UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

EFEITOS DA RESISTÊNCIA À INSULINA NA TRANSCRIÇÃO E TRADUÇÃO DE PROTEÍNAS EM ESTRUTURAS CEREBRAIS DE RATOS

HUGO BOCK

Porto Alegre, dezembro de 2014.

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“Persevere e triunfarás!”

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PARTE I

RESUMO

A resistência encefálica à insulina tem sido implicada na etiologia de doenças neurodegenerativas já que, em indivíduos com diabete melittus, o risco de desenvolver Doença de Alzheimer e outras demências quase duplica. O objetivo geral do presente trabalho é avaliar os efeitos da resistência à insulina em parâmetros metabólicos e na expressão gênica e proteica de proteínas neurais envolvidas nos processos de sinalização da insulina em um modelo experimental de resistência à insulina em ratos submetidos a uma dieta hiperpalatável**.** O presente trabalho mostrou que há interação entre os genes envolvidos na via de sinalização da insulina e de resistência à insulina com fatores ambientais, neste caso, a dieta. O perfil lipídico e a tolerância à glicose nos animais submetidos à dieta hiperpalatável foi avaliado e intolerância à glicose foi desenvolvida. Os genes envolvidos na via de sinalização da insulina avaliados no córtex se mostraram menos expressos no grupo de ratos submetidos à dieta hiperpalatável, resistentes à insulina (IR), quando comparados ao grupo controle. Acreditamos que estes resultados sugerem uma menor proteção durante uma fase de privação de glicose devido à resistência à insulina. No hipocampo, observamos um aumento geral nos níveis de mRNA, especialmente os níveis dos genes Pi3kr1, Creb1 e Camk2a, o que pode estar associados a um efeito compensatório da sinalização prejudicada da insulina na estrutura.Um aumento dos níveis de CREB nesta estrutura parece ser essencial para a manutenção dos níveis normais de mRNA das outras moléculas envolvidas na via de sinalização da insulina a fim de evitar lesões graves no hipocampo. A influência do estado de resistência à insulina aumentou apenas a expressão de Grin2B no hipocampo de ratos IR. No córtex observamos os níveis mais baixos de mRNA em Grin1, Grin2A e Grin3A no grupo IR. Também foi investigado se as proteínas 14-3-3 não seriam o ponto chave desta diversidade, já que estão diretamente relacionadas com a via de sinalização da insulina no cérebro e

desempenham um papel chave na fosforilação e interação com IRS-1 e IRS-2, com GSK3 e com PI3K. Demonstramos que isoformas 14-3-3 são diferencialmente expressas no SNC de ratos com a resistência à insulina. Os níveis de mRNA de 14-3-3 isoforma η demonstrou ser significativamente menor no córtex grupo IR. O nível de proteína 14-3-3 θ foi significativamente maior no grupo de hipocampo de IR, estas mudanças são devido a uma resposta na tentativa de proteger o hipocampo, aumentando os níveis de mRNA de 14-3-3 θ e seu efeito protetor. Uma alteração da via insulina/IR/IRS/PI3K/AKT/FOXO1-CREB está presente na maioria dos pacientes com a síndrome metabólica, mas também pode haver o envolvimento da via IGF/PI3K/CAMK2/CREB. O nosso estudo mostra alterações nestas duas vias, no hipocampo e no córtex respectivamente. Estas hipóteses devem ser confirmadas por estudos bioquímicos e/ou histológicos em futuras investigações para saber se todas essas alterações moleculares podem ser traduzidas em ganho ou perda de função normal das proteínas envolvidas. Há que se investigar também o papel da família de proteínas 14-3-3 como possível alvo terapêutico, bem como a interação destas vias de sinalização com os receptores N-Metil-D-Aspartato e as implicações na memória e plasticidade sináptica de pacientes com doenças neurológicas como a Doença de Alzheimer.

ABSTRACT

Brain insulin resistance has been implicated in the etiology of neurodegenerative diseases since in individuals with diabetes mellitus, the risk of developing Alzheimer Disease and other dementias nearly doubles. The overall objective of this study is to evaluate the effects of insulin resistance in metabolic parameters, gene and protein expression of neural proteins involved in insulin signaling processes in an experimental model. This work shows interaction between genes involved in the signaling pathway of insulin and insulin resistance with environmental factors resulting in changes in mRNA levels, in the amount of protein in the central nervous system (CNS) and in the development of obesity and insulin resistance. The lipid profile and glucose tolerance test in animals subjected to the palatable diet was evaluated and shows development of glucose intolerance. Determination of expression of some genes directly or indirectly involved in insulin signaling route was evaluated. In general, almost all the genes were less expressed in the group of rats subjected to highly palatable, insulin resistant (IR) diet compared to the control group in the cortex. These results suggest that this brain structure is less protected during a period of deprivation of glucose due to insulin resistance. In the hippocampus, general increases in mRNA levels were observed, particularly the levels of Pi3kr1, CREB1 and CAMK2A genes. These results may be associated with a compensatory effect of the impaired insulin signaling in structure and increased levels of CREB appears to be essential for the maintenance of normal levels of all other molecules involved in the insulin signaling pathway to avoid serious damage to the hippocampus. The influence of the state of insulin resistance in synaptic plasticity and neuronal survival was also evaluated and only the expression of Grin2B was increased in the hippocampus of rats IR. In the cortex we observed lower levels of mRNA in Grin1, Grin2A and Grin3A in IR group. We decided to investigate if the 14-3-3 proteins were not the key

point of this diversity since they are directly related to the insulin signaling pathway in the brain and play a key role in the phosphorylation and interacts with IRS-1 and IRS- 2 with GSK3 and PI3K. 14-3-3 isoforms are differentially expressed in the CNS of rats with insulin resistance. mRNA levels of 14-3-3 η isoform shown to be significantly lower in the IR group in cortex. The level of 14-3-3 θ was significantly higher in the hippocampus of IR. We believe that these changes are due to an attempt to protect the hippocampus by increasing mRNA levels of 14-3-3 θ and its protective effect. Taking all together, alterated signaling in the insulin/IR/IRS/PI3K/AKT/FOXO1-CREB pathway is present in most patients with metabolic syndrome. It also may involve IGF/PI3K/CAMK2/CREB pathway. Our study shows changes in both pathways, in the hippocampus and cortex, respectively. This hypothesis should be confirmed by biochemical and/or histological investigations in future studies to see if all these molecular changes can be translated into a gain or loss of normal function of proteins involved. An investigation of the role of 14-3-3 family should be made to evaluate them as a possible therapeutic target, as well as the interaction with these signaling pathways and whith N-Metil-D-Aspartate receptors, and implications in memory, synaptic plasticity and neurological diseases like patients with Alzheimer Disease.

LISTA DE ABREVIATURAS

CaMKII - proteína-cinase dependente de Ca2+/calmodulina II;

CO - grupo controle;

CREB - proteína de ligação ao elemento de resposta ao AMPc;

DA - Doença de Alzheimer;

DM1 - Diabetes Mellitus tipo 1;

DM2 - Diabetes mellitus tipo 2;

FOXO1 - *forkhead box subgroup O1*;

Gapdh - gliceraldeideo-3-fosfato desidrogenase;

Glu - glutamato;

GLUT - transportadores de glicose;

Grin - *glutamate receptor, ionotropic, N-methyl-D-aspartate*;

GSK - glicogênio-sintase-cinase;

HDL - lipoproteína de alta densidade (*High Density Lipoprotein*);

icv - intracerebroventricular;

IGF - fator de crescimento semelhante à insulina;

IR - grupo resistente à insulina;

IRS - substratos do receptor de insulina;

LDL - lipoproteína de baixa densidade (*Low Density Lipoprotein*);

LTD - depressão de longa duração (*Long-term depression*);

LTP - potenciação de longa duração (*Long-term potentiation*);

mTOR - proteína alvo de rapamicina em mamíferos;

NMDAR - receptor NMDA;

PI3K - fosfoinositídeo-3-cinase;

PKB - proteína-cinase B, também chamada de Akt;

RI - receptor de insulina;

Ser/Thr - serina/treonina;

SNC - sistema nervoso central;

STZ - estreptozotocina;

TNFα - fator de necrose tumoral-alfa;

Tyr - tirosina;

WB - *Western Blot.*

1 INTRODUÇÃO

1.1 Dieta e Obesidade

O estilo de vida baseado no sedentarismo e no consumo de dietas palatáveis com alto conteúdo de carboidratos (principalmente sacarose) e lipídeos tem sido associado ao aumento dos índices de obesidade da população. A obesidade é o resultado das discrepâncias entre o consumo de energia e o seu gasto, e portanto, quando o consumo é maior que o gasto ocorre um balanço energético positivo e, consequentemente, um aumento da massa de gordura. As crianças e adolescentes, influenciados pelo meio ambiente, estão adotando um estilo de vida vida cada vez mais sedentário.

A mudança na dieta, ingestão de alimentos altamente energéticos e um estilo de vida sedentário transformou a obesidade, anteriormente considerado um fenômeno do mundo desenvolvido, em uma epidemia global (Kelly *et al*., 2008; Cabalero, 2007). Segundo a Organização Mundial da Saúde a prevalência da obesidade quase duplicou desde 1980. 35 % dos adultos apresentam sobrepeso e 11 % são obesos. Estes são os dois maiores fatores globais de risco de morte, matando cerca de 3,4 milhões de poessoas por ano (OMS, 2014). No Brasil, cerca de 52 % da população está em sobrepeso e 19,5 % é considerada obesa (OMS, 2014). O aumento da prevalência da obesidade é, provavelmente, o resultado de fatores ambientais e de estilo de vida contemporâneos, tais como maior acesso a alimentos palatáveis e redução do exercício físico. Apesar dos grandes avanços no conhecimento dos mecanismos fisiológicos que regulam o peso corporal e o gasto energético, a obesidade continua a ser um grande problema de saúde em todo o mundo com uma série de consequências vasculares, metabólicas e psicossociais (Field *et al*., 2001).

A menor utilização de gordura como fonte de energia pelo organismo está entre os mecanismos biológicos possivelmente envolvidos no ganho de peso. A preferência do organismo em oxidar carboidratos em detrimento de gorduras pode ser expressa por um elevado coeficiente respiratório, calculado pela razão entre a produção de dióxido de carbono (resultante da oxidação de carboidratos) e o consumo de oxigênio (determinado por calorimetria) (Marra *et al*., 2004).

A obesidade é caracterizada por hiperfagia que conduz a um aumento da massa de gordura corporal, a uma perda de sensibilidade à insulina e um aumento dos níveis de glicose no sangue (Berthoud *et al*., 2011). Obesidade associada a depósitos de gordura abdominal é um importante fator de risco para a resistência à insulina, diabetes mellitus tipo 2 (DM2) e síndrome metabólica (Guh *et al*., 2009).

1.2 Diabetes Mellitus

O diabetes é uma doença heterogênea com múltiplas etiologias, sendo caracterizada por altos níveis de glicose no sangue como resultado de uma quantidade insuficiente de insulina para as necessidades do corpo. O diabetes mellitus tipo 1 (DM1) é uma doença autoimune que resulta em destruição das células β pela presença de anticorpos nas ilhotas, evento que resulta em necessade de insulina ao longo de toda a vida do paciente. O DM2, forma mais comum da doença, é influenciado por fatores de estilo de vida, tais como a idade, a gravidez e a obesidade, mas também tem um forte componente genético (Ashcroft & Rorsman, 2012).

O DM2 é considerado uma doença crônica, associada a uma redução progressiva da massa de células β ou a uma insuficiência irreversível das células β em secretar insulina, sendo considerada uma doença incurável. Na história recente, o DM2 é dita como uma doença

de dois estágios, com a resistência à insulina desempenhando um papel primário seguido por disfunção das células β que leva à síndrome clínica de diabetes com hiperglicemia (Khardori, 2013).

Nos últimos dez anos, dois estudos independentes têm desafiado essa visão de que o DM2 é incurável, pois foi observado que a restrição energética grave reverteu o DM2 dentro de 1 semana (Lim *et al*., 2011). Em outro estudo, obsevou-se que os pacientes que se submeteram à cirurgia bariátrica apresentaram remissão do diabetes muito antes da perda substancial de peso (Karra *et al*., 2010).

1.3 Resistência à Insulina

A insulina regula os níveis de glicose no sangue, em grande parte, através da modulação direta da produção de glicose hepática pela inibição da glicogenólise, mas também por inibição da lipólise e por diminuição dos níveis de glucagon (Saltiel & Kahn, 2001). Outro hormônio importante é a leptina, derivada do adipócito, que exerce funções de modulação em várias regiões do SNC. O alvo principal destes hormônios parece ser o hipotálamo, que compreende uma rede fortemente regulada e complexa de neuropeptídeos e neurotransmissores, os quais influenciam os parâmetros de homeostase energética (Leibowitz & Wortley, 2004).

A resistência à insulina se caracteriza pela diminuição da capacidade da insulina de exercer as suas funções fisiológicas normais. A resistência à insulina inicialmente induz mecanismos de compensação, de modo que a hipersecreção de insulina mantém os níveis de glicose no sangue sob controle por algum tempo. Esta etapa, que pode ser chamada de pré-diabetes, é clinicamente difícil de detectar, porque os valores normais de glicose no sangue são mantidos. No entanto, a situação se agrava progressivamente devido à ocorrência da

chamada insuficiência pancreática, em que as células β não são capazes de manter a hipersecreção de insulina, pois começam a se deteriorar e, consequentemente, a secreção de insulina diminui. Este é o ponto em que a maioria dos casos de DM2 e de síndrome metabólica começa a ser diagnosticado (Solomon *et al.*, 2014; Wong *et al*., 2014).

Em ensaios de sensibilidade à insulina, a resistência à insulina levou às seguintes características: hiperinsulinemia e hiperglicemia no estado de jejum, aumento de hemoglobina glicosilada, hiperglicemia pós-prandial, hiperlipidemia, tolerância à glicose diminuída, tolerância à insulina deficiente, diminuição da taxa de infusão de glicose, aumento da produção de glicose hepática e aumento de marcadores inflamatórios no plasma entre outras (Leibowitz & Wortley, 2004; He *et al*., 2011; Filippi *et al*., 2013; Ye *et al*., 2013).

A resistência à insulina está intimamente associada com algumas das doenças clínicas crônicas muito prevalentes: DM2, síndrome metabólica, aterosclerose, síndrome do ovário policístico e esteatose hepática. A incidência de morbidade e mortalidade em toda a população atribuível à resistência à insulina é grande e crescente nestas doenças (Semple *et al*., 2011).

A resistência à insulina também pode ocorrer devido a outros fatores que são capazes de interferir ou modificar de alguma forma as moléculas envolvidas nesta via de sinalização. Alguns dados epidemiológicos associam a resistência à insulina a marcadores inflamatórios. Citocinas pró-inflamatórias, tais como fator de necrose tumoral-alfa (TNFα), estimulam a fosforilação de IRS em resíduos de serina, causam defeitos na atividade tirosina-cinase do receptor de insulina e diminuem a atividade de IRS e PI3K, inibindo, assim, a via de sinalização de insulina (Hotamisligil *et al*., 1993).

1.4 Insulina no Sistema Nervoso Central (SNC)

A insulina tem muitas atividades fisiológicas em que a redução de glicose no sangue é a função primária. Além disso, a insulina estimula a síntese de ácidos graxos e de glicogênio, promove a função mitocondrial, melhora a microcirculação e induz proliferação celular (He et al., 2011; Ye et al., 2013).

O diabetes era considerado uma doença periférica. Entretanto, há alguns anos, estudos demonstraram o envolvimento do SNC no diabetes, indicando que o DM2 é um fator de risco para demências, DA e comprometimento cognitivo, por exemplo (Figura 1) (Ott *et al*., 1996; MacKnight *et al.*, 2002; Cukierman *et al*., 2005; Ohara *et al*., 2011).

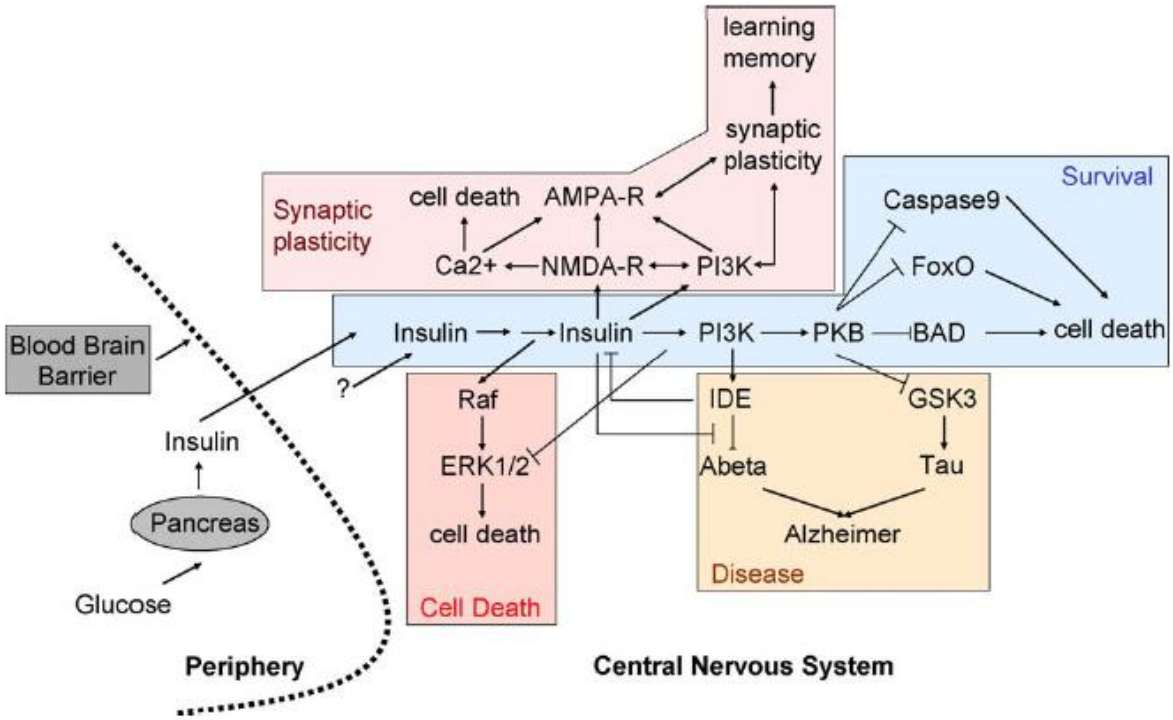

Figura 1 – Efeitos da insulina no SNC. A insulina ativa a rota de sinalização de PI3K que afeta a plasticidade sináptica, a sobrevivência ou morte celular e doenças (adaptado de van der Heide *et al.*, 2006).

O SNC tem um papel central na integração de sinais hormonais dos tecidos periféricos, tais como o pâncreas, o tecido adiposo e o intestino, para regular homeostase da energia. A alteração da sensibilidade a esses hormônios no SNC pode levar a um

comportamento de alimentação descontrolada e, consequentemente, a um aumento no peso corporal e adiposidade (Filippi *et al*., 2013).

Durante muito tempo, o cérebro foi descrito como um órgão insensível à insulina. No entanto, os efeitos tróficos importantes para o desenvolvimento e sobrevivência de células neurais são bastante conhecidos (Park, 2001). O receptor de insulina (RI) está presente principalmente nos neurônios de áreas cerebrais relacionadas com aprendizado, memória e cognição, como hipocampo, córtex e amígdala (Hill *et al*., 1986; Park, 2001).

A importância da sinalização encefálica da insulina na regulação do comportamento da alimentação foi demonstrada em estudos genéticos em que ratos nocaute para o RI neuronal. Esse animais apresentaram um aumento da ingestão de alimentos e do desenvolvimento de obesidade sensível à dieta com o aumento da gordura corporal, da leptina plasmática e dos níveis de insulina, resistência à insulina leve e hipertrigliceridemia (Bruning *et al*., 2000).

O prejuízo da ação da insulina no cérebro vem sendo associado a alterações no peso corporal, na ingestão de alimentos, na homeostase da glicose e na função cognitiva. Estudos indicam que a insulina periférica chega ao cérebro por meio de transporte mediado por receptores e que, ratos alimentados com uma dieta com alto teor de gordura mostram diminuição deste transporte (Heni *et al*., 2014; Woods *et al*., 2003).

