2016

- ✓ **Artigos científicos publicados durante o doutorado**

1. Protein malnutrition blunts the increment of taurine transporter expression by a high-fat diet and impairs taurine reestablishment of insulin secretion. Branco RCS, Camargo RL, Batista TM, **Vettorazzi JF**, Borck PC, Dos Santos-Silva JCR, Boschero AC, Zoppi CC, Carneiro EM. FASEB J. 2017 Jun 1. pii: fj.201600326RRR. doi: 10.1096/fj.201600326RRR.

2. Protein malnutrition potentiates the amplifying pathway of insulin secretion in adult obese mice. Leite NC, de Paula F, Borck PC, **Vettorazzi JF**, Branco RC, Lubaczeuski C, Boschero AC, Zoppi CC, Carneiro EM. Sci Rep. 2016 Sep 16;6:33464. doi: 10.1038/srep33464.

3. Acute Exercise Improves Insulin Clearance and Increases the Expression of Insulin-Degrading Enzyme in the Liver and Skeletal Muscle of Swiss Mice. Kurauti MA, Freitas-Dias R, Ferreira SM, **Vettorazzi JF**, Nardelli TR, Araujo HN, Santos GJ, Carneiro EM, Boschero AC, Rezende LF, Costa-Júnior JM. PLoS One. 2016 Jul 28;11(7):e0160239. doi: 10.1371

4. Benefits of L-alanine or L-arginine supplementation against adiposity and glucose intolerance in monosodium glutamate-induced obesity. Araujo TR, Freitas IN, **Vettorazzi JF**, Batista TM, Santos-Silva JC, Bonfleur ML, Balbo SL, Boschero AC, Carneiro EM, Ribeiro RA. Eur J Nutr. 2016 Jun 17.

5. Day-restricted feeding during pregnancy and lactation programs glucose intolerance and impaired insulin secretion in male rat offspring. de Almeida Faria J, de Araújo TM, Mancuso RI, Meulman J, da Silva Ferreira D, Batista TM, **Vettorazzi JF**, da Silva PM, Rodrigues SC, Kinote A, Carneiro EM, Bordin S, Anhê GF. Acta Physiol (Oxf). 2016 Jul;217(3):240-53. doi: 10.1111/apha.12684

6. The bile acid TUDCA increases glucose-induced insulin secretion via the cAMP/PKA pathway in pancreatic beta cells. **Vettorazzi JF**, Ribeiro RA, Borck PC, Branco RC, Soriano S, Merino B, Boschero AC, Nadal A,

Quesada I, Carneiro EM. Metabolism. 2016 Mar;65(3):54-63. doi: 10.1016/j.metabol.2015.10.021

7. Chronic use of pravastatin reduces insulin exocytosis and increases β-cell death in hypercholesterolemic mice. Lorza-Gil E, Salerno AG, Wanschel AC, **Vettorazzi JF**, Ferreira MS, Rentz T, Catharino RR, Oliveira HC. Toxicology. 2016 Feb 17;344-346:42-52. doi: 10.1016/j.tox.2015.12.007

8. Taurine supplementation ameliorates glucose homeostasis, prevents insulin and glucagon hypersecretion, and controls β, α, and δ-cell masses in genetic obese mice. Santos-Silva JC, Ribeiro RA, **Vettorazzi JF**, Irles E, Rickli S, Borck PC, Porciuncula PM, Quesada I, Nadal A, Boschero AC, Carneiro EM. Amino Acids. 2015 Aug;47(8):1533-48. doi: 10.1007/s00726-015-1988-z.

9. Effects of paternal hypothalamic obesity and taurine supplementation on adiposity and vascular reactivity in rat offspring. de Fátima Leão V, Raimundo JM, Ferreira LL, Santos-Silva JC, **Vettorazzi JF**, Bonfleur ML, Carneiro EM, Ribeiro RA. Adv Exp Med Biol. 2015;803:749-63. doi: 10.1007/978-3-319-15126-7_60

10. The effect of taurine supplementation on glucose homeostasis: the role of insulin-degrading enzyme. Camargo RL, Branco RC, de Rezende LF, **Vettorazzi JF**, Borck PC, Boschero AC, Carneiro EM. Adv Exp Med Biol. 2015;803:715-24. doi: 10.1007/978-3-319-15126-7_57