Em resumo, cérebro é um órgão sensível à insulina e, em associação com outros nutrientes como ácidos graxos, aminoácidos e leptina, podem desempenhar um papel importante na regulação do equilíbrio de energia e homeostase da glicose. Assim, uma deficiência dos mecanismos de sensoriamento no SNC pode levar ao ganho de peso e a desregulação dos níveis de glicose no plasma. A dieta, ao invés de obesidade por si só, parece desempenhar um papel mais importante na indução de um estado de resistência à insulina central (Wang *et al*., 2001).

1.5 Cascata de Sinalização de Insulina

Em 1995 surgiram as primeiras evidências da cascata de sinalização insulina / fosfatidilinositol-3-cinase / proteína cinase B / glicogênio sintase cinase-3-β (insulina/PI3K/Akt/GSK3β) (Cross *et al*., 1995). De maneira geral, a insulina age quando se liga ao domínio extracelular dos RI, resultando na ativação da atividade tirosina cinase do receptor. Após a autofosforilação do RI, ocorre a fosforilação de uma série de substratos intracelulares para iniciar várias vias de sinalização. Estes incluem os substratos do receptor de insulina (IRS), os quais atuam como locais de ligação para outras proteínas, como a fosfoinositídeo-3-cinase (PI3K) e a proteína-cinase B (PKB, também chamada de Akt), ativando suas cascatas de sinalização pró-sobrevivência neuronal. A Akt é capaz de fosforilar a glicogênio-sintase-cinase (GSK3), diminuindo assim a atividade desta cinase e abolindo a inibição da síntese de glicogênio via glicogênio sintase. Também ativa fatores de transcrição de genes envolvidos na sobrevivência neuronal, através da fosforilação da proteína de ligação ao elemento de resposta ao AMPc (CREB), e inibe fatores de transcrição de morte celular, como a *forkhead box subgroup O* (FOXO1). A ativação da Akt induzida por insulina também leva a mobilização dos transportadores de glicose (GLUT) e a captação de glicose em diversos tecidos (van der Heide *et al*., 2006; Taniguchi *et al*., 2006; Kadowaki *et al*., 2012; Oh *et al*., 2013).

O efeito sistêmico final da insulina é o de facilitar o transporte de glicose através do GLUT4. Camundongos *knockout* heterozigotos para o gene que codifica GLUT4 apresentaram intolerância à glicose, resistência à insulina e hipertensão, mas não desenvolveram diabetes. Além disso, os camundongos *knockout* homozigotos para GLUT4 não desenvolveram diabetes nem em idade precoce. Isto sugere que outros mecanismos

compensatórios ocorrem em idades precoces, uma vez que apenas 50 a 60% dos camundongos que chegam à idade adulta desenvolvem diabetes (Stenbit *et al*., 1997).

Apesar da causa da resistência à insulina ainda ser objeto de muitos estudos pela carência de maiores esclarecimentos, a ocorrência de um componente genético poligênico, incluindo o meio ambiente, parece ser amplamente aceita. Numa tentativa inicial para determinar os moduladores da sensibilidade à insulina, as primeiras abordagens utilizaram modelos animais com deleções nos genes envolvidos na cascata de sinalização de insulina (Ros Pérez & Medina-Gómez, 2011). Camundongos *knockout* para o receptor de insulina morrem dentro de uma semana de vida, devido à marcada cetoacidose (Accili *et al*., 1996).

A resistência à insulina pode ocorrer devido a alguma anormalidade na cascata de sinalização de insulina. O RI ativado fosforila diretamente os IRS em vários resíduos de tirosina (Tyr), sendo que o IRS-1 e o IRS-2 são os mais importantes no transporte de glicose. A resistência à insulina pode surgir devido à ativação inapropriada de cinases de serina/treonina (Ser/Thr), que fosforilam estes resíduos ao nível de IRS inibindo a sinalização da insulina. No entanto, este conceito simples não explica a grande complexidade de funções de IRS (Boura-Halfon & Zick, 2009; Siddle, 2011). Camundongos *knockout* para IRS-1 são pequenos e apresentam resistência à insulina no músculo, o que é compensado pela hiperplasia de células β do pâncreas (Tamemoto *et al*., 1994). Ratos *knockout* para ISR2 têm um fenótipo de resistência à insulina grave no fígado e diabetes numa idade precoce (Kubota *et al*., 2000).

O próximo nível de sinalização da insulina envolve PI3K, elemento-chave na resposta metabólica à insulina, com efeito antilipolítico na síntese de ácidos graxos e na síntese de glicogênio. No SNC, os principais neurorreguladores, entre eles a insulina, interagem no cérebro via ativação direta ou indireta de PI3K e este diálogo de peptídeos e neurotransmissores é de grande importância na fisiopatologia do SNC e, especificamente, na

regulação do comportamento alimentar (Gerozissis, 2008). Existem várias isoformas de subunidades reguladoras (p85α, P55α, P50α, P85β, P55γ) e subunidades catalíticas (p110 α, β). Em camundongos, o *knockout* de subunidades regulatórias p85 provoca a morte após o nascimento (Ueki *et al*., 2002), e os heterozigotos com diminuição das subunidades p85α e β, p55α e p50α, apresentam um aumento da sensibilidade à insulina, levando a hipoglicemia (Mauvais-Jarvis *et al*., 2002).

A Akt está em um nível mais abaixo da cascata de regulação e sinalização da insulina. Existem três isoformas distintas desta enzima (Akt1, Akt2 e Akt3), sendo que no músculo esquelético e no fígado, a Akt2 parece mediar a sensibilidade à insulina. A deleção de Akt2 causa resistência à insulina nestes tecidos e camundongos desenvolvem diabetes. Por outro lado, a deleção de Akt1 não gera resistência à insulina ou intolerância à glicose, mas provoca o atraso de crescimento destes animais (Cho *et al*., 2001). No SNC, uma vez fosforilada, a Akt atua como um regulador chave para a sobrevivência neuronal inibindo proteínas pró-apoptóticas como a GSK3ß e caspases (Miyawaki *et al*., 2009). Além disso, a Akt é capaz de ativar fatores de transcrição de genes envolvidos na sobrevivência neuronal como CREB e a proteína alvo de rapamicina em mamíferos (mTOR) 2 e inativa fatores de transcrição de genes que promovem morte celular como a FOXO1 (van der Heide *et al*., 2006).

Uma via de sinalização alternativa da insulina é a que envolve o receptor do fator de crescimento semelhante à insulina (IGF). A insulina é capaz de se ligar a receptores IGF, embora com menor afinidade do que o próprio receptor de insulina. Desta forma, quando a insulina está presente no soro em concentrações elevadas, é possível a sua ligação a receptores IGF causando sua ativação (Boucher *et al*., 2010). O aumento da sinalização de insulina via receptor IGF é potencialmente importante para a modulação da massa das ilhotas

pancreáticas. A ativação do receptor leva a fosforilação do IRS-2 e consequente ativação da sinalização através da via da PI3K/Akt (Rhodes, 2005).

Outro ponto importante na sinalização mediada por insulina é que um aumento de Ca^{2+} é geralmente reconhecido como um componente chave na absorção de glicose mediada por contração do músculo esquelético. Por exemplo, a proteína-cinase dependente de Ca^{2+}/calmodulina II (CaMKII) atua como um componente intermediário da cascata de sinalização induzida por contração que resulta na translocação de vesículas GLUT4 para a membrana do plasma e aumento da absorção de glicose (Wright *et al*., 2004) . A insulina aumenta o influxo de Ca^{2+} nas células do músculo esquelético, indicando que a insulina induz um influxo de Ca^{2+} tanto em tecido muscular normal como em tecidos de animais resistentes à insulina (Lanner *et al*., 2006). Esta exocitose de GLUT4 mostra muitas semelhanças com a liberação de neurotransmissor, um processo geralmente induzido por aumentos transitórios na concentração de Ca^{2+} nos locais de liberação (Rizzuto & Pozzan, 2006). Por isso, é surpreendente que alterações na concentração de Ca^{2+} não são, em geral, consideradas como envolvidas na absorção de glicose mediada por insulina.

A função mitocondrial também desempenha um papel muito importante no mecanismo de secreção de insulina pelas células β do pâncreas em resposta ao aumento dos níveis de glicose. Disfunções como alterações de número, de tamanho e na capacidade oxidativa de mitocôndrias também foram associadas com a resistência à insulina e relatados em pacientes com diabetes ou resistentes à insulina (Kim *et al*., 2008).

1.6 Sinalização de Insulina e Doenças Neurológicas

Os estudos realizados em animais para avaliar a disfunção na sinalização encefálica da insulina são baseados em modelos de resistência à insulina induzidos por

obesidade associada ao consumo de dieta hiperlipídica e/ou hiperpalátável (Schwartz & Porte, 2005) ou pela administração intracerebroventricular (icv) de estreptozotocina (STZ) (Hoyer & Lannert, 2007). No primeiro modelo, o objetivo é mimetizar o consumo de alimentos com alto teor de gordura, hiperpalatáveis e de fácil acesso da sociedade ocidental. O segundo modelo mimetiza alguns achados bioquímicos em pacientes e modelos animais geneticamente modificados de doença de Alzheimer (DA) como hiperfosforilação da proteína Tau e da GSK3ß (Grunblatt *et al*., 2007).

A resistência encefálica à insulina tem sido associada com a etiologia de doenças neurodegenerativas, uma vez que os estudos epidemiológicos relatam que, em indivíduos com DM, o risco de desenvolver DA e outras demências quase duplica (Ott *et al*., 1996b). Em roedores, a alteração na sinalização encefálica da insulina, pela administração icv de STZ, causa prejuízo cognitivo e dano neuronal por mecanismos que podem potencialmente estar associados a hiperativação do sistema glutamatérgico e, assim como ocorre com os humanos, o envelhecimento provoca diminuição dos RI no cérebro de roedores (Hoyer & Lannert, 2007). Da mesma maneira, dietas ricas em açúcares ou ácidos graxos saturados também produzem resistência periférica a insulina e efeitos similares ao da administração icv de STZ no cérebro (Steen *et al*., 2005). A administração periférica de insulina em doses que não alterem consideravelmente a glicemia é capaz de reverter algumas perdas cognitivas em pacientes com a DA (Neumann *et al*., 2008). Quando essas observações são realizadas em modelos animais, a insulina causa na maioria das vezes uma melhora da função cognitiva (Park, 2001; Neumann *et al*., 2008; Babri *et al*., 2007). Assim, a insulina parece ser um importante neuromodulador da atividade cerebral e sua interação com os componentes do sistema glutamatérgico tem sido pouco explorado (van der Heide *et al*., 2005).

A GSK3 é uma ser/thr cinase citoplasmática que fosforila e inibe a glicogênio sintase, inibindo a síntese de glicogênio a partir da glicose. No entanto, ela é também

conhecida por regular vários processos celulares através de vias de sinalização importantes para a proliferação celular, para a renovação das células estaminais, para a apoptose e para o desenvolvimento. A GSK3 está também sujeita a regulação aguda por insulina, podendo causar sua inativação também *in vivo* (Cross *et al*., 1995, 1997). A desregulação de vias de sinalização envolvendo a GSK3 está associada com a patogênese de vários distúrbios neurológicos e psiquiátricos. A utilização de ratos que superexpressam GSK3 são um meio eficaz para estudar a função de GSK3 no desenvolvimento do cérebro, na morfologia, na neurogênese, na memória e na aprendizagem, na função motora sensorial, sociabilidade, emoções, bem como nos comportamentos depressivos (Kaidanovich-Beilin & Woodgett, 2011).

1.7 Receptor tipo N-metil-D-aspartato (NMDAR)

Os receptores do tipo N-metil-D-aspartato (NMDARs) são canais iônicos com grande permeabilidade a íons $Ca2^{+}$ e estão associados a várias funções e mecanismos de desenvolvimento do SNC pré e pós-natal, incluindo diferenciação celular, crescimento, migração e proliferação de axônios e degeneração de neurônios que não são mais usados (Ikonomidou et al., 1999; Ikonomidou et al., 2001). Os NMDARs têm um papel importante na plasticidade sináptica referente ao aprendizado e memória e são alvos de fármacos usados em terapias relacionadas ao manejo da dor e de doenças neurológicas (Lalonde & Joyal, 1993). A ativação deficiente ou excessiva desses receptores está relacionada com alterações do desenvolvimento normal do SNC (Lalonde & Joyal, 1993). A morte neuronal devido à excitotoxicidade observada após traumatismo craniano, eventos isquêmicos, hipóxia e hipoglicemia está relacionada a uma ativação excessiva dos NMDARs (Choi, 1988).

Um NMDAR funcional é um complexo heteromultimérico composto por uma combinação de subunidades que são codificados por uma família gênica: Grin1, Grin2A, Grin2B, Grin2C, Grin2D, Grin3A e Grin3B, sendo que, a subunidade Grin1 está sempre presente. A partir do isolamento dos genes responsáveis pela síntese de proteínas formadoras dos NMDARs, vários estudos foram realizados em modelos animais de estados patológicos mostrando a importância dos NMDARs no SNC. Esses receptores estão envolvidos em várias funções do SNC, desde a vida embrionária até a morte neuronal. Esta diversidade de funções pode ser parcialmente explicada pela composição das subunidades presentes no receptor funcional. Alguns dados apontam para disfunções neurológicas e doenças psiquiátricas quando uma ou mais subunidades estão ausentes ou super-expressas (Monyer et al., 1992; Lynch et al., 1994; Pérez-Otaño et al., 2001).

1.8 Proteínas 14-3-3

As proteínas 14-3-3 são uma família de moléculas diméricas altamente conservadas e que são expressos em todas as células eucarióticas. A família consiste de sete isoformas (β, γ, ε, ζ, η, θ e σ). Embora sendo descritas pela primeira vez em 1967, elas começaram a atrair a atenção de cientístas em meados de 1990 quando se tornou claro que eles interagem e afetar a função de um grande número de outras proteínas. As proteínas 14-3-3 podem afetar a proteína alvo de várias maneiras, por exemplo, alterando a sua estrutura, a atividade enzimática, a localização subcelular, a estabilidade, o estado de fosforilação ou interações moleculares (Moore & Perez, 1967).

As proteínas 14-3-3 estão envolvidas na regulação e coordenação de muitos processos celulares, incluindo as vias de sinalização, metabolismo (como a glicólise, ácidos graxos e síntese de nucleotídeos), a regulação da transcrição de níveis de mRNA, apoptose, entre outros,

devido à grande número e diversidade de parceiros interagindo (Pozuelo Rubio *et al*., 2004). Juntos, os resultados de vários estudos indicam que as proteínas 14-3-3 podem interagir diretamente e modificando funções de enzimas que são de importância central na regulação metabólica. As proteínas 14-3-3 estão diretamente relacionadas com a via de sinalização da insulina no cérebro, visto que desempenham um papel chave na fosforilação e interação com IRS-1 e IRS-2, com GSK3 e com PI3K entre outras (Kleppe *et al*., 2011).

Entre as primeiras funções relatados de proteínas 14-3-3 está a regulação da tirosina hidroxilase (TH) sugerindo um possível envolvimento de proteínas 14-3-3 na doença de Parkinson. Desde então, observou-se a relevância de proteínas 14-3-3 em doenças crônicas, bem como em doenças neurodegenerativas agudas, incluindo a doença de Alzheimer, doenças da poliglutamina, a esclerose lateral amiotrófica e acidente vascular cerebral. A função das proteínas 14-3-3, neste contexto, são tão diversas como os mecanismos envolvidos na neurodegeneração, atingindo desde processos celulares basais como apoptose, estabilização e agregação de proteínas, a processos muito específicos responsáveis pela vulnerabilidade seletiva de populações celulares em doenças neurodegenerativas únicas (Steinacker *et al*., 2011).

As proteínas 14-3-3 também modulam a cascata de sinalizaçãocerebras de insulina através da fosforilação e a interacção com proteínas reguladoras-chave, tais como o receptor IRS-1 e IRS-2, GSK3 e PI3K (Ogihara *et al.*,1997, Neukamm *et al.*, 2013, Agarwal-Mawal *et al.*, 2003, Dubois *et al.*, 2009). Muitas proteínas que se ligam a 14-3-3 em resposta à insulina ativando vias de sinalização foram identificadas. Em geral, há uma rede de interações de 14-3-3 com fosfoproteínas que fornecem informações mecanicistas demonstrando sua associação com a captação de glicose estimulada por insulina, bem como outros efeitos em eventos intracelulares (Chen *et al.*, 2011).

O perfil cerebral de transcrição e tradução de genes até proteínas é uma ampla área de estudo, já que os mecanismos moleculares da maioria das doenças cerebrais são pouco

conhecidos e multifatoriais. Estudos desse tipo podem gerar informações de grande interesse na área de saúde pública, uma vez que contribuem para explicar mecanismos fisiopatológicos associados doenças neuropsiquiátricas (Marx, 2005).

2 OBJETIVOS

2.1 Objetivo geral

O objetivo geral do presente trabalho foi avaliar os efeitos da resistência à insulina em parâmetros metabólicos e na expressão gênica e proteica de proteínas neurais envolvidas nos processos de sinalização cerebral da insulina em um modelo experimental de resistência à insulina em ratos submetidos a uma dieta hiperpalatável**.**

2.2 Objetivos específicos

- Verificar a expressão gênica e proteica de isoformas da proteína 14-3-3 em estruturas cerebrais de ratos submetidos à dieta hiperpalatável e de um grupo controle;

- Determinar a expressão de genes envolvidos direta ou indiretamente na rota de sinalização da insulina no córtex e no hipocampo de ratos submetidos à dieta hiperpalatável em relação a um grupo controle;

- Comparar a quantidade de proteínas envolvidas na rota de sinalização da insulina (CREB, PI3K e CaMKII) em estruturas cerebrais de ratos submetidos à dieta hiperpalatável e de um grupo controle;

- Avaliar, nos mesmos grupos acima, a expressão gênica das subunidades Grin1, Grin2A, Grin2B, Grin2C, Grin2D e Grin3A do receptor N-metil-D-aspartato (NMDA) em estruturas cerebrais de ratos submetidos à dieta hiperpalatável e de ratos normais.

PARTE II

CAPÍTULO I

Artigo a ser submetido ao periódico Neuropharmacology

Induced Insulin Resistance Modulates Levels of mRNA and Proteins of Insulin Signaling Pathway in Brain Structures of Rats

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Abstract

Insulin molecular pathway involves a lot of signaling molecules leading to different metabolism outcomes. Thus, understanding the pathogenesis of insulin resistance has become increasingly important to guide the development of future therapies and inform health and economic policy. The purpose of the present study was to investigate if insulin resistance modulates the levels of mRNA and proteins involved in the insulin signaling pathway in cortex and hippocampus of rats with insulin resistance induced by high palatable diet (also known as *cafeteria diet* or *Western Style Diet*). In the cortex, six genes (Insr, Irs-2, Akt1, Akt3, Casp3 and Sod2) showed a significant decrease of mRNA levels, in hippocampus only three (Pi3kr1, Camk2a and Creb1). We also measure the protein content of PI3K p85, CaMK2 and CREB. We observed a significant increase of CaMK2 protein levels in cortex, CREB and PI3K in hippocampus. In the cortex, glycemia was found to be negatively correlated with mRNA levels of Irs-2, Akt1, Akt3, Casp3 and Sod2. In hippocampus, only Creb1 was found to have a positive correlation with glycemia. The levels of TC and LDL were increased in the IR group. These two lipid parameters were negatively correlated with cortex mRNA levels of Irs-2, Akt1 and Akt3. A defect in the canonical insulin/IR/IRS/PI3K/AKT/FOXO1-CREB pathway is present in most patients with the metabolic syndrome, or the insulin resistance is mediated by signaling via an alternative pathway such as that involving the IGF1R/PI3K/CaMK2/CREB. So, insulin resistant state is leading the cortex to trying to maintain it normal functions increasing the protein levels. In hippocampus we believe that this profile of mRNA and protein alterations may be due to a compensatory effect of impaired insulin signaling in that structure.

Key words: insulin resistance; mRNA expression; rat brain; high palatable diet.

1 Introduction

The ingestion of diets rich in sugar and fat, along with a sedentary lifestyle, is associated with increased obesity prevalence (Adam and Epel, 2007). Obesity is most simply defined as a state in which the total amount of triglyceride stored in adipose tissue is abnormally increased. This condition is strongly associated with a wide variety of adverse health outcomes, including diabetes, vascular disease, and certain types of cancers (Kopelman, 2000). Obesity levels have been increasing in children and adolescents, and it could modify maturation of neuronal circuits, leading to dysfunction or diseases during adulthood (Sisk and Zehr, 2005).

Diabetes is a condition defined by a state of chronic elevation of plasma glucose levels. Type 2 Diabetes mellitus (T2DM) is characterized by chronic hyperglycemia, and initially hyperinsulinemia due to relative insulin resistance of insulin-target tissues (Biddinger and Kahn, 2006). It has long been clear that people with T2DM are very often obese or overweight. The majority of T2DM patients present impaired metabolic response to administer or endogenous insulin, and obesity tends to lead to insulin resistance, even in non-diabetic individuals (Petersen and Shulman, 2006).

Insulin resistance is related with the pathogenesis for many modern diseases. Thus, comprehension of insulin resistance pathogenesis has become increasingly important to guide the development of future therapies as well as health and economic policies. Insulin action essentially provides an integrated set of signals that allow the balance between nutrient availability and demand (Samuel and Shulman, 2012). Insulin resistance is subtle and affects a far greater number of people. By some estimates, within 40 years, one in every three Americans will have type 2 diabetes (Boyle *et al*., 2010).

Insulin resistance is a major feature of many common metabolic diseases and has high heritability. It is perhaps surprising that so few genes having an impact on insulin action have thus far emerged from genome-wide approaches (Rung, *et al*., 2009). The molecular mechanisms of insulin action in liver and both muscle and fat are known to show key differences leading to interindividual alterations in basal and post-meal insulin sensitivity (Lindgren *et al*., 2009).

Insulin is well known as the major and immediate regulator of blood glucose levels in peripheral tissues and has emerged as a major regulatory substance within the central nervous system (CNS). Insulin signaling in the CNS is essential for energy regulation and glucose homeostasis as well as for reproduction (Bruning, *et al*., 2000). For instance, two major brain areas of behavior affected by CNS insulin are those related to feeding and cognition (Banks *et al*. 2012). The brain clearly has a leading role in the control of energy balance, and obesity is a major risk factor for T2DM (Samuel and Shulman, 2012). High risk of dementia or significant cognitive decline has been associated with T2DM as well as with insulin resistance without T2DM (Cole *et al*., 2007; Cukierman *et al*., 2005). Therefore, insulin resistance possibly enhances neurodegeneration progression or synaptic loss responsible for symptoms of cognitive decline and dementia (Williamson *et al*., 2012).

At the molecular level, binding of insulin to the extracellular domain of the insulin receptor results in activation of receptor tyrosine kinase activity. Following autophosphorylation, the insulin receptor phosphorylates a number of intracellular substrates to initiate a series of intracellular signaling pathways. These substrates include insulin receptor substrates-1 and -2 (IRS-1, IRS-2). The phosphotyrosine residues in both IRS-1 and IRS-2 act as docking sites for other proteins such as phosphatidylinositol 3-kinase (PI3K). PI3K is activated when its regulator subunit PI3K regulatory class IA bounds to IRS-1 or IRS-2. PI3K activation appears to be essential for many insulin effects, including protein

kinase cascades involving v-akt murine thymoma viral oncogene homolog 1 (AKT). AKT is able to phosphorylate glycogen synthase kinase 3 beta (GSK3-β), thereby decreasing the activity of this kinase. As a consequence, AKT abolishes glycogen synthesis inhibition, and activates translation via the regulatory activity of initiation factor of translation eukaryotic translation initiation factor 2 (eIF2) (Kido *et al*., 2000; Murata, *et al*., 2003; Proud, 2005; Samuel and Shulman, 2012; Welsh, *et al*., 1998).

Regardless of whether IRS, PI3K, and AKT isoforms are activated in response to insulin, all these branches converge upon forkhead box O proteins (FOXO), cAMP response element binding protein (CREB) and its co-activators, such as peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC-1α), among others (Matsumoto *et al*., 2006; Oh *et al*., 2013).

As described above, insulin provides an integrated set of signals to balance nutrient absorption and utilization, regulating carbohydrate and fat metabolism in the body. Insulin molecular pathway involves a lot of signaling molecules leading to different metabolism outcomes.

Therefore, the aim of this study was to investigate levels of mRNA and proteins involved in the insulin signaling pathway in cortex and hippocampus of rats with insulin resistance induced by high palatable diet.

More specifically to quantify mRNA expression of Insr, Irs-1, Irs-2, Akt1, Akt2, Akt3, Pi3kr1, Gsk3b, Creb1, Camk2a, Ppargc1a, Cdk5, Casp3, Foxo1, Fyn, Sod2 and Ide genes; and CREB, PI3K and CaMK2 protein levels in this model of insulin resistance.

2 Experimental Procedures

2.1 Animals and Diet

Eight (n = 8) adult male Wistar rats were housed under controlled temperature (22 ± 2 ºC) and humidity (55 ± 10 %) conditions on a 12 h light-dark cycle (lights on at 7 am), with food and water offered *ad libitum*. All experiments were performed in agreement with the international standards and with the Brazilian College of Animal Experimentation for animal protection. The Ethical Committee on animal use of the Universidade Federal do Rio Grande do Sul, Brazil, approved all experiments (number 19446). When animals were 2 months-old (ranging between 200-250 g of weight), rats were divided into 2 experimental groups: control (CO) and insulin resistant (IR). High palatable diet (also known as *cafeteria diet* or *Western Style Diet*) was used to induce insulin resistance in IR group for 130 days (Dietrich *et al*. 2007; Schwartz and Porte, 2005). CO group was fed with a standard laboratory diet for the same period.

After 130 days we evaluate biochemical parameters. For glucose tolerance test rats were deprived from food overnight (8 h) and blood samples were taken by a small puncture on the tail. For plasma lipid profile animals were anesthetized with ketamine/xylazine intraperitoneal (100/10 mg/kg) and blood was collected. They were than decapitated, cortex and hippocampus were rapidly dissected and each structure was divided into two. One part was rapidly frozen, and stored at -80ºC for proteome analysis. The other part was submerged in RNAlater® (Ambion, USA) and stored at –20ºC for mRNA analysis.

2.2 Biochemical Profile

For the glucose tolerant test, rats received an injection of glucose solution at 50% (w/v) (2 g/kg of body weight). Blood samples were taken before the injection, and 30, 60, and

120 minutes after glucose overload. Glycemia was measured using the commercial glucometer AccuChek Active (Roche, USA).

Plasma lipid profile was performed by measuring levels of total content of triglycerides (TG), total cholesterol (TC) as well as fractions of high density lipoprotein (HDL) and low density lipoprotein (LDL). Analyzes were performed with commercial kits (Katal®, MG, Brazil).

2.3 Evaluation of mRNA and protein levels

mRNA levels were measured by quantitative real-time PCR (qPCR) using gene-specific TaqMan® FAM/MGB inventoried or made to order assays (Table 1), using glyceraldehyde-3-phosphate dehydrogenase (Gapdh) as endogenous control (inventoried assay Rn99999916_s1, Applied Biosystems, USA). Total RNA was isolated using TRIreagent® solution (Ambion, USA) according to manufacturer's protocol. RNA concentration was estimated using the fluorimetric method Quant-iT® RNA Assay (Invitrogen, USA) in the Qubit™ (Invitrogen, USA) equipment. cDNA was synthesized by reverse transcription (RT) reaction using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Two hundred ng of total RNA was placed in a total reaction volume of 20 µl containing 1x RT buffer, 4 mM of each dNTP, 1x RT Random Primers, and 0,05 units of MultiScribe™ Reverse Transcriptase. RT reaction was performed as follows: 10 min at 25 ºC, 2 h at 37 ºC and 5 s at 85 ºC. Subsequently, cDNA was kept at – 20 ºC until further use.

Table 1 – List of the assays used for mRNA relative quantification.

Gene	Assay number	RefSeq	Amplicon size (bp)
Insr	Rn00690703_m1	NM_017071.2	68
Irs1	Rn02132493_s1	NM_012969.1	147
Irs2	Rn01482270_s1	NM_001168633.1	61
Pik3r1	Rn00564547_m1	NM_013005.1	97
Akt1	Rn00583646_m1	NM_033230.1	87
Akt2	Rn00690900_m1	NM_017093.1	138
Akt3	Rn00442194_m1	NM_031575.1	71
Gsk3b	Rn00583429_m1	NM_032080.1	71
Creb1	Rn00578828_g1	NM_031017.1; NM_134443.1	69
Foxo1	Rn01494868_m1	NM_001191846.1	99
Ppargc1a	Rn00580241_m1	NM_031347.1	94
Casp3	Rn00563902_m1	NM_012922.2	93
Ide	Rn00565839_m1	NM_013159.1	122
Cdk5	Rn04219635_m1	NM_080885.1	95
Camk2a	Rn01258147_m1	NM_012920.1	93
Fyn	Rn00562616_m1	NM_012755.1	105
Sod2	Rn00690588_g1	NM_017051.2	64

* All assays are from Applied Biosystems, USA.

cDNA solution was diluted 1:5 in water and the reaction was carried out in a total volume of 12 µl containing 1 µl of diluted cDNA solution, 1x of gene specific TaqMan® assay, 1x of endogenous control TaqMan® assay and 1x PCR Master Mix (Applied Biosystems, USA). Cycling program was 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Reactions of each sample were performed in triplicate in an ABI Prism 7500 Fast Sequence Detector System (Applied Biosystems, USA).

Transcriptional profile of each gene was determined in both brain structures. Relative mRNA levels were calculated by the ΔΔCt method according to Livak and Schmittgen (2001), using Gapdh as endogenous control, and control group of each structure as calibrator.

For protein analysis, total protein extracts of the four groups were analyzed by Western Blot (WB). Twenty μg of protein were mixed with Bolt™ LDS Sample Buffer (4x) (Novex, USA) and Bolt™ Sample Reducing Agent (10x) (Novex, USA). Samples were then heated for 5 min at 95°C and separated on a precasted Bolt™ Bis-Tris Plus Gel 4 to 12% polyacrylamide gradient (Novex, USA) using Bolt™ MES SDS Running Buffer (Novex, USA) for 35 min. at 165 V. After gel electrophoresis, bands were transferred to a nitrocellulose membrane using iBlot® Transfer Stack (Novex, USA) in the iBlot® Gel Transfer Device (Invitrogen, USA) using the P3 - 7 min program.

After blotting the free sites were blocked with 5 % non-fat milk-powder in Tris Buffered Saline containing 0.5 % Tween 20 (TBS-T), pH 7.3 to 7.5 for 1 h. Membranes were rinsed once for 10 minutes and 3 more times for 5 minutes with TBS-T solution and then incubated overnight at 4°C with CaMK2 (MAB8699, Chemicon Internacional, USA), CREB (#9104, Cell Signaling Technology) and PI3K p85 (#4257P, Cell Signaling Technology) primary antibodies diluted 1:1.000 in TBS-T with 1% BSA solution. Following incubation with primary antibodies, the membranes were rinsed again as previous described and then incubated with the secondary antibody conjugated to horseradish peroxidase (anti-rabbit IgG HRP-linked, #7074, 1:4.000; Cell Signaling Technology, USA) diluted in TBS-T with 1% BSA solution for 1 h. The blots were developed using the Western Lightning ECL Pro (PerkinElmer, USA) and the membranes were then imaged using the ImageQuant LAS 500 (General Electric Healthcare, USA). The same membranes were washed, incubated with housekeeping anti β-actin-HRP linked antibody (#A3854, 1:30.000; Sigma-Aldrich, USA) and the bands detected as described above. Immunoreactive bands were quantified using ImageJ software v.1.47 (available from National Institutes of Health, USA).

2.4 Statistical Analysis

Statistical analyses were performed using student t-test. The correlation between variables was calculated by Pearson correlation. The differences were considered statistically significant when $p < 0.05$.

3 Results

3.1 Evaluation of mRNA and protein levels

In the cortex, six genes showed a significant decrease of mRNA levels: Insr (p = 0.0291), Irs-2 (p = 0.0222), Akt1 (p = 0.0114), Akt3 (p = 0.0003), Casp3 (p = 0.0256) and Sod2 (p = 0.0244) (Figure 1). Three genes show significant differences in mRNA expression in hippocampus IR group when compared to the CO, Pi3kr1 (p = 0.0440), Camk2a (p = 0.0363) and Creb1 (p = 0.0341) (Figure 2). For further investigations we decide to measure the protein content of PI3K p85, CaMK2 and CREB, all of them with altered levels of mRNA in hippocampus.

We observed a significant increase of protein immunodetection of CREB in hippocampus IR group (p = 0.0090), when compared to CO group (Figure 3). Cortex does not show significant changes. Curiously when we analyzed CaMK2 protein levels we do not see any changes in the hippocampus but we observe a significant increase (p = 0.0169) in cortex IR group (Figure 4). PI3K show no changes in cortex and a significant increase in hippocampus IR group (p = 0.0125) similar to CREB (Figure 5).

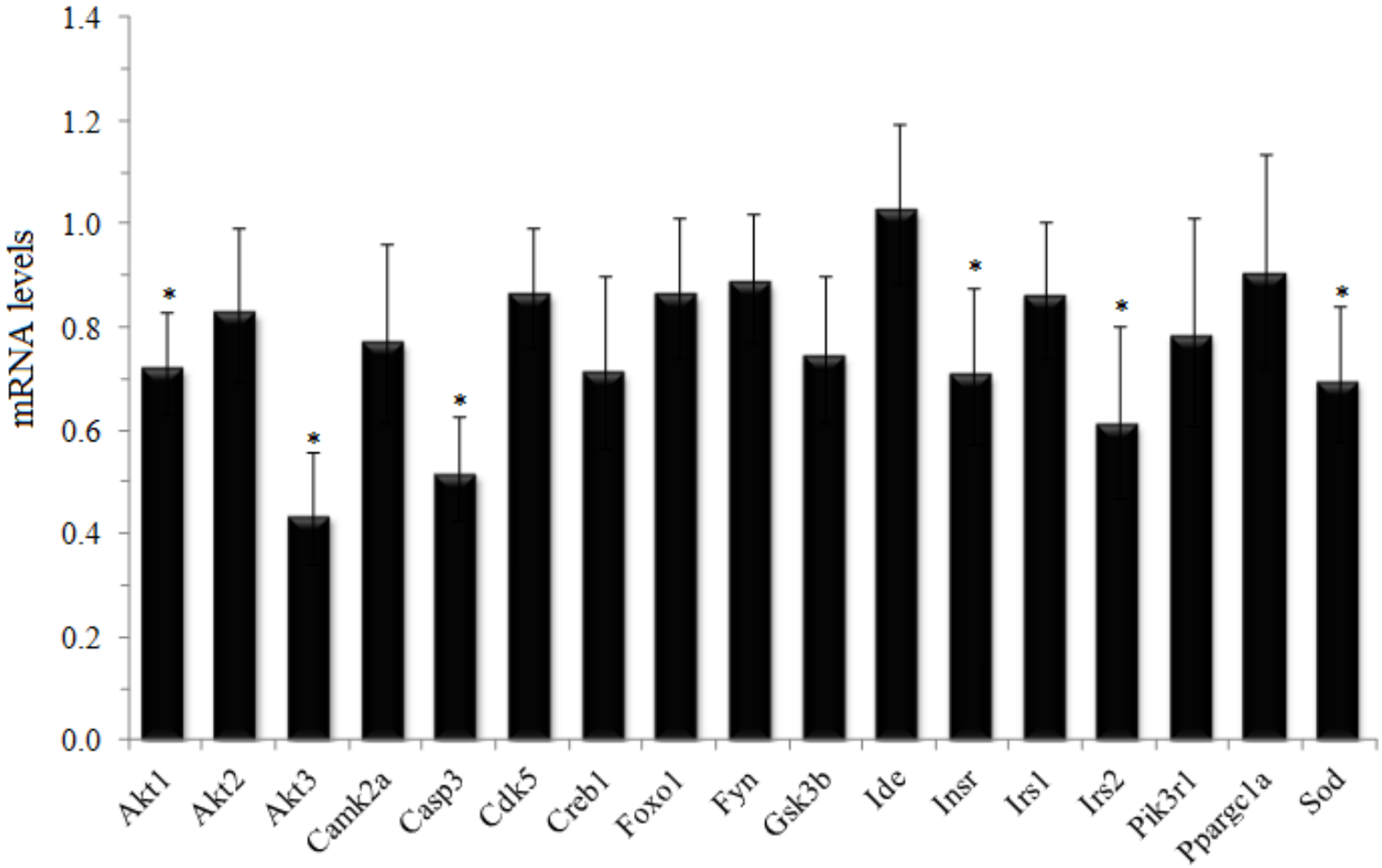

Figure 1 – Relative levels of mRNA in cortex of insulin resistant group. Results are presented as mean ± SD (n = 4 in triplicate) and compared using student t-test. Significant levels (*) were considered when $p < 0.05$

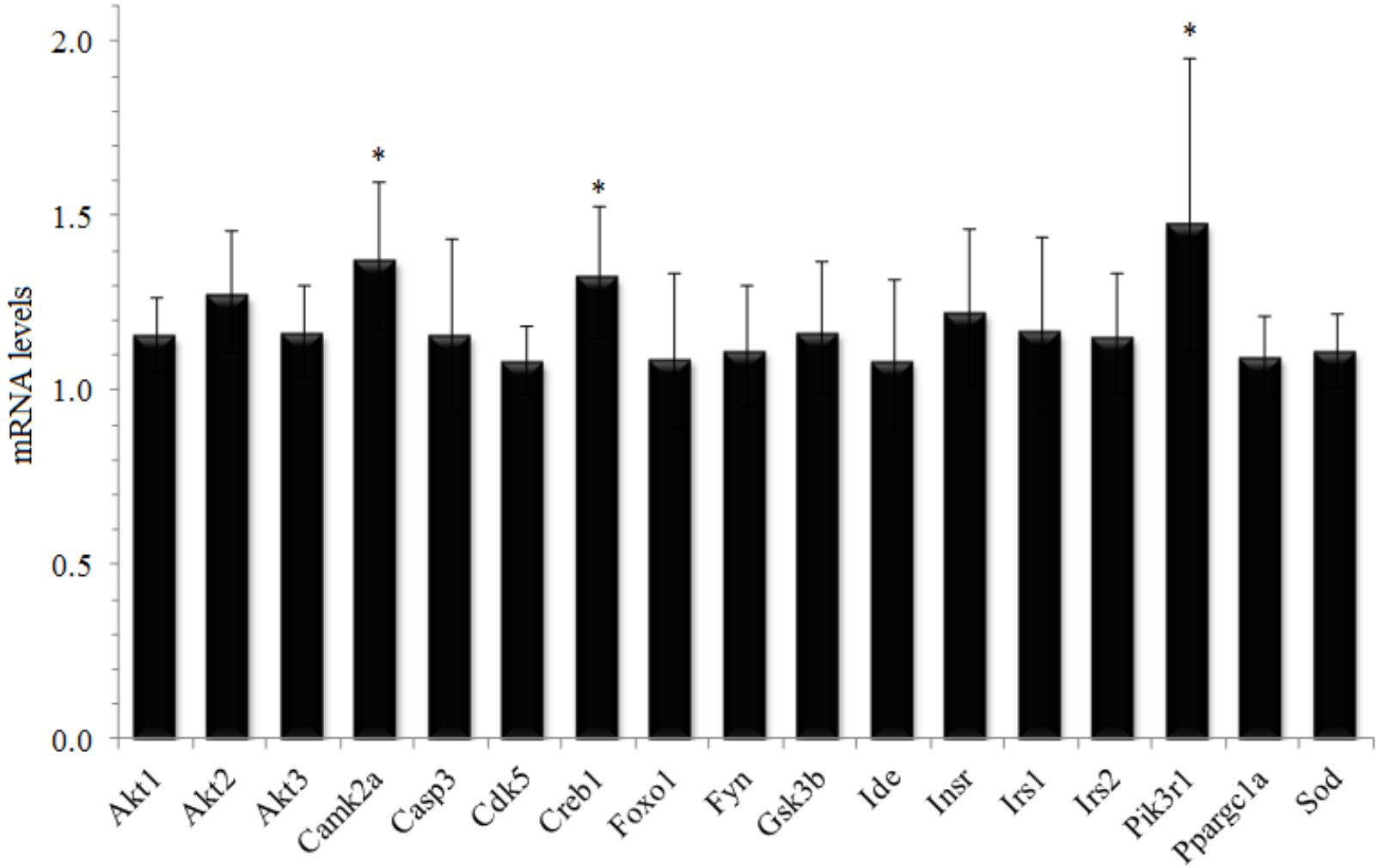

Figure 2 – Relative levels of mRNA in hippocampus of insulin resistant group. Results are presented as mean ± SD (n = 4 in triplicate) and compared using student t-test. Significant levels (*) were considered when $p < 0.05$.