11. Taurine supplementation regulates pancreatic islet function in response to potentiating agents in leptin-deficient obese mice. Santos-Silva JC, Ribeiro RA, **Vettorazzi JF**, Borck PC, Boschero AC, Carneiro EM. Adv Exp Med Biol. 2015;803:371-85. doi: 10.1007/978-3-319-15126-7_28

12. Taurine Supplementation Enhances Insulin Secretion Without Altering Islet Morphology in Non-obese Diabetic Mice. Ribeiro RA, Santos-Silva JC, **Vettorazzi JF**, Cotrim BB, Boschero AC, Carneiro EM. Adv Exp Med Biol. 2015;803:353-70. doi: 10.1007/978-3-319-15126-7_27

13. Vagotomy ameliorates islet morphofunction and body metabolic homeostasis in MSG-obese rats. Lubaczeuski C, Balbo SL, Ribeiro RA, **Vettorazzi JF**, Santos-Silva JC, Carneiro EM, Bonfleur ML. Braz J Med Biol Res. 2015 May;48(5):447-57. doi: 10.1590

14. Enhanced glucose-induced intracellular signaling promotes insulin hypersecretion: pancreatic beta-cell functional adaptations in a model of genetic obesity and prediabetes. Irles E, Ñeco P, Lluesma M, Villar-Pazos S, Santos-Silva JC, **Vettorazzi JF**, Alonso-Magdalena P, Carneiro EM, Boschero AC, Nadal Á, Quesada I. Mol Cell Endocrinol. 2015 Mar 15;404:46-55. doi: 10.1016

15. Augmented β-Cell Function and Mass in Glucocorticoid-Treated Rodents Are Associated with Increased Islet Ir-β /AKT/mTOR and Decreased AMPK/ACC and AS160 Signaling. Protzek AO, Costa-Júnior JM, Rezende LF, Santos GJ, Araújo TG, **Vettorazzi JF**, Ortis F, Carneiro EM, Rafacho A, Boschero AC. Int J Endocrinol. 2014;2014:983453. doi: 10.1155/2014/983453

16. Taurine supplementation increases K(ATP) channel protein content, improving Ca2+ handling and insulin secretion in islets from malnourished mice fed on a high-fat diet. **Vettorazzi JF**, Ribeiro RA, Santos-Silva JC, Borck PC, Batista TM, Nardelli TR, Boschero AC, Carneiro EM. Amino Acids. 2014 Sep;46(9):2123-36. doi: 10.1007/s00726-014-1763-6

✓ **Artigos científicos submetidos ou em fase de resposta**

1. Vagotomy reduces insulin clearance in metabolic programmed mice. Lubaczeuski C, **Vettorazzi JF**, Costa-Junior JM, Boschero AC, Carneiro EM. Journal of Endocrinology

2. The bile acid TUDCA improves insulin clearance increasing the insulin-degrading enzyme expression in the liver of obese mice. **Vettorazzi JF**, Kurauti MA, Soares GM , Borck PC, Ferreira SM, Souto Branco RC,

Boschero AC, Michelone LSL,Costa Junior JM* Carneiro EM. Scientific Reports

3. Cortistatin regulates glucose-induced electrical activity and insulin secretion in mouse pancreatic beta-cells. Sergi Soriano, Alex Rafacho, Manuel Castellano-Muñoz, Paloma Alonso-Magdalena, Antonia Ruiz-Pino, Eva Bru-Tarí, Beatriz Merino, Esperanza Irles, Melisa Bello, Pau Iborra, Sabrina Villar-Pazos, Jean Vetorazzi, Montserrat Nacher, Eduard Montanya, Raúl M. Luque, Ángel Nadal, Iván Quesada. Scientific Reports

4. Low-protein diet after weaning disrupts peripheral clock and daily insulin secretion in mice. Patricia Cristine Borck, Thiago Martins Batista, **Jean Franciesco Vettorazzi**, Rafael Ludermann Camargo, Antonio Carlos Boschero, *Elaine Vieira, *Everardo Magalhães Carneiro. The Journal of Nutritional Biochemistry

5. Anti-contractile effects of perivascular adipose tissue in thoracic aorta from rats fed a high-fat diet: role of aerobic exercise training. Hygor N. Araujo, Jamaira A. Victório, Carmem P. Valgas da Silva, Amanda C. S. Sponton, **Jean F Vettorazzi**, Camila de Moraes, Ana P. C. Davel, Angelina Zanesco, Maria A. Delbin. Clinical and Experimental Pharmacology and Physiology