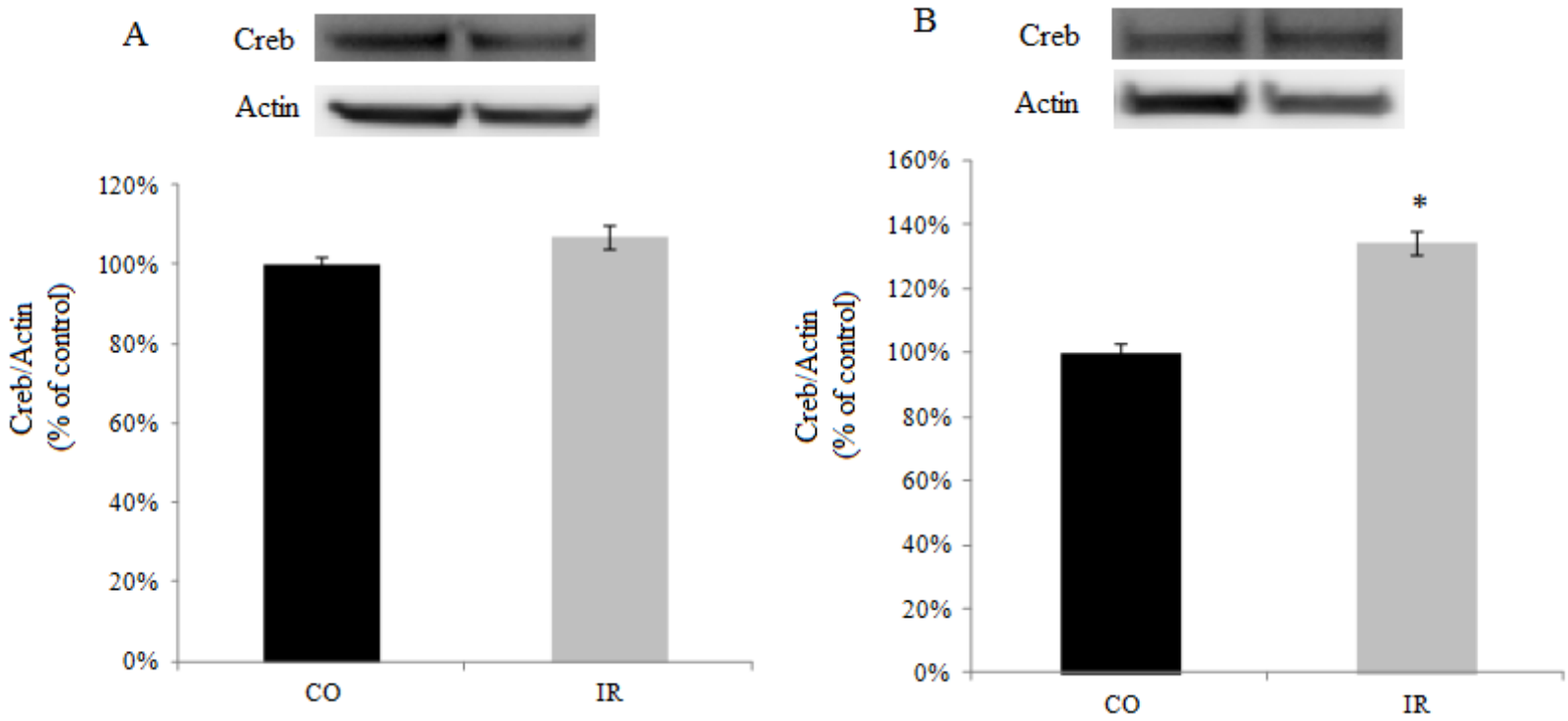

Figure 3 – Western blot analysis of Creb in (A) Cortex and (B) Hippocampus. Creb1 was immunoprecipitated with a specific antibody. Immunoreactive bands were quantified by scanning densitometry. To correct variations in protein loading, Creb band densities were normalized by dividing by their corresponding housekeeping Actin band densities. Results are presented as mean ± SD (n=4). Means were compared with student t-test and * was used for $p < 0.05$.

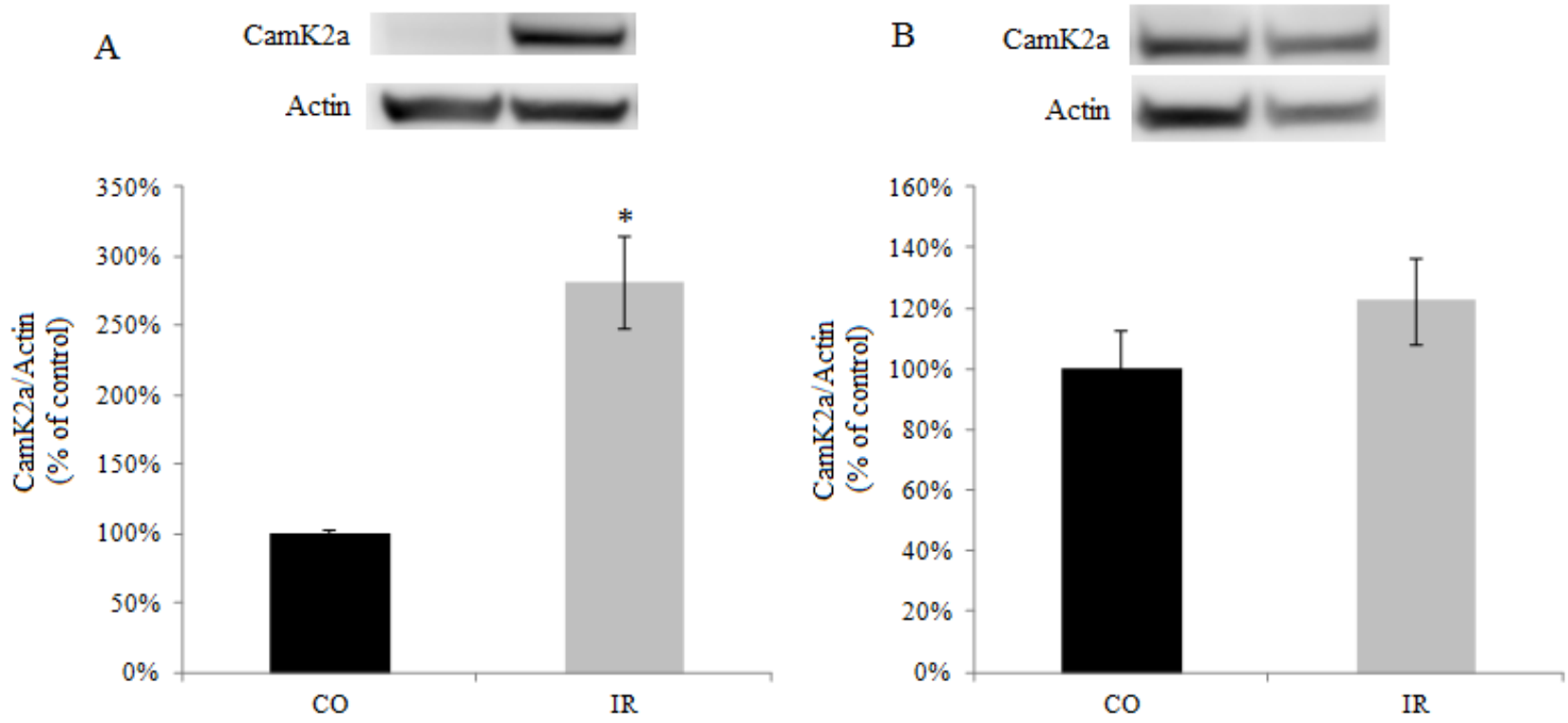

Figure 4 – Western blot analysis of CamK2a in (A) Cortex and (B) Hippocampus. CamK2a was immunoprecipitated with a specific antibody. Immunoreactive bands were quantified by scanning densitometry. To correct variations in protein loading, CamK2a band densities were normalized by dividing by their corresponding housekeeping Actin band densities. Results are presented as mean ± SD (n=4). Means were compared with student t-test and * was used for $p < 0.05$.

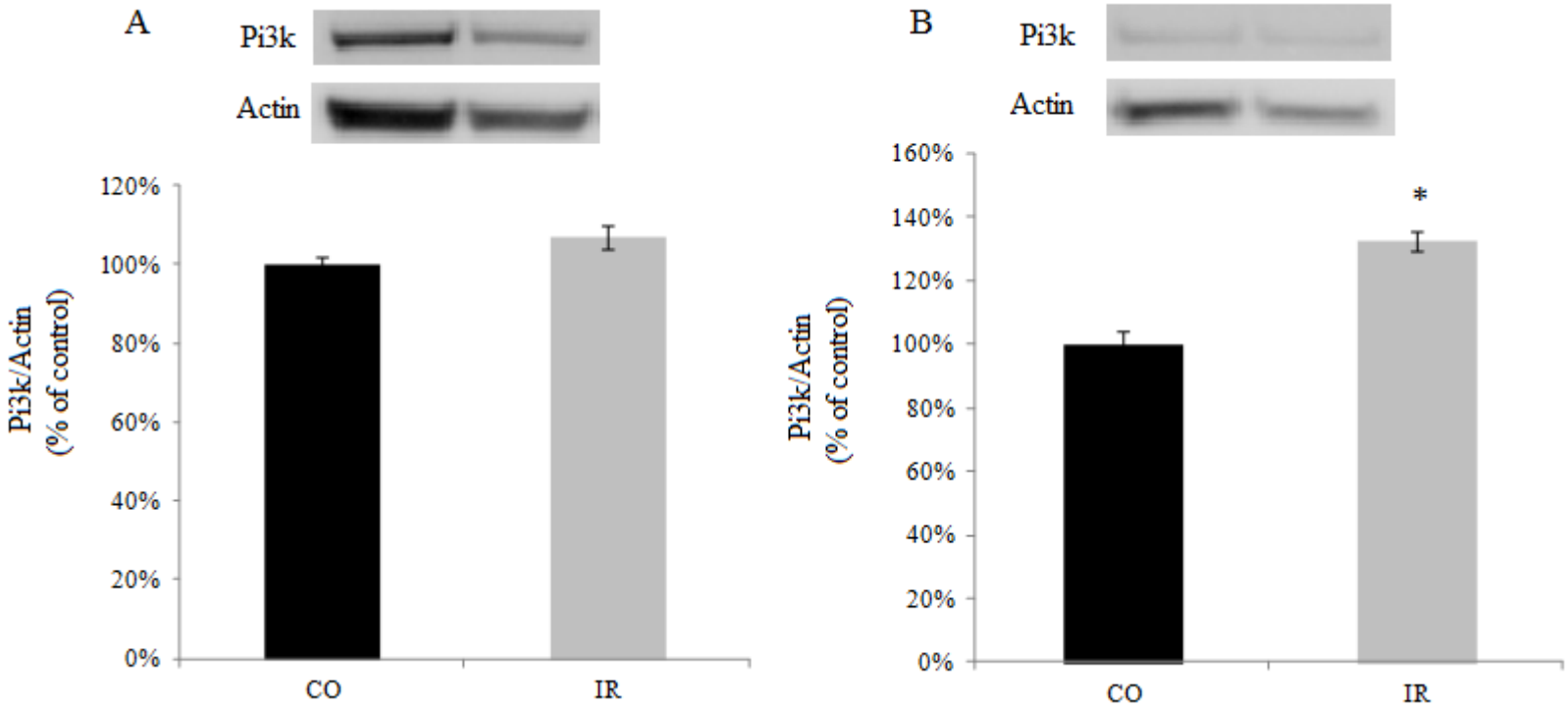

Figure 5 – Western blot analysis of Pi3K in (A) Cortex and (B) Hippocampus. Pi3K was immunoprecipitated with a specific antibody. Immunoreactive bands were quantified by scanning densitometry. To correct variations in protein loading, Pi3K band densities were normalized by dividing by their corresponding housekeeping Actin band densities. Results are presented as mean ± SD (n=4). Means were compared with student t-test and * was used for $p < 0.05$.

3.2 Correlation of Biochemical Profile *versus* mRNA levels

Blood glucose levels at 0, 30, 60, and 120 minutes were measured (data not shown), plotted and the area under the curve (AUC) was used to correlate with mRNA levels. The AUC of blood glucose levels were significantly increased ($p < 0.0001$) in the IR group when compared to CO group (Figure 6A). In the cortex, glycemia was found to be negatively correlated with mRNA levels of Irs-2 (r = -0.8605, $p = 0.0061$), Akt1 (r = -0.8251, $p = 0.0117$), Akt3 (r = -0.928, $p = 0.0009$), Casp3 (r = -0.7218, $p = 0.0432$) and Sod2 (r = -0.7487, $p = 0.0326$) when compared to CO group (Figure 6B). In hippocampus, only Creb1 was found to have a correlation with glycemia (r = 0.7232, $p = 0.0426$) (Figure 6C). All others correlations were not statistically significant.

The levels of TG, TC, HDL and LDL were measured (data not shown) and only TC and LDL were increased in the IR group when compared to CO group. These two lipid parameters were negatively correlated with cortex mRNA levels of Irs-2, Akt1 and Akt3. In hippocampus only TG show a positive correlation with mRNA levels of Pi3kr1 and Creb1 (data not shown).

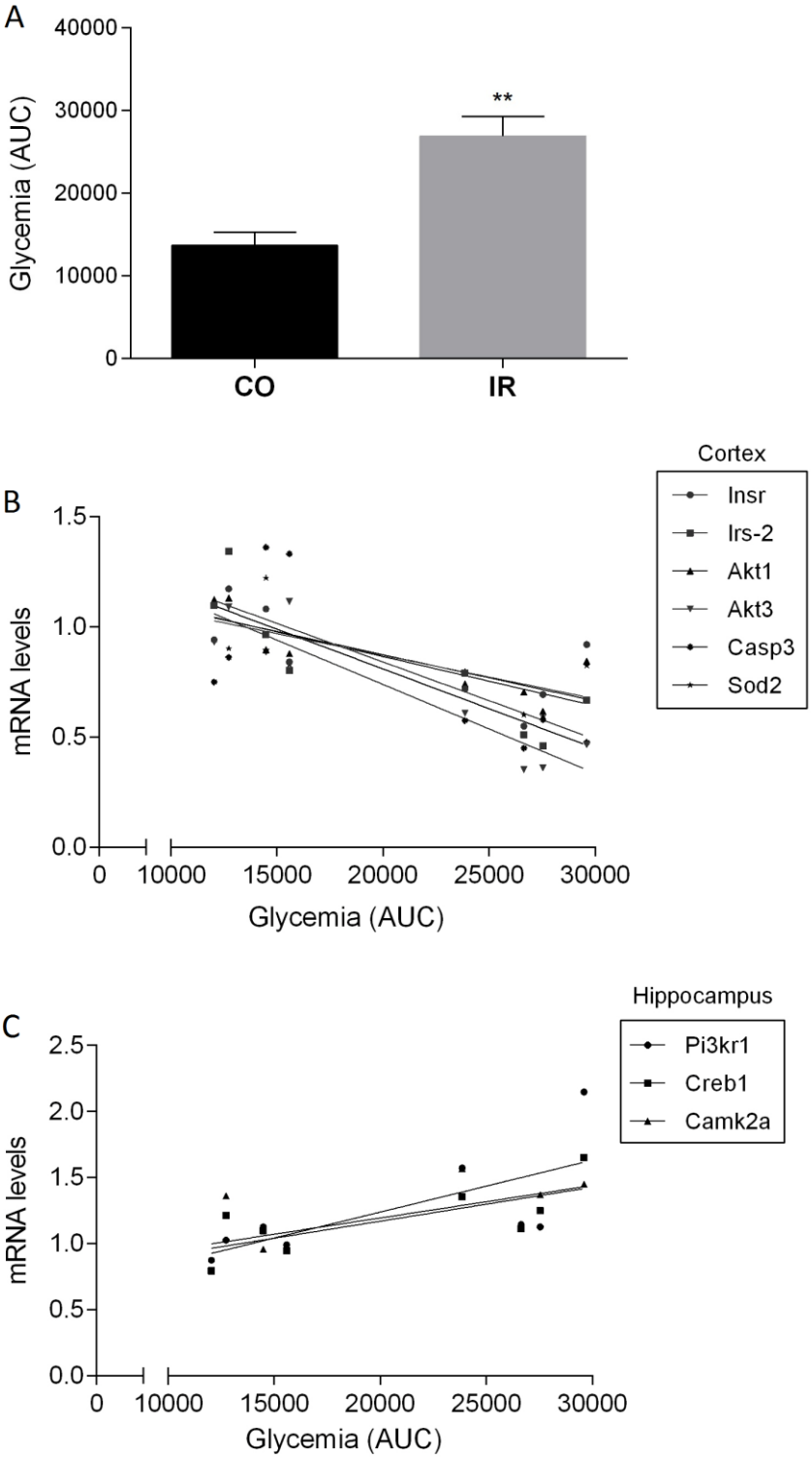

Figure 6 – Correlation of blood glucose *versus* mRNA levels. (A) Glycemia (AUC) levels of CO and IR groups. Results are presented as mean ± SD (n=4) with ** for $p < 0.0001$. Correlation of glycemia and mRNA levels in (B) cortex and (C) hippocampus.

4 Discussion

The central nervous system (CNS) plays a key role to balance the energy equation. Environmental and internal signals are integrated in CNS circuits. Insulin is transported from blood into the brain and stimulates receptors to reduce food intake (Ryan, 2012). Abnormal insulin signaling, like insulin resistance state, plays a fundamental role in the pathogenesis of a host of metabolic diseases, ranging from T2DM to hypertension, lipid disorders, atherosclerosis and reproductive abnormalities (Matsumoto *et al*., 2006). In this study we demonstrate that genes involved in insulin signaling pathway and insulin resistance interact with environmental factors (increased fat/caloric intake through high palatable diet), resulting in changes in mRNA levels and protein content in CNS, and in the development of obesity and insulin resistance as described previously (Kahn *et al*., 2006).

In cortex a general decrease in mRNA levels were observed when we compared CO and IR group. Statistically significant changes were observed in Insr, Irs2, Akt1, Akt3, Sod2, and Casp3 with lower levels in IR group. Insulin sensitivity is also determined by the number of insulin receptors on the plasma membrane and the steady state levels of insulin receptor mRNA effectively control that (Lee and Gorospe, 2010). Thus, lower mRNA levels of Insr comply with the insulin resistant state and it also leads to lower mRNA levels of some downstream effectors (Irs2, Akt1 and Akt3). The insulin resistant state affects the amount of mRNA at mitochondrial level, showed by reduced mRNA level of Sod2, a protector against high oxidative stress, which it is in agreement to our hypothesis that cortex is more affected by insulin insult (impaired signal). Considering protein levels, we observed an increase in CaMK2 levels only. It has been shown that in exercise-induced glucose transporter type 4 (GLUT4) up regulation is mediated by influx of calcium via CaMK2 (Ojuka *et al*., 2012).

Taking in account that insulin, when not bended to Insr, remains in the extracellular cleft, an increase in this protein concentration activates IGF-1 in turn releasing calcium from intracellular stores leading to activation of CREB by CaMK2 (Stears *et al*., 2012; Zuloaga *et al*., 2013). Therefore it is likely that insulin resistant state at cortex is trying to maintain normal functions by increasing protein levels of these proteins. Findings presented here indicate up regulation of translation by other factors in order to maintain cortex glucose homeostasis.

In hippocampus a general increase in mRNA levels was observed when CO group was compared to IR group. However, statistically significant higher levels were reached by 3 genes in IR group: Pi3kr1, Camk2a, and Creb1. When protein levels were taken into account, alterations were observed in CREB and PI3K only. This may be due to a compensatory effect of impaired insulin signaling in the structure. Up regulation of Creb, at mRNA and protein levels, is plausible with the results of general up regulation in mRNA levels since it is a well known as a physiological transcriptional regulator of gluconeogenesis *in vivo*. Furthermore, Creb is not only important in the direct transcriptional activation of gluconeogenic genes, but is also responsible for the progression of hepatic insulin resistance in the diet-induced or the genetic mouse models of obesity by impairing the transcriptional activity of Creb and results in transcriptional repression of gluconeogenic genes (Jitrapakdee, 2012). Despite the importance of Creb in diverse tissues, our study shows that Creb is also important in the CNS insulin resistant state, mainly in hippocampus. In turn, an increase of Creb levels seems to be essential to maintain in normal levels all other molecules involved in the insulin signaling pathway to avoid severe injuries in hippocampus. Thus, further studies are necessary to delineate the diverse roles of Creb in the transcriptional control of energy homeostasis at CNS level.

Taking all together, a defect in the canonical insulin/IR/IRS/PI3K/AKT/FOXO1-CREB pathway is present in most patients with the metabolic syndrome, as well as in patients with insulin receptoropathies and patients with lipodystrophy, and/or that even partial loss of signaling via this pathway is sufficient to allow constitutive FOXO1 activation and stimulates even more glucoses production through glcose-6-phosphatase and phosphoenolpyruvate carboxykinase 1 (Stears *et al*., 2012). Or the insulin resistance is mediated by signaling via an alternative pathway such as that involving the IGF1R/PI3K/CaMK2/CREB (Zuloaga *et al*., 2013). Insulin is known to be capable of binding IGF receptors albeit with lower affinity than to the insulin receptor itself, so when present in the serum in high concentrations it remains entirely feasible for it to bind and activate the IGF receptors (Boucher *et al*., 2010). Our study corroborate, at molecular level in CNS, with those two signaling pathways described above, one, the classic via, in the hippocampus and the other, the alternative via, in cortex.

These hypotheses must be confirmed by a biochemical and/or histological studies in future investigations to know if all of those molecular alterations can be translated into gain and loss of protein function.

5 Acknowledgments

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6 Conflict of Interest Statement

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

7 References

Adam TC, Epel ES. Stress, eating and the reward system. Physiol Behav 2007; 91:449–458.

Banks WA, Owen JB, Erickson MA. Insulin in the brain: there and back again. Pharmacol Ther 2012; 136(1):82-93.

Biddinger SB, Kahn CR. From mice to men: insights into the insulin resistance syndromes. Ann. Rev Physiol 2006; 68:123-158.

Boucher J, Tseng YH, Kahn CR. Insulin and insulin-like growth factor-1 receptors act as ligand-specific amplitude modulators of a common pathway regulating gene transcription. Journal of Biological Chemistry 2010; 285(22):17235–17245.

Boyle JP, Thompson TJ, Gregg EW, Barker LE, Williamson DF. Projection of the year 2050 burden of diabetes in the US adult population: dynamic modeling of incidence, mortality, and prediabetes prevalence. Popul Health Metr 2010; 8:29.

Brüning JC, Gautam D, Burks DJ, Gillette J, Schubert M, Orban PC, Klein R, Krone W, Müller-Wieland D, Kahn CR. Role of brain insulin receptor in control of body weight and reproduction. Science 2000; 289:2122–2125.

Cole A, Astell A, Green C, Sutherland C. Molecular connections between dementia and diabetes. Neurosci Biobehav Rev 2007; 31:1046–1063.

Cukierman T, Gerstein HC, Williamson JD. Cognitive decline and dementia in diabetes-systematic overview of prospective observational studies. Diabetologia 2005; 48:2460–2469.

Dietrich MO, Muller A, Bolos M, Carro E, Perry ML, Portela LV, Souza DO, Torres-Aleman I. Western style diet impairs entrance of blood-borne insulin-like growth factor-1 into the brain. Neuromolecular Med 2007; 9(4):324-30.

Jitrapakdee S. Transcription factors and coactivators controlling nutrient and hormonal regulation of hepatic gluconeogenesis. Int J Biochem Cell Biol 2012; 44(1):33-45.

Kahn SE, Hull RL, Utzschneider KM. Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature 2006; 444(7121):840-846.

Kido Y, Burks DJ, Withers D, Bruning JC, Kahn CR, White MF, Accili D. Tissue-specific insulin resistance in mice with mutations in the insulin receptor, IRS-1, and IRS-2. The Journal of clinical investigation. 2000; 105(2):199-205.

Kopelman, PG. Obesity as a medical problem. Nature 2000; 404:635–643.

Lee EK, Gorospe M. Minireview: posttranscriptional regulation of the insulin and insulin-like growth factor systems. Endocrinology 2010; 151(4):1403-1408.

Lindgren CM, Heid IM, Randall JC, Lamina C, Steinthorsdottir V, Qi L, Speliotes EK, Thorleifsson G, Willer CJ, Herrera BM, Jackson AU, Lim N, Scheet P, Soranzo N, Amin N, Aulchenko YS, Chambers JC, Drong A, Luan J, Lyon HN, Rivadeneira F, Sanna S, Timpson NJ, Zillikens MC, Zhao JH, Almgren P, Bandinelli S, Bennett AJ, Bergman RN, Bonnycastle LL, Bumpstead SJ, Chanock SJ, Cherkas L, Chines P, Coin L, Cooper C, Crawford G, Doering A, Dominiczak A, Doney AS, Ebrahim S, Elliott P, Erdos MR, Estrada K, Ferrucci L, Fischer G, Forouhi NG, Gieger C, Grallert H, Groves CJ, Grundy S, Guiducci C, Hadley D, Hamsten A, Havulinna AS, Hofman A, Holle R, Holloway JW, Illig T, Isomaa B, Jacobs LC, Jameson K, Jousilahti P, Karpe F, Kuusisto J, Laitinen J, Lathrop GM, Lawlor DA, Mangino M, McArdle WL, Meitinger T, Morken MA, Morris AP, Munroe P, Narisu N, Nordström A, Nordström P, Oostra BA, Palmer CN, Payne F, Peden JF, Prokopenko I, Renström F, Ruokonen A, Salomaa V, Sandhu MS, Scott LJ, Scuteri A, Silander K, Song K, Yuan X, Stringham HM, Swift AJ, Tuomi T, Uda M, Vollenweider P, Waeber G, Wallace C, Walters GB, Weedon MN; Wellcome Trust Case Control Consortium, Witteman JC, Zhang C, Zhang W, Caulfield MJ, Collins FS, Davey Smith G, Day IN, Franks PW, Hattersley AT, Hu FB, Jarvelin MR, Kong A, Kooner JS, Laakso M, Lakatta E, Mooser V, Morris AD, Peltonen L, Samani NJ, Spector TD, Strachan DP, Tanaka T, Tuomilehto J, Uitterlinden AG, van Duijn CM, Wareham NJ, Hugh Watkins; Procardis Consortia, Waterworth DM, Boehnke M, Deloukas P, Groop L, Hunter DJ, Thorsteinsdottir U, Schlessinger D, Wichmann HE, Frayling TM, Abecasis GR, Hirschhorn JN, Loos RJ, Stefansson K, Mohlke KL, Barroso I, McCarthy MI; Giant Consortium. Genome-wide association scan meta-analysis identifies three loci influencing adiposity and fat distribution. PLoS Genet 2009; 5:e1000508.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25(4):402-408.

Matsumoto M, Han S, Kitamura T, Accili D. Dual role of transcription factor FoxO1 in controlling hepatic insulin sensitivity and lipid metabolism. J Clin Invest 2006; 116(9):2464-2472.

Murata H, Hresko RC, Mueckler M. Reconstitution of phosphoinositide 3-kinase-dependent insulin signaling in a cell-free system. J Biol Chem 2003; 278(24):21607-21614.

Oh KJ, Han HS, Kim MJ, Koo SH. CREB and FoxO1: two transcription factors for the regulation of hepatic gluconeogenesis. BMB Rep 2013; 46(12):567-574.

Ojuka EO, Goyaram V, Smith JA. The role of CaMKII in regulating GLUT4 expression in skeletal muscle. Am J Physiol Endocrinol Metab. 2012; 303(3):E322-331.

Petersen KF, Shulman GI. Etiology of insulin resistance. Am J Med 2006; 119:S10–S16.

Proud CG. eIF2 and the control of cell physiology. Semin Cell Dev Biol. 2005; 16(1):3-12.

Rung J, Cauchi S, Albrechtsen A, Shen L, Rocheleau G, Cavalcanti-Proença C, Bacot F, Balkau B, Belisle A, Borch-Johnsen K, Charpentier G, Dina C, Durand E, Elliott P, Hadjadj S, Järvelin MR, Laitinen J, Lauritzen T, Marre M, Mazur A, Meyre D, Montpetit A, Pisinger C, Posner B, Poulsen P, Pouta A, Prentki M, Ribel-Madsen R, Ruokonen A, Sandbaek A, Serre D, Tichet J, Vaxillaire M, Wojtaszewski JF, Vaag A, Hansen T, Polychronakos C, Pedersen O, Froguel P, Sladek R. Genetic variant near IRS1 is associated with type 2 diabetes, insulin resistance and hyperinsulinemia. Nature Genet 2009; 41, 1110–1115 (2009).

Ryan KK, Woods SC, Seeley RJ.Central nervous system mechanisms linking the consumption of palatable high-fat diets to the defense of greater adiposity. Cell Metab 2012; 15(2):137-149.

Samuel VT, Shulman GI. Mechanisms for insulin resistance: common threads and missing links Cell 2012; 148(5):852-871.

Schwartz MW, Porte D Jr. Diabetes, obesity, and the brain. Science 2005; 307(5708):375-379.

Sisk CL, Zehr JL. Pubertal hormones organize the adolescent brain and behavior. Front Neuroendocrinol 2005; 26:163–174.

Stears A, O'Rahilly S, Semple RK, Savage DB. Metabolic insights from extreme human insulin resistance phenotypes. Best Pract Res Clin Endocrinol Metab 2012; 26(2):145-157.

Welsh GI, Miller CM, Loughlin AJ, Price NT, Proud CG Regulation of eukaryotic initiation factor eIF2B: glycogen synthase kinase-3 phosphorylates a conserved serine which undergoes dephosphorylation in response to insulin. FEBS letters 1998; 421(2):125-130

Williamson R, McNeilly A, Sutherland C. Insulin resistance in the brain: an old-age or new-age problem? Biochem Pharmacol 2012; 84(6):737-745.

Zuloaga R, Fuentes EN, Molina A, Valdés JA. The cAMP response element binding protein (CREB) is activated by insulin-like growth factor-1 (IGF-1) and regulates myostatin gene expression in skeletal myoblast. Biochem Biophys Res Commun 2013; 440(2):258-64.

CAPÍTULO II

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Changes in Brain 14-3-3 Proteins in Response to Insulin Resistance Induced by a High Palatable Diet

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Abstract The 14-3-3 protein family takes part in a wide range of cellular processes and is expressed in all eukaryotic organisms. In mammals, seven isoforms (β, ε, η, γ, τ, ζ, and σ) have been identified. 14-3-3 proteins are suggested to modulate the insulin-signaling cascade in the brain. The aim of this study was to investigate whether insulin resistance state induced by high palatable diet modulates expression of the 14-3-3 proteins in brain. Wistar male rats (n=8) were divided into two experimental groups: insulin resistant (IR), induced by high palatable diet, and control (CO) group. Biochemical parameters (glucose tolerance test and plasma lipid profile) were evaluated after 130 days. Brain structures (cortex and hippocampus) were dissected for evaluation of messenger RNA (mRNA) and protein levels of different 14-3-3 proteins. Statistical analyses included Student t test and Pearson correlation. Significant decrease was observed in Ywhah and in Ywahq mRNA levels in the cortex of IR group, while no changes were observed in the hippocampus. Significant increase of θ isoform was observed in hippocampus IR group by immunodetection, while no differences were detected in the remaining isoforms. Inverse correlation was observed between blood glucose levels in cortex IR group and both Ywhah and Ywhaq mRNA levels. Protein levels of Creb and phosphatidylinositide 3-kinases (PI3K) showed to be increased in the hippocampus. These alterations may be due to a compensatory effect of impaired insulin signaling. We demonstrated differential expression of 14-3-3 isoforms throughout brain regions of rats with IR. As a whole, our results indicate that brain 14-3-3 levels are influenced by different diets.

Keywords 14-3-3 · Insulin resistance · Protein expression · mRNA levels · Rat brain

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Introduction

The 14-3-3 protein family consists of highly conserved regulatory molecules expressed in all eukaryotic cells [1, 2]. In mammals, seven isoforms (β, γ, ε, ζ, η, θ, and σ) have been identified to date, and they seem to be highly abundant in the mammalian nervous system [3]. 14-3-3 proteins participate in a wide range of cellular processes through binding interactions with hundreds of structurally and functionally diverse proteins [4]. These proteins are implicated in regulation and coordination of many cellular processes, including signaling pathways, transcriptional regulation of messenger RNA (mRNA) levels, apoptosis, and signaling cascades among others [5, 6].

In the brain, 14-3-3 proteins are suggested to modulate the insulin signaling cascade pathway through phosphorylation and interaction with key regulatory proteins such as insulin

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receptor substrate-1 (IRS-1) and IRS-2, glycogen synthase kinase 3 (GSK3), and phosphatidylinositide 3-kinases (PI3K) [7–10].

Insulin is well known as the major and immediate regulator of blood glucose levels in peripheral tissues and has emerged as a major regulatory substance within the brain [11]. For instance, two major brain areas of behavior affected by insulin are those related to feeding and cognition [12]. Depressive-like behavior in rats can be explained by downregulation of hypothalamic insulin receptor signaling [13]. In addition, insulin receptor protein levels decrease with age, which suggests that insulin signaling and insulin receptor levels are involved in the aging process [14, 15]. Disease states such as dementia and Alzheimer are related to insulin resistance and to other aspects of metabolic syndrome [16]. High risk of dementia or significant cognitive decline has been associated to both insulin resistance alone and type 2 diabetes mellitus (T2DM) [17, 18]. Therefore, insulin resistance possibly enhances the synaptic loss and neurodegeneration associated with cognitive decline and dementia [19].

Many proteins that bind to 14-3-3 in response to insulin-activated signaling pathways have been identified [20]. Overall, a network of 14-3-3-phosphoprotein interactions has been shown to provide mechanistic insights into glucose uptake stimulated by insulin as well as other effects in intracellular events [21]. IRS proteins serve as intracellular docking and adapter molecules that integrate stimuli from different cellular pathways. IRS2 and 14-3-3 have been shown to interact on high cAMP levels, upon insulin and IGF-1 stimulation [8]. Recently, 14-3-3 mRNA and protein levels were shown to be affected in an isoform- and tissue-specific manner in a streptozotocin-induced diabetic animal model [22].

It is well recognized that insulin resistance and disrupted glucose metabolism occurs in peripheral tissues. However, recent publications support the view that insulin resistance is also present in the brain. This perspective coincides with the current concept that brain insulin receptors/signaling have physiological relevance for neuroplasticity and neuromodulation mechanisms [23–26]. We have previously demonstrated in an insulin resistance model that in addition to peripheral parameters, some neurological aspects are also modified. All of these observations point to the fact that brain insulin resistance is reasonably reliable [11, 27]. Thus, impaired signaling modifies the expression of a wide variety of other related proteins, including 14-3-3 proteins, taking into account that insulin is a pro-survival molecule. The extent of mRNA and protein reflects a dynamic balance among all cell processes, although the way this balance is achieved remains a challenge. Emerging evidence is changing the role for many regulatory mechanisms occurring after mRNA are manufactured. This has been examined to date in almost every organism; amount of transcript does not fully predict protein extent [28, 29].

Therefore, the purpose of the present study was to investigate whether insulin resistance state induced by high palatable diet modulates the expression of 14-3-3 isoforms in the brain of rats.

Experimental Procedures

Animals and Diet

Eight (n=8) adult male Wistar rats were housed under controlled temperature (22±2 °C) and humidity (55±10 %) conditions on a 12-h light-dark cycle (lights on at 7 a.m.), with food and water offered ad libitum. All experiments were performed in agreement with international standards and the Brazilian College of Animal Experimentation for animal protection. The project was approved by the Ethical Committee on animal use of the Universidade Federal do Rio Grande do Sul, Brazil (#19446). At 2 months old (ranging from 200 to 250 g of weight), animals were divided into two experimental groups (n=4): control (CO) and insulin resistant (IR). Administration of high palatable diet (also known as *cafeteria diet* or *Western-style diet*) during 130 days was used to induce insulin resistance in IR group [27, 28], while CO group was fed with standard laboratory diet.

Biochemical parameters were evaluated after 130 days. For glucose tolerance test, rats were deprived from food overnight (8 h) and blood samples were collected through a small puncture on the tail. Animals were anesthetized with ketamine/xylazine intraperitoneally (100/10 mg/kg), and blood was collected for plasma lipid profile. They were then decapitated, the cortex and hippocampus were rapidly dissected, and each structure was divided into two pieces. One was rapidly frozen and stored at −80 °C for proteome analysis; the other was submerged in RNAlater® (Ambion, USA) and stored at −20 °C for mRNA analysis.

Biochemical Profile

Glucose solution at 50 % (w/v) was administrated into rats (2 g/kg of body weight) for glucose tolerance test (GTT). Blood samples were collected at fasting and at 30, 60, and 120 min after glucose overload, and glycemia was measured with a commercial glucometer, Accu-Chek Active (Roche, USA).

Plasma lipid profile was performed by measuring levels of total content of triglycerides (TG), total cholesterol (TC), and fractions of high-density lipoprotein (HDL) and low-density lipoprotein (LDL). Analyses were performed using commercial kits (Katal®, MG, Brazil).

Evaluation of Protein Profile by Mass Spectrometry

Pieces of frozen tissue were homogenized in PBS using a homogenizer, and proteins were extracted with 10 % TCA in cold acetone. Protein extracts of all the groups (cortex IR, cortex CO, hippocampus IR, and hippocampus CO groups) were analyzed by mass spectrometry (MS) to identify differentially expressed proteins. A total of 100 μg of protein was digested with trypsin (Sigma-Aldrich, USA) at 1:50 (*w*/*w*) enzyme/protein ratio for 1 h at 37 °C. Reaction was then quenched, and tryptic digest was separated using a nanoLC Ultra-1D plus system (Eksigent, USA), followed by direct elution to a nanospray ion source connected to a hybrid mass spectrometer (LTQ-XL and LTQ Orbitrap Discovery, Thermo, USA). Flow rate was set at 300 nL/min using 2–98 % acetonitrile/0.1 % formic acid gradient during 5 h.

Tandem mass spectra were obtained by Proteome Discoverer 1.0 software (Thermo Fisher Scientific, USA), and all MS/MS samples were analyzed using SEQUEST software assuming the digestion by trypsin. Scaffold software v. 3.1.2 (Proteome Software Inc., USA) was used to validate MS/MS-based peptide and protein identifications. Proteomes were compared using DeconTools software v. 1 (Pacific Northwest National Laboratory, USA) and MultiAlign v. 5.0.3 (Pacific Northwest National Laboratory, USA). Protein quantitation was assessed by spectral counting, where the number of observed spectral counts for each protein is a frequency-based analysis approach that provides a rough measure of protein levels in complex protein mixtures, especially for more abundant proteins [30, 31].

Evaluation of mRNA Levels of 14-3-3 Isoforms

mRNA levels were measured by quantitative real-time PCR (qPCR) using gene-specific TaqMan® FAM/MGB inventoried or made to order assays (14-3-3 isoform β/α, gene Ywhab, assay Rn00695953_m1; ζ/δ, Ywhaz, Rn00755072_m1; η, Ywhah, Rn00755085_m1; θ, Ywhaq, Rn00820723_g1; Applied Biosystems, USA), using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as endogenous control (inventoried assay Rn99999916_s1, Applied Biosystems, USA). Total RNA was extracted using TRI Reagent® solution (Ambion, USA) according to manufacturer's protocol. RNA concentration was estimated using the fluorimetric method Quant-iT® RNA Assay (Invitrogen, USA) in the Qubit™ (Invitrogen, USA) equipment. Complementary DNA (cDNA) was synthesized by reverse transcription (RT) reaction using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Two hundred nanograms of total RNA was placed in a total reaction volume of 20 μL containing 1× RT buffer, 4 mM of each dNTP, 1× RT Random Primers, and 0.05 units of MultiScribe™ Reverse Transcriptase. RT reaction was performed as follows: 10 min at 25 °C, 2 h at 37 °C, and 5 s at 85 °C. Subsequently, cDNA was kept at −20 °C until further use.

cDNA solution was diluted 1:5 in water, and reaction was carried out in a total volume of 12 μL containing 1 μL of diluted cDNA solution, 1× of gene-specific TaqMan® assay, 1× of endogenous control TaqMan® assay, and 1× PCR Master Mix (Applied Biosystems, USA). Cycling program was 2, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Reactions of each sample were performed in triplicate in an ABI Prism 7500 Fast Sequence Detector System (Applied Biosystems, USA).

Transcriptional profile of each 14-3-3 isoform was determined in both brain structures. The relative mRNA levels were calculated by the ΔΔCt method according to Livak and Schmittgen [32], using GAPDH as endogenous control and the control group of each structure as calibrator.

Evaluation of Protein Levels by Western Blot

Total protein extracts of each group were analyzed by Western blot (WB) for protein analysis. Twenty micrograms of protein were mixed with Bolt™ LDS Sample Buffer (4×) (Novex, USA) and Bolt™ Sample Reducing Agent (10×) (Novex, USA). Samples were then heated for 5 min at 95 °C and separated on a precasted Bolt™ Bis-Tris Plus Gel 4 to 12 % polyacrylamide gradient (Novex, USA) using Bolt™ MES SDS Running Buffer (Novex, USA) for 35 min. at 165 V. Following electrophoresis, protein content was transferred to a nitrocellulose membrane using iBlot® Transfer Stack (Novex, USA) in the iBlot® Gel Transfer Device (Invitrogen, USA) using the P3–7-min program.

Free sites were blocked with 5 % nonfat milk powder in tris-buffered saline containing 0.5 % Tween 20 (TBS-T) pH 7.3 to 7.5 for 1 h after blotting. Membranes were rinsed once for 10 min followed by three times for 5 min with TBS-T solution and then incubated for 1 h with 14-3-3 isoform primary antibodies (14-3-3 β/α, #9636, 1:2.000; 14-3-3 ζ/δ, #7413, 1:2.000; 14-3-3 θ, #9638, 1:1.1000; 14-3-3 η, #5521, 1:1.000; Cell Signaling Technology, USA) diluted in TBS-T with 1 % BSA solution. For Creb (#9104, Cell Signaling Technology) and PI3K p85 (#4257P, Cell Signaling Technology), the primary antibodies were diluted 1:1.000 and incubated overnight at 4 °C. Next to incubation with primary antibodies, membranes were rinsed again as previously described and incubated for 1 h with secondary antibody conjugated to horseradish peroxidase (anti-rabbit IgG HRP-linked, #7074, 1:4.000; Cell Signaling Technology, USA) diluted in TBS-T with 1 % BSA solution. All incubations and washes were performed at room temperature with gentle

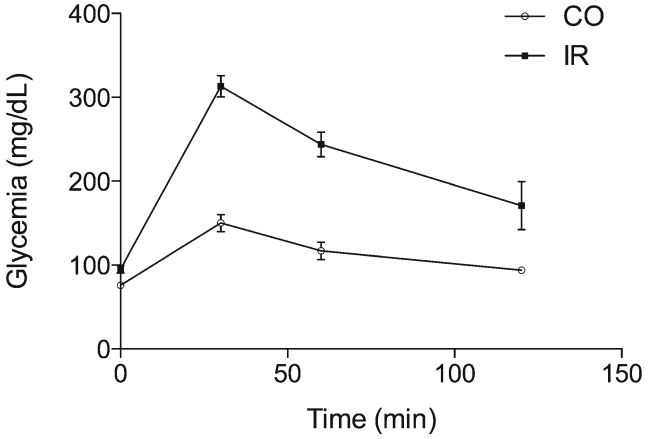

Fig. 1 Glucose tolerance test. High palatable diet during 130 days altered the glucose tolerance test (GTT). Measures were at fasting and at 30, 60, and 120 min after glucose overload. *CO* represents control group, and *IR* represents insulin-resistant group (n=4 per group)

agitation. Blots were developed using the Western Lightning ECL Pro (PerkinElmer, USA), and membranes were then imaged using the ImageQuant LAS 500 (General Electric Healthcare, USA). Same membranes were rinsed and blotted to housekeeping anti-β-actin HRP-linked antibody (#A3854, 1:30.000; Sigma-Aldrich, USA) and followed by the same treatment described above. Immunoreactive content was quantified using ImageJ software v. 1.47 (available from the National Institutes of Health, USA).

Statistical Analysis

Statistical analyses were performed using Student t test. Correlation between variables was calculated by Pearson correlation, and differences were considered statistically significant when $p<0.05$.

Results

Biochemical Profile Demonstrates Insulin Resistance in Animal Model

We demonstrated that the treatment with high palatable diet during 130 days altered results of the GTT. Blood glucose levels at 0, 30, 60, and 120 min after glucose overload were measured and are shown in Fig. 1. Area under the curve (AUC) showed a significant higher value in the IR group when compared to CO group ($p<0.0001$) (data not shown). We have also evaluated lipid parameters (TG, TC, HDL, and LDL), and TC as well as LDL was significantly increased in the IR group when compared to CO (Fig. 2).

Evaluation of mRNA Levels of 14-3-3 Isoforms and Correlation with Biochemical Parameters

Significant decrease of mRNA levels in cortex IR group was observed in Ywhah (14-3-3 η, $p=0.0118$) and Ywahq (14-3-3 θ, $p=0.0156$). Ywhab (14-3-3 β/α) and Ywhaz (14-3-3 ζ/δ) genes do not show significant differences in mRNA levels in cortex IR group, when compared to CO group. mRNA levels of all 14-3-3 isoforms analyzed do not show any significant difference in hippocampus (Fig. 3).

In the cortex, AUC of IR group in GTT has shown negative correlation to mRNA levels of Ywhah ($r=-0.7587$, $p=0.0291$) and Ywhaq ($r=-0.8063$, $p=0.0156$) when compared to the CO group. All remaining correlations with glucose levels were not statistically significant (Fig. 4).

No differences were found between each lipid parameters evaluated (TG, TC, HDL, and LDL) and mRNA levels of the four 14-3-3 isoforms in the cortex and hippocampus (Fig. 5).

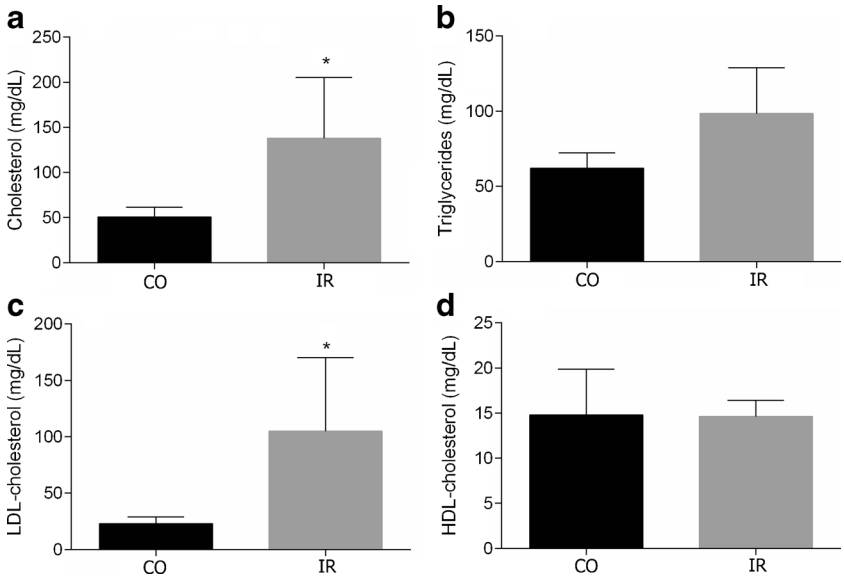

Fig. 2 Plasma lipid profile. Levels of total cholesterol (**a**), triglycerides (**b**), LDL-cholesterol (**c**), and HDL-cholesterol (**d**). *CO* represents control group (n=4), and *IR* represents insulin-resistant group (n=4). Means were compared by Student t test; *$p<0.05$

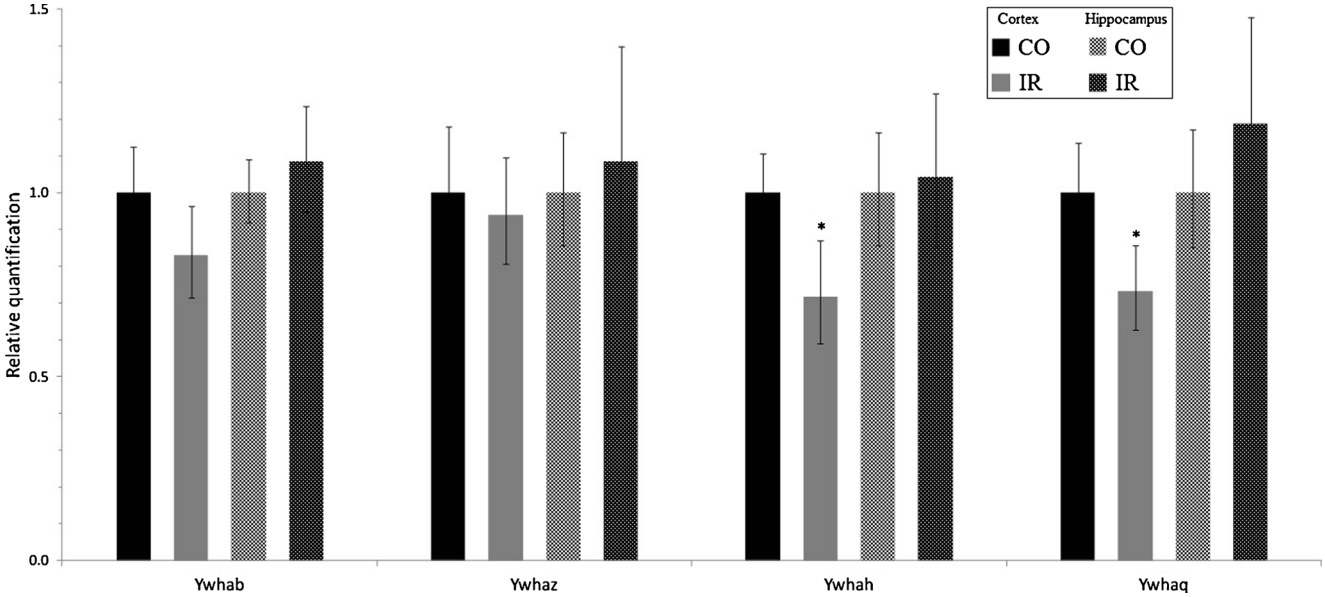

Fig. 3 Relative mRNA levels of 14-3-3 isoforms. Results are presented as mean±SD (n=4). Significant levels (*) were considered when $p<0.05$ Student t test. *CO* represents control group, and *IR* represents insulin resistant group

Evaluation of Protein Content of 14-3-3 Isoforms, Creb, and PI3K

Two (α/β and ζ/δ) of the four isoforms of 14-3-3 proteins analyzed showed alteration of expression pattern between test (IR) and control (CO) groups in MS experiment. Creb and PI3K were not detected, although total number of peptides found in MS was lower than expected for each group.

We observed significant increase of protein immunodetection in WB of the 14-3-3 θ isoform (p=0.0428) in hippocampus IR group, when compared to CO. No differences were found in the hippocampus for the other isoforms analyzed. In the cortex, all four isoforms analyzed do not show significant differences between the groups as well (Fig. 6). Creb and PI3K show a significant increase of protein immunodetection only in the hippocampus of IR group when compared to CO group (p=0.0090 and 0.0125, respectively). The cortex does not show significant changes (Fig. 7).

Discussion

In the present study, we have evaluated the effect of insulin resistance induced by high palatable diet on lipid and glucose parameters and on protein and mRNA levels of four 14-3-3 isoforms in adult male rats. Levels of TC and LDL and glucose intolerance showed to be significantly affected by diet, as expected.

mRNA levels and protein immunodetection of the four 14-3-3 isoforms (α/β, ζ/δ, η, and θ) were evaluated in the cortex and hippocampus of rats with induced insulin resistance and compared to rats fed with normal diet. We have then demonstrated that 14-3-3 isoforms

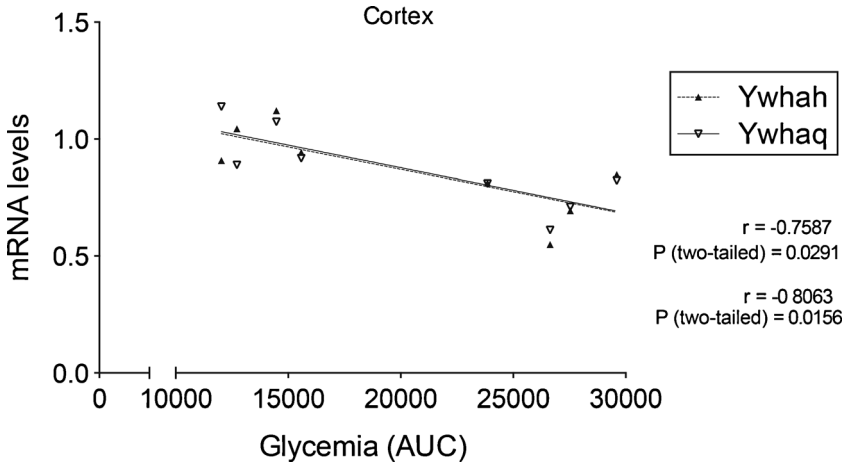

Fig. 4 Correlation of blood glucose versus mRNA levels. Correlation of glycemia (AUC) and mRNA levels of Ywhah and Ywhaq in cortex (Pearson correlation test)

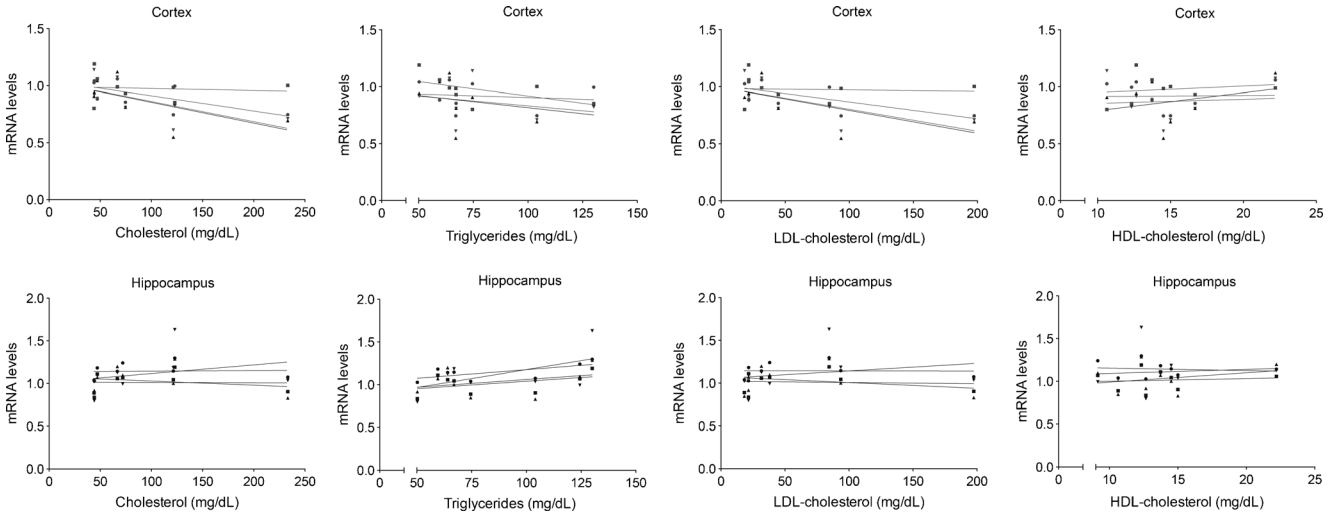

Fig. 5 Correlation of mRNA levels versus plasma lipid profile. mRNA levels of 14-3-3 isoforms (*black circle* Ywhab; *black square* Ywhaz; *black triangle* Ywhah; *black inverted triangle* Ywhaq) and total cholesterol, triglycerides, LDL cholesterol, and HDL cholesterol in the cortex (*upper set*) and in the hippocampus (*lower set*) per animal (n=4)

are differently expressed in the brain of rats with insulin resistance. As previously stated, 14-3-3 proteins are a family that can modulate interaction between proteins. Regulation of interaction usually involves phosphorylation of the interacting protein, and phosphorylation of 14-3-3 isoforms themselves may modulate interaction in some cases [2, 6, 33].

Proteome analysis revealed a decrease from 2 to 1 in peptide spectral count of 14-3-3 α/β in hippocampus IR group. These isoforms interact with the tuberous sclerosis 1/tuberous sclerosis 2 dimmer (TSC1/TSC2) and is involved in cell growth and morphology [34]. They form a protein complex that inhibits signal transduction to downstream effectors of the mammalian target of rapamycin (mTOR) [35]. The

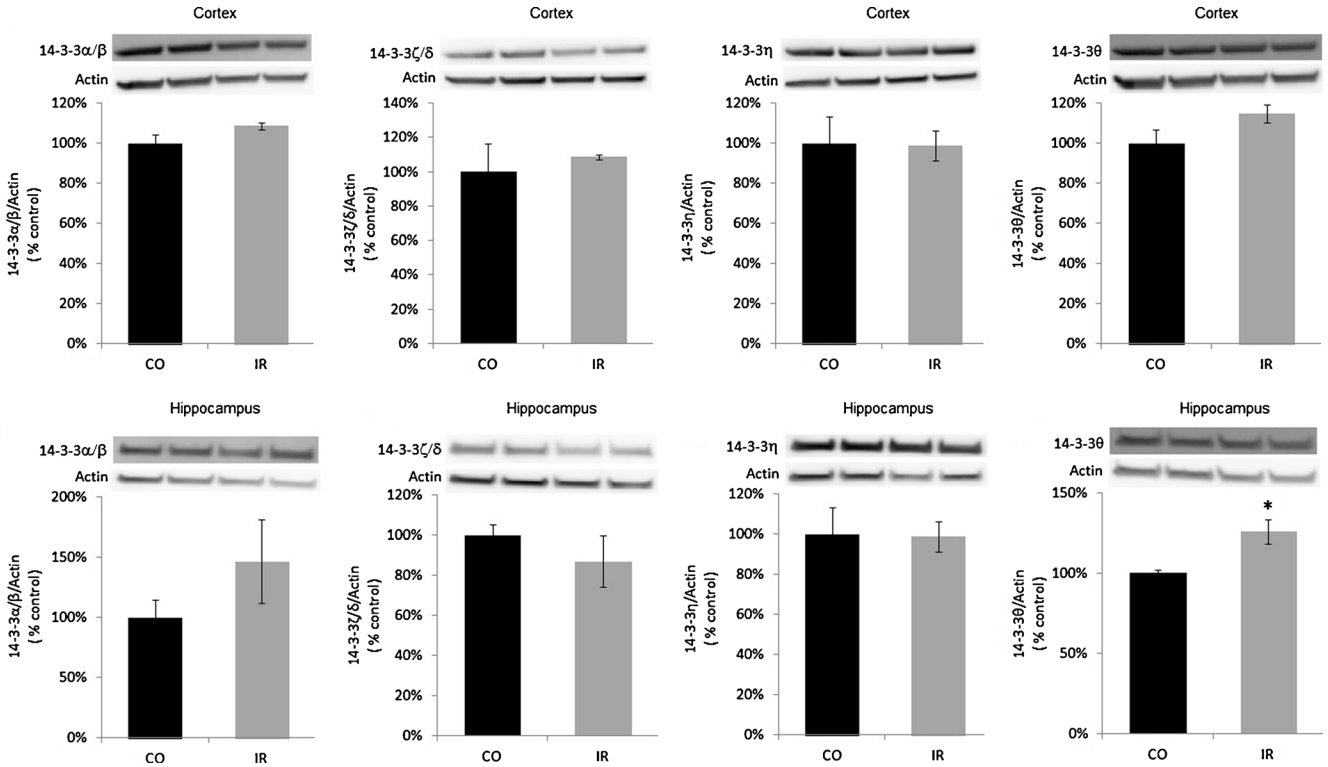

Fig. 6 Western blot analysis of 14-3-3 isoforms. Immunoreactive content was quantified by scanning densitometry, and density of each 14-3-3 antibody was normalized by actin content, in order to correct variations in protein loading. Upper panels represent results from the cortex, and lower panels represent results in the hippocampus. Results are presented as mean±SD (n=4). Means were compared using Student t test; *p<0.05

mTOR pathway is deregulated in diabetes, and a decrease of this isoform in insulin resistance state may be a consequence of the reduced signaling of insulin in the hippocampus that might be leading to a decreased inhibition of mTOR signaling [36].

We have also seen alterations in 14-3-3 ζ/δ isoforms in the proteome analysis. In cortex IR group, 3 counts in 14-3-3 ζ/δ isoforms were identified in peptide spectral while none were found in the CO. A small difference in peptide spectral count was also shown in the hippocampus, which increased from 4 to 5 counts when insulin resistance was induced. Interestingly, mRNA levels and immune content (WB) of this isoform remained unchanged. A recent study suggests that increasing 14-3-3 ζ/δ levels or activity could be a novel approach to the prevention of beta cell death that occurs in diabetes [37]. Other studies correlated 14-3-3 ζ/δ with IRS1 and IRS2 and with protein kinase B (Akt), but not in brain structures [7, 38]. IRS1, IRS2, and Akt are essential proteins in insulin pathway and may have a close interaction with 14-3-3 ζ/δ due to upregulation of this protein in insulin-resistant state.

mRNA levels of 14-3-3 η isoform were demonstrated to be significantly lower in cortex IR group. This isoform was previously shown to be important in long-term potentiation (LTP), more specifically as a downstream component of the pathway involved in presynaptic LTP [39]. Furthermore, 14-3-3 η has been associated with psychotic bipolar disorder [40]. In our study, despite no alterations in peptide and protein levels were present, we have demonstrated negative correlation of blood glucose levels and mRNA levels of this isoform. Therefore, we believe that this isoform can be involved in a fine tune regulation in the insulin pathway taking into account that variation was only found in mRNA levels in the cortex while no significant alteration was shown in the hippocampus.

mRNA levels of 14-3-3 θ isoform were shown to be downregulated in the present study. This isoform is involved in apoptosis and cell proliferation and promotes assembly and stability of microtubules. In addition, a protective effect in Parkinson disease has been demonstrated [41], and decrease observed here might be responsible for an increase in α-synuclein inclusion formation. It has been shown that overexpression of 14-3-3 θ protects against dopaminergic cell loss in a α-synuclein transgenic C. elegans model [42]. We demonstrate here a decrease in mRNA levels of Ywhaq only in the cortex, which is negatively correlated to AUC of blood

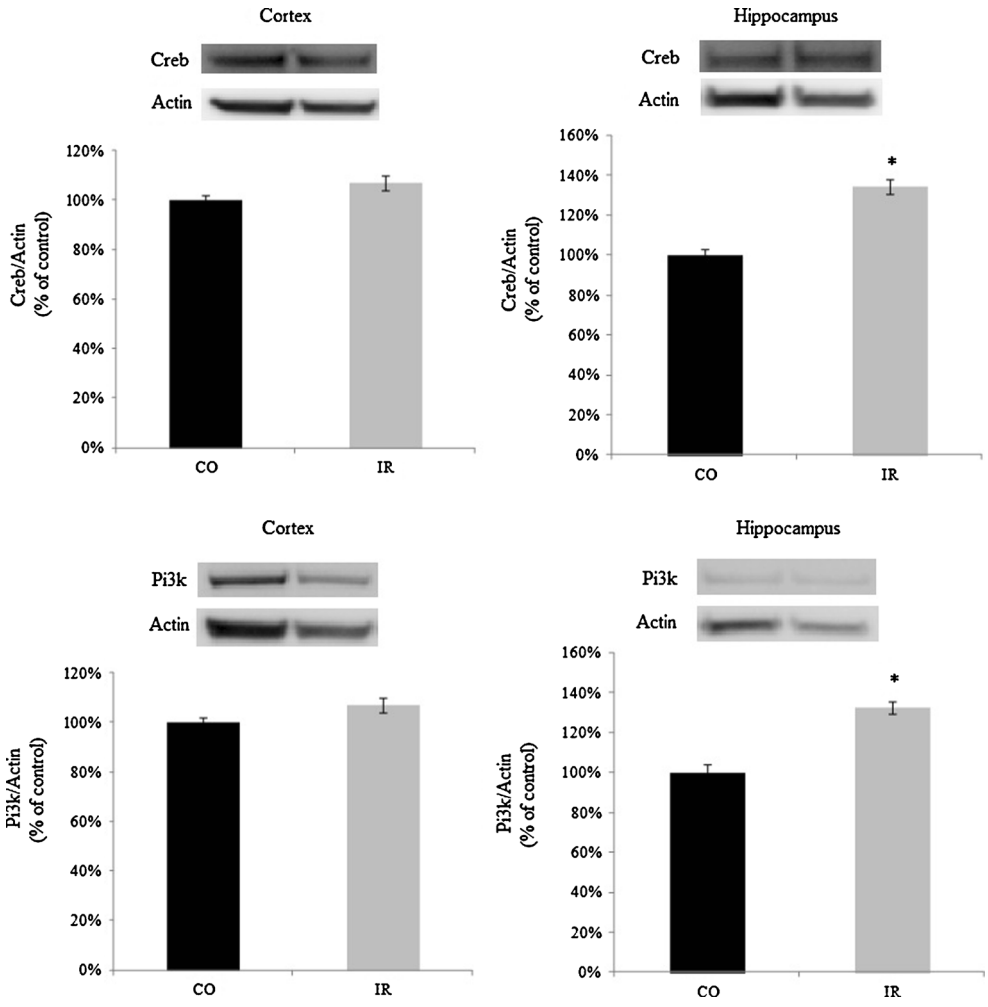

Fig. 7 Western blot analysis of Creb and PI3K in the cortex and hippocampus. Immunoreactive content was quantified by scanning densitometry, and density of each Creb (*upper set*) and PI3K (*lower set*) antibody was normalized by actin content, in order to correct variations in protein loading. Results are presented as mean±SD (n=4). Means were compared using Student t test; *p<0.05

glucose levels. Others studies have also shown downregulation in the cortex in association to bipolar disorder and schizophrenia [41]. Further, we have also seen an increase of almost 19 % in Ywhaq levels in the hippocampus, although no statistically significant difference was detected. Moreover, levels of 14-3-3 θ isoform detected by WB were significantly higher in hippocampus IR group. Involvement of 14-3-3 θ in the signaling pathway of growth factors, such as insulin, by stimulating the PI3K-Akt signal has been shown [43]. We then propose that insulin-resistant state may injure neurons, and these changes are an attempt to protect hippocampus by increasing 14-3-3 θ mRNA levels due to its protective effect. This data suggest that 14-3-3 θ isoform can play a key role in the insulin signaling pathway.

A recent work evaluated mRNA and protein levels of 14-3-3 proteins in a streptozotocin rat model of early DM1. Although they have assessed all forebrain and the model was of acute DM1, some results are in agreement with those presented in this work that show a decrease in mRNA levels of 14-3-3 3 η and θ and unchanged mRNA and protein levels of other isoforms. They demonstrated a decrease in protein levels of 14-3-3 θ in the brain cytosol while we observed an increase in protein levels of 14-3-3 θ in the hippocampus [22].

mRNA concentrations had represented concentrations and activities of the corresponding proteins, thereby assuming that transcript abundances are the main determinant of protein abundances. However, we observed a decrease in mRNA levels of 14-3-3 η and θ in the cortex and an increase in protein levels of 14-3-3 θ in the hippocampus. We also need to consider that when dealing with four isoforms and two distinct brain regions, it is plausible to expect differences in the molecular levels dependent on the isoform or the brain region. Insulin-resistant state can produce different responses in different structures, at different molecular levels, and in different molecules. There are many regulatory processes between transcription and translation, and protein stability is also an important issue. Data from transcription level can indicate whether the protein is present or not and, roughly, the levels of this protein. So, transcription data is useful for identifying potential candidates for follow-up at the protein level. However, changes in gene expression do not necessarily reflect alterations in protein level [44]. On the other hand, the discrepancies between mRNA and protein expression could be partially explained by differential tissue sensitivity pattern caused by insulin resistance.

We also evaluated the protein levels of Creb and PI3K by WB. Interestingly, the immunocontent of Creb and PI3K were increased in the hippocampus, but not in the cortex. This may be due to a compensatory effect of impaired insulin signaling in the structure. Creb is not only important in the direct transcriptional activation of gluconeogenic genes but also responsible for the progression of hepatic insulin resistance in the diet-induced or the genetic mouse models of obesity [45]. Despite the importance of Creb in diverse tissues, our study shows that Creb is also important in the CNS insulin-resistant state, mainly in the hippocampus. In turn, an increase of Creb levels seems to be essential to maintain CNS insulinergic pathway. Thus, further studies are necessary to delineate the diverse roles of Creb in the brain insulin resistance.

14-3-3 proteins have a number of binding partners involved in regulation of important basal functions in all cell types. The relevance of 14-3-3 proteins in the nervous tissue is based on the relevance of certain processes for proper neuronal development and function, such as synapse formation, and neuronal plasticity and development [46].

Improving brain insulin sensitivity might be a strategy for preventing or treating neurodegenerative disorders that correlate with brain insulin resistance [47]. Thus, drugs designed to increase the peripheral insulin sensitivity may potentially have similar effects in the brain; however, few studies have evaluated this hypothesis. In addition, 14-3-3 proteins are very important to many normal neurological functions as well as in neurological disorders. Connection between altered 14-3-3/ligand interaction and neural diseases suggests a pathological role for 14-3-3 proteins. It remains to be demonstrated alterations of specific isoforms in specific disease and/or injuries. However, results presented here provide further evidences to the involvement of 14-3-3 isoforms in the insulin signaling pathway.

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References

1. Wang W, Shakes DC (1996) Molecular evolution of the 14-3-3 protein family. J Mol Evol 43:384–398
2. Ferl RJ, Manak MS, Reyes MF (2002) The 14-3-3s. Genome Biol 3(7):3010.1–3010.7
3. Fu H, Subramanian RR, Masters SC (2000) 14-3-3 proteins: structure, function, and regulation. Annu Rev Pharmacol Toxicol 40:617–647
4. Obsil T, Obsilova V (2011) Structural basis of 14-3-3 protein functions. Semin Cell Dev Biol 22(7):663–672
5. Pozuelo Rubio M, Geraghty KM, Wong BH, Wood NT, Campbell DG, Morrice N, Mackintosh C (2004) 14-3-3-affinity purification of over 200 human phosphoproteins reveals new links to regulation of cellular metabolism, proliferation and trafficking. Biochem J 379: 395–408
6. Kleppe R, Martinez A, Døskeland SO, Haavik J (2011) The 14-3-3 proteins in regulation of cellular metabolism. Semin Cell Dev Biol 22:713–719
7. Ogihara T, Isobe T, Ichimura T, Taoka M, Funaki M, Sakoda H, Onishi Y, Inukai K, Anai M, Fukushima Y, Kikuchi M, Yazaki Y, Oka Y, Asano T (1997) 14-3-3 protein binds to insulin receptor substrate-1, one of the binding sites of which is in the phosphotyrosine binding domain. J Biol Chem 272:25267–25274

8. Neukamm SS, Ott J, Dammeier S, Lehmann R, Häring HU, Schleicher E, Weigert C (2013) Phosphorylation of serine 1137/1138 of mouse insulin receptor substrate (IRS) 2 regulates cAMP-dependent binding to 14-3-3 proteins and IRS2 protein degradation. J Biol Chem 288(23):16403–16415
9. Agarwal-Mawal A, Qureshi HY, Cafferty PW, Yuan Z, Han D, Lin R, Paudel HK (2003) 14-3-3 connects glycogen synthase kinase-3 beta to tau within a brain microtubule-associated tau phosphorylation complex. J Biol Chem 278(15):12722–12728
10. Dubois F, Vandermoere F, Gernez A, Murphy J, Toth R, Chen S, Geraghty KM, Morrice NA, MacKintosh C (2009) Differential 14-3-3 affinity capture reveals new downstream targets of phosphatidylinositol 3-kinase signaling. Mol Cell Proteomics 8(11):2487–2499
11. Muller AP, Gnoatto J, Moreira JD, Zimmer ER, Haas CB, Lulhier F, Perry ML, Souza DO, Torres-Aleman I, Portela LV (2011) Exercise increases insulin signaling in the hippocampus: physiological effects and pharmacological impact of intracerebroventricular insulin administration in mice. Hippocampus 21(10):1082–1092
12. Banks WA, Owen JB, Erickson MA (2012) Insulin in the brain: there and back again. Pharmacol Ther 136(1):82–93
13. Grillo CA, Piroli GG, Kaigler KF, Wilson SP, Wilson MA, Reagan LP (2011) Downregulation of hypothalamic insulin receptor expression elicits depressive-like behavior in rats. Behav Brain Res 222:230–235
14. Chung YH, Shin CM, Joo KM, Kim MJ, Cha CI (2002) Region-specific alterations in insulin-like growth factor receptor type I in the cerebral cortex and hippocampus of aged rats. Brain Res 946:307–313
15. Bosco D, Fava A, Plastino M, Montalcini T, Pujia A (2011) Possible implications of insulin resistance and glucose metabolism in Alzheimer's disease pathogenesis. J Cell Mol Med 15:1807–1821
16. Luschsinger JA, Tang MX, Shea S, Mayeux R (2004) Hyperinsulinemia and the risk of Alzheimer's disease. Neurology 63:1187–1192
17. Cole A, Astell A, Green C, Sutherland C (2007) Molecular connections between dementia and diabetes. Neurosci Biobehav Rev 31:1046–1063
18. Cukierman T, Gerstein HC, Williamson JD (2005) Cognitive decline and dementia in diabetes-systematic overview of prospective observational studies. Diabetologia 48:2460–2469
19. Williamson R, McNeilly A, Sutherland C (2012) Insulin resistance in the brain: an old-age or new-age problem? Biochem Pharmacol 84(6):737–745
20. Larance M, Rowland AF, Hoehn KL, Humphreys DT, Preiss T, Guilhaus M, James DE (2010) Global phosphoproteomics identifies a major role for AKT and 14-3-3 in regulating EDC3. Mol Cell Proteomics 9(4):682–694
21. Chen S, Synowsky S, Tinti M, MacKintosh C (2011) The capture of phosphoproteins by 14-3-3 proteins mediates actions of insulin. Trends Endocrinol Metab 22(11):429–436
22. Taurino F, Stanca E, Vonghia L, Siculella L, Sardanelli AM, Papa S, Zanotti F, Gnoni A (2014) Short-term type-1 diabetes differentially modulates 14-3-3 proteins in rat brain and liver. Eur J Clin Investig 44(4):350–358
23. Talbot K, Wang HY, Kazi H, Han LY, Bakshi KP, Stucky A, Fuino RL, Kawaguchi KR, Samoyedny AJ, Wilson RS, Arvanitakis Z, Schneider JA, Wolf BA, Bennett DA, Trojanowski JQ, Arnold SE (2012) Demonstrated brain insulin resistance in Alzheimer's disease patients is associated with IGF-1 resistance, IRS-1 dysregulation, and cognitive decline. J Clin Invest 122:1316–1338
24. Kern W, Peters A, Fruehwald-Schultes B, Deininger E, Born J, Fehm HL (2001) Improving influence of insulin on cognitive functions in humans. Neuroendocrinology 74:270–280
25. Bigornia SJ1, Farb MG, Mott MM, Hess DT, Carmine B, Fiscale A, Joseph L, Apovian CM, Gokce N (2012) Relation of depot-specific adipose inflammation to insulin resistance in human obesity. Nutr Diabetes 2((3):e30
26. Spielman LJ, Little JP, Klegeris A (2014) Inflammation and insulin/IGF-1 resistance as the possible link between obesity and neurodegeneration. J Neuroimmunol 273(1):8–21
27. Dietrich MO, Muller A, Bolos M, Carro E, Perry ML, Portela LV, Souza DO, Torres-Aleman I (2007) Western style diet impairs entrance of blood-borne insulin-like growth factor-1 into the brain. Neruomol Med 9(4):324–330
28. Schwartz MW, Porte D Jr (2005) Diabetes, obesity, and the brain. Science 307(5708):375–379
29. de Sousa AR, Penalva LO, Marcotte E, Vogel C (2009) Global signatures of protein and mRNA expression levels. Mol BioSyst 5:1512–1526
30. Liu H, Sadygov RG, Yates JR 3rd (2004) A model for random sampling and estimation of relative protein abundance in shotgun proteomics. Anal Chem 76(14):4193–4201
31. Fu X, Gharib SA, Green PS, Aitken ML, Frazer DA, Park DR, Vaisar T, Heinecke JW (2008) Spectral index for assessment of differential protein levels in shotgun proteomics. J Proteome Res 7(3):845–854
32. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. Methods 25(4):402–408
33. Aitken A (2006) 14-3-3 proteins: a historic overview. Semin Cancer Biol 16:162–172
34. Shumway SD, Li Y, Xiong Y (2003) 14-3-3-beta binds to and negatively regulates the tuberous sclerosis complex 2 (TSC2) tumor suppressor gene product, tuberin. J Biol Chem 278:2089–2092
35. Inoki K, Li Y, Zhu T, Wu J, Guan K-L (2002) TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol 4:648–657
36. Zoncu R, Efeyan A, Sabatini DM (2011) mTOR: from growth signal integration to cancer, diabetes and ageing. Nat Rev Mol Cell Biol 12(1):21–35
37. Lim GE, Piske M, Johnson JD (2013) 14-3-3 proteins are essential signalling hubs for beta cell survival. Diabetologia 56(4):825–837
38. Powell DW, Rane MJ, Chen Q, Singh S, McLeish KR (2002) Identification of 14-3-3-zeta as a protein kinase B/Akt substrate. J Biol Chem 277:21639–21642
39. Simsek-Duran F, Linden DJ, Lonart G (2004) Adapter protein 14-3-3 is required for a pre-synaptic form of LTP in the cerebellum. Nat Neurosci 7:1296–1298
40. Grover D, Verma R, Goes FS, Mahon PL, Gershon ES, McMahon FJ (2009) Family based association of YWHAH in psychotic bipolar disorder. Am J Med Genet B Neuropsychiatr Genet 150B:977–983
41. Foote M, Zhou Y (2012) 14-3-3 proteins in neurological disorders. Int J Biochem Mol Biol 3(2):152–164
42. Yacoubian TA, Slone SR, Harrington AJ, Hamamichi S, Schieltz JM, Caldwell KA, Standaert DG (2010) Differential neuroprotective effects of 14-3-3 proteins in models of Parkinson's disease. Cell Death Differ 1(e2):1–13
43. Kakinuma N, Roy BC, Zhu Y, Wang Y, Kiyama R (2008) Kank regulates RhoA-dependent formation of actin stress fibers and cell migration via 14-3-3 in PI3K-Akt signaling. J Cell Biol 181(3):537–549
44. Vogel C, Marcotte EM (2012) Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. Nat Rev Genet 13(4):227–232
45. Jitrapakdee S (2012) Transcription factors and coactivators controlling nutrient and hormonal regulation of hepatic gluconeogenesis. Int J Biochem Cell Biol 44(1):33–45
46. Steinacker P, Aitken A, Otto M (2011) 14-3-3 proteins in neurodegeneration. Semin Cell Dev Biol 22:696–704
47. Park CR (2001) Cognitive effects of insulin in the central nervous system. Neurosci Biobehav Rev 25:311–323

CAPÍTULO III

Artigo a ser submetido ao periódico Journal of Neurochemistry

Expression of N-Methyl-D-Aspartate (NMDA) Glutamate Receptor Subunits Genes in Cortex and Hippocampus of Rats Submitted to High Palatable Diet

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Running title: NMDA Glutamate Receptor Subunits Genes Expression levels.

Key words: Glutamate, NMDA, high palatable diet, insulin resistance, NMDA receptor, mRNA expression, rat brain, real time PCR.

Abbreviations

Glu – glutamate

GluR – glutamate receptor

Grin – glutamate receptor, ionotropic, N-methyl-D-aspartate

Gapdh – glyceraldehyde-3-phosphate dehydrogenase

Abstract

N-methyl-D-aspartate receptor (NMDAR) constitutes a subfamily identified by specific molecular composition and unique pharmacological and functional properties. NMDAR subtypes differ in their sensitivity to ligands as well as interaction with intracellular signaling proteins. The aim of the present study was to evaluate expression levels of NMDAR subunits in cortex and hippocampus of rats. Adult male Wistar rats were divided into 2 groups (n = 4 each): insulin resistant group, fed with high palatable diet for 130 days, and control group, fed with regular diet. mRNA levels were measured by quantitative real-time PCR (qPCR) using gene-specific TaqMan® FAM/MGB inventoried assays normalized by glyceraldehyde-3-phosphate dehydrogenase (Gapdh) as endogenous control. We observed downregulation of Grin1, Grin2A, and Grin3A in cortex and upregulation of Grin2B in hippocampus. We showed in this study the effects of high palatable diet at the molecular level. Impaired brain insulin signaling modulates mRNA levels of NMDAR subunits in cortex and hippocampus. Results presented here bring novel insights for a better elucidation of connection between insulin signaling pathway and NMDAR activation in the CNS.

1 Introduction

Glutamate (Glu) is considered the most abundant excitatory neurotransmitter of the CNS, and is involved in important physiological functions such as learning/memory processes and neurodevelopment (Debane *et al*., 2003; Genoux and Montgomery, 2007). However, Glu may also acts as a potent neurotoxin that leads to neural death observed in several acute and chronic CNS disorders (Dingledine *et al*., 1999) including traumatic brain injury and ischemic stroke as well as Parkinson and Alzheimer diseases (Maragakis and Rothstein, 2004; Sheldon and Robinson, 2007).

Glu can acts on two distinct classes of glutamate receptors (GluR), metabotropic and ionotropic, when released into the synaptic cleft (Simeone *et al*., 2004). N-methyl-D-aspartate receptor (NMDAR) constitutes a subfamily identified by specific molecular composition and unique pharmacological and functional properties within the family of excitatory ionotropic glutamate receptors. Of particular interest is the high permeability to calcium ions, which confers a central role to both synaptic plasticity under physiological conditions and neuronal death under excitotoxic pathological conditions (Dingledine *et al*., 1999; Meldrum, 2000).

Functional NMDARs are tetramers composed by different subunits. Typically, endogenous NMDARs are di-heteromers comprising two ubiquitously expressed NMDAR1 subunit and two out of four distinct NMDAR2 (A, B, C, D) or out of two NMDAR3 (A, B), which assemble as a dimmer of dimmers (Sanz-Clemente *et al*., 2013). They are codified by the glutamate receptor, ionotropic, N-methyl-D-aspartate (Grin) gene family; Grin1, Grin2A, Grin2B, Grin2C, Grin2D, Grin3A, and Grin3B (Monyer *et al*., 1992). For instance, these

NMDAR subtypes differ in their sensitivity to ligands, permeation and blockage by divalent ions as well as interaction with intracellular signaling proteins (Cull-Candy *et al*., 2001).

A number of endogenous substances, mainly those that are key players in metabolic processes such as glucose and insulin, have been known to have a direct effect on memory enhancing. Recent studies suggest that insulin may provide memory enhancement also in humans. In addition, some of those studies indicate that enhancing effects of glucose may be entirely due to an increase of insulin levels stimulated by glucose (Stern and Alberini, 2013; Benedict *et al*., 2004).

CNS integrates peripheral signals and progressively adapts to changes in order to maintain energy balance (Morton *et al*., 2006). Synaptic vesicle release (Zhang *et al*., 2011) and continual plasticity in feeding circuits may be a key component in energy balance control (Dietrich and Horvath, 2009). Detailed understanding of intracellular signaling cascades of hormones has begun to accumulate, and these studies indicate that leptin, insulin, and ghrelin play important roles in synaptic functions (Gao and Horvath, 2007). Involvement of synaptic regulation in feeding behavior has been reveled by cellular and circuitry analysis, and highlights the relevance of investigating the effects of hormones on synaptic transmission for the understanding of how CNS controls energy homoeostasis (Pang and Han, 2012).

Circulating insulin can penetrate the blood–brain barrier and binds to insulin receptors to regulate glucose levels and energy balance (Banks, 2006). Defective insulin signaling in the CNS contributes to obesity and diabetes mellitus type 2. Several epidemiological studies suggest that insulin resistance, along with chronic inflammation, may be underlying links between diabetes and dementia as well as neurodegeneration (Takeda *et al*., 2010).

As a whole, the expression of NMDAR subunits can vary in neurological diseases as well as during development. To date, the transcriptional pattern of expression of different subunits in brain structures of rats submitted to high palatable diet was not evaluated yet. Here we compare NMDAR subunits gene transcriptional expression in cortex and hippocampus of rats submitted to high palatable diet and regular diet.

2 Experimental Procedures

2.1 Animals and Diet

Eight (n = 8) adult male Wistar rats were housed under controlled temperature (22 ± 2 °C) and humidity (55 ± 10 %) conditions on a 12 h light-dark cycle (lights on at 7 am), with food and water offered *ad libitum*. All experiments performed were in agreement with international standards and according to of the Brazilian College of Animal Experimentation for animal protection. The Ethical Committee on animal use of the Universidade Federal do Rio Grande do Sul, Brazil, approved all experiments (number 19446). When animals were 2 months-old (ranging between 200-250 g of weight), rats were divided into 2 experimental groups: control (CO) and insulin resistant (IR). High palatable diet (also known as *cafeteria diet* or *Western Style Diet*) was used to induce insulin resistance in IR group for 130 days (Dietrich *et al*. 2007; Schwartz and Porte, 2005). CO group was fed with a standard laboratory diet during the same period.

After 130 days, a glucose tolerance test was performed. Rats were deprived from food overnight (8 h) and blood samples were taken by a small puncture on the tail. Rats were then decapitated, cortex and hippocampus were rapidly dissected, submerged in RNAlater® (Ambion, USA), and stored at –20ºC for mRNA analysis.

2.2 RNA extraction and cDNA synthesis

Total RNA was extracted using TRIreagent® solution (Ambion, USA) according to manufacturer's protocol. RNA concentration was estimated using the fluorimetric method Quant-iT® RNA Assay (Invitrogen, USA) in the Qubit™ (Invitrogen, USA) equipment. cDNA was synthesized by reverse transcription (RT) reaction using High Capacity cDNA

Reverse Transcription Kit (Applied Biosystems, USA). Two hundred ng of total RNA was placed in a total reaction volume of 20 µl containing 1x RT buffer, 4 mM of each dNTP, 1x RT Random Primers, and 0,05 units of MultiScribe™ Reverse Transcriptase. RT reaction was performed as follows: 10 min at 25 ºC, 2 h at 37 ºC and 5 s at 85 ºC. Subsequently, cDNA was kept at – 20 ºC until further use.

2.3 Quantitative real-time PCR (qPCR)

mRNA levels were measured by quantitative real-time PCR (qPCR) using gene-specific TaqMan® FAM/MGB inventoried assays (Grin1, Rn01436038_m1; Grin2A, Rn00561341_m1; Grin2B, Rn00561352_m1; Grin2C, Rn00561364_m1; Grin2D, Rn00575638_m1; Grin3A, Rn_01448553_m1; Grin3B, Rn_00591133_m1; Applied Biosystems, USA). Expression values of the target gene were normalized using glyceraldehyde-3-phosphate dehydrogenase (Gapdh) as endogenous control (inventoried assay Rn99999916_s1, Applied Biosystems, USA).

cDNA solution was diluted 1:5 in water before qPCR. Reaction was carried out in a total volume of 8.0 µl containing 1 µl of diluted cDNA solution, 1x of gene specific TaqMan® assay, 1x of endogenous control TaqMan® assay and 1x PCR Master Mix (Applied Biosystems, USA). Cycling program was 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Reactions were performed in triplicate in an ABI Prism 7500 Fast Sequence Detector System (Applied Biosystems, USA). Transcriptional profile of each NMDAR subunit was determined in both brain structures. Relative mRNA levels were calculated by the ΔΔCt method according to Livak and Schmittgen (2001), using Gapdh as endogenous control and the control group of each structure as calibrator.

2.4 Statistical analysis

Statistical analyses were performed using student t-test. The differences were considered statistically significant when $p < 0.05$.

3 Results

A decrease in mRNA levels of NMDAR subunits in the cortex was observed when CO group and IR group were compared. Statistically significant lower levels were observed in IR group of Grin1 (p = 0.0437), Grin2A (p = 0.0223), and Grin3A (p = 0.0002) (Figure 1).

In hippocampus, Grin2B was the unique subunit up regulated (p = 0.0437). Other subunits in this structure do not show any significant difference in mRNA levels between the two groups (Figure 2). We were not able to evaluate mRNA levels of Grin3B in the present study due to undetectable levels.

4 Discussion

Despite structural similarity of NMDAR subunits in cortex and hippocampus of rats, there are dramatic functional differences among NMDAR subunits. In a variety of neurological disorders, neuronal damage is associated to excitotoxicity induced by overactivation of NMDAR as a consequence of the release of high Glu levels into the extracellular space (Lipton and Rosenberg, 1994; Nishizawa, 2001). However, NMDAR activation has been reported to produce either neuronal survival and synaptic plasticity or death-promoting actions, and it has been claimed that this dual action is mediated by receptor subunit composition (Liu *et al*., 2007).

Neuron survival and synaptic plasticity cannot be viewed as two independent parallel processes. These two insulin-dependent processes are intertwined by the PI3K-pathway. Therefore, signals acting on neuronal survival should alter synaptic plasticity, and synaptic activity should alter neuronal survival. It is known that insulin modulates hippocampal activity-dependent synaptic plasticity, facilitating long-term potentiation (LTP) and long-term (LTD) induction, in a NMDAR dependent manner. This provides a mechanistic link between insulin and synaptic plasticity, and explains insulin function as a neuromodulator. In addition, those facts suggest that both NMDA receptor and insulin signaling are required for changes in synaptic plasticity to occur (van der Heide *et al*., 2005). Thus, the functionality and expression of NMDAR seem to be closely correlated. Different brain areas may have distinct levels of expression and function as well as vulnerability to insults, even considering the brain as an integrated system (Vizi, 2000).

We demonstrated here effects of high palatable diet at the molecular level. Impaired brain insulin signaling modulates mRNA levels of NMDAR subunits in cortex and

hippocampus. Upregulation of Grin2B was observed in hippocampus, and this was the unique subunit that showed statistically different expression levels in this brain area. It is likely that this involves calcium-calmodulin-dependent kinase II (CaMKII), which is also increased in this condition (unpublished data). Previous report shows that CaMKII binds to Grin2A *in vitro*. However, that interaction is much weaker than the well-documented association between CaMKII and Grin2B (Bayer *et al*., 2001). Calcium entry through NMDARs activates CaMKII that can then bind to NMDAR2B. This association is presumed to be an important requirement for LTP maintenance, since disrupting this interaction reverses the potentiation (Sanhueza *et al*., 2011).

Grin2B expression tends to decrease with age, being the major subunit at immature synapses while Grin2A predominates in mature synapses (Flint *et al*., 1997). Therefore, disruption of Grin2A and Grin2B pattern of expression may have detrimental effects. In this study, we observe an abnormal state that modulates the expression in an opposite direction. The insulin resistant state was associated to an increase in mRNA levels of Grin2B in hippocampus.

In cortex, we observe lower mRNA levels of Grin1, Grin2A, and Grin3A in IR group. Taking all together, that general reduced mRNA expression of NMDAR subunits in cortex indicate that impaired insulin signaling may affect this structure more severely than hippocampus, in which most of NMDAR subunits levels remains unchanged, except higher expression levels of Grin2B.

Results presented here bring novel insights for a better elucidation of connection between insulin signaling pathway and NMDAR activation in the CNS. However, we are aware that further studies are required to assemble a complete comprehension of the whole

process, mainly carrying out studies at protein level. This knowledge in different conditions such as diabetes mellitus type 2 is even more relevant to guide new therapies.

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6 References

Banks, W.A., 2006. Blood-brain barrier and energy balance. Obesity. 14 (Suppl. 5), 234S–237S.

Bayer, K.U., De Koninck, P, Leonard, A.S., Hell, J.W. and Schulman, H., 2001. Interaction with the NMDA receptor locks CaMKII in an active conformation. Nature 411(6839), 801–805.

Benedict, C., Hallschmid, M., Hatke, A., Schultes, B., Fehm, H.L., Born, J. and Kern, W., 2004. Intranasal insulin improves memory in humans. Psychoneuroendocrinology. 29, 1326–1334.

Choi, D.W., 1988. Glutamate neurotoxicity and diseases of the nervous system. Neuron, 1, 623-634.

Conn, P.J. and Pin, J.P., 1997. Pharmacology and functions of metabotropic glutamate receptors. Annu. Rev. Pharmacol. Toxicol. 37, 205-237.

Cull-Candy, S., Brickley, S. and Farrant, M., 2001. NMDA receptor subunits: diversity, development and disease. Curr. Opin. Neurobiol. 11, 327–335.

Danbolt, N.C., 2001. Glutamate uptake. Prog. Neurobiol. 65, 1-105.

Debanne, D., Daoudal, G., Sourdet, V. and Russier, M., 2003. Brain plasticity and ion channels. J. Physiol. Paris. 97, 403-414.

Dietrich, M.O., Muller, A., Bolos, M., Carro, E., Perry, M.L., Portela, L.V., Souza, D.O. and Torres-Aleman, I., 2007. Western style diet impairs entrance of blood-borne insulin-like growth factor-1 into the brain. Neuromolecular Med. 9(4), 324-330.

Dietrich, M.O. and Horvath, T.L., 2009. Feeding signals and brain circuitry. Eur. J. Neurosci. 30, 1688–1696.

Dingledine, R., Borges, K., Bowie, D. and Traynelis, S.F., 1999. The glutamate receptor ion channels. Pharmacol. Rev. 51, 7-61.

Flint, A.C., Maisch, U.S., Weishaupt, J.H., Kriegstein, A.R. and Monyer, H., 1997. NR2A subunit expression shortens NMDA receptor synaptic currents in developing neocortex. J. Neurosci. 17, 2469–2476.

Gao, Q. and Horvath, T.L., 2007. Neurobiology of feeding and energy expenditure. Annu. Rev. Neurosci. 30, 367–398.

Genoux, D. and Montgomery, J.M., 2007. Glutamate receptor plasticity at excitatory sinapses in the brain. Clin. Exp. Pharmacol. Physiol. 34, 1058-1063.

Lipton, S.A. and Rosenberg, P.A., 1994. Excitatory amino acids as a final common pathway for neurologic disorders. N. Engl. J. Med. 330, 613-622.

Liu, Y., Wong, T.P., Aarts, M., Rooyakkers, A., Liu, L., Lai, T.W., Wu, D.C., Lu, J., Tymianski, M., Craig, A.M. and Wang, Y.T., 2007. NMDA receptor subunits have differential roles in mediating excitotoxic neuronal death both in vitro and in vivo. J. Neurosci. 27, 2846–2857.

Lynch, D.R., Anegawa, N.J., Verdoorn, T. and Pritchett, D.B., 1994. N-methyl-D-aspartate receptors: different subunit requirements for binding of glutamate antagonists, glycine antagonists, and channel-blocking agents. Mol. Pharmacol. 45, 540-545.

Maragakis, N.J. and Rothstein, J.D., 2004. Glutamate transporters: animal models to neurologic disease. Neurobiol. Dis. 15, 461-473.

Meldrum, B.S., 2000. Glutamate as a neurotransmitter in the brain: review of physiology and pathology. J. Nutr. 130, 1007S-1015S.

Monyer, H., Sprengel, R., Schoepfer, R., Herb, A., Higuchi, M., Lomeli, H., Burnashev, N., Sakmann, B. and Seeburg, P.H., 1992. Heteromeric NMDA receptors: molecular and functional distinction of subtypes. Science. 256, 1217-1221.

Morton, G.J., Cummings, D.E., Baskin, D.G., Barsh, G.S. and Schwartz, M.W., 2006. Central nervous system control of food intake and body weight. Nature. 443, 289–295.

Nishizawa, Y., 2001. Glutamate release and neuronal damage in ischemia. Life Sci. 69, 369-381.

Ozawa, S., Kamiya, H. and Tsuzuki, K., 1998. Glutamate receptors in the mammalian central nervous system. Prog. Neurobiol. 54, 581-618.

Pang, Z.P. and Han, W., 2012. Regulation of synaptic functions in central nervous system by endocrine hormones and the maintenance of energy homoeostasis. Biosci. Rep. 32(5), 423-432.

Pérez-Otaño, I., Schulteis, C.T., Contractor, A., Lipton, S.A., Trimmer, J.S., Sucher, N.J. and Heinemann, S.F., 2001. Assembly with the NR1 subunit is required for surface expression of NR3A-containing NMDA receptors. J. Neurosci. 21, 1228-1237.

Quinlan, E.M., Philpot, B.D., Huganir, R.L. and Bear, M.F., 1999. Rapid, experience-dependent expression of synaptic NMDA receptors in visual cortex in vivo. Nat. Neurosci. 2, 352–357.

Sanhueza, M., Fernandez-Villalobos, G., Stein, I.S., Kasumova, G., Zhang, P., Bayer, K.U., Otmakhov, N., Hell, J.W. and Lisman, J., 2011. Role of the CaMKII/NMDA receptor complex in the maintenance of synaptic strength. J. Neurosci. 31(25), 9170–9178.

Sanz-Clemente, A., Nicoll, R.A. and Roche, K.W., 2013. Diversity in NMDA receptor composition: many regulators, many consequences. Neuroscientist. 19(1), 62-75.

Schwartz, M.W. and Porte, D., 2005. Diabetes, obesity, and the brain. Science. 307(5708), 375-379.

Segovia, G., Porras, A., Del Arco, A. and Mora, F., 2001. Glutamatergic neurotransmission in aging: a critical perspective. Mech. Ageing. Dev. 122, 1-29.

Sheldon, A.L. and Robinson, M.B., 2007. The role of glutamate transporters in neurodegenerative diseases and potential opportunities for intervention. Neurochem. Int. 51, 333-355.

Simeone, T.A., Sanchez, R.M. and Rho, J.M., 2004. Molecular biology and ontogeny of glutamate receptors in the mammalian central nervous system. J. Child. Neurol. 19, 343-360.

Stern, S.A. and Alberini, C.M., 2013. Mechanisms of memory enhancement.Wiley Interdiscip. Rev. Syst. Biol. Med. 5(1), 37-53.

Takeda, S., Sato, N., Uchio-Yamada, K., Sawada, K., Kunieda, T., Takeuchi, D., Kurinami, H., Shinohara, M., Rakugi, H. and Morishita, R., 2010. Diabetes-accelerated memory dysfunction via cerebrovascular inflammation and Abeta deposition in an Alzheimer mouse model with diabetes. Proc. Natl. Acad. Sci. U.S.A. 107, 7036–7041.

van der Heide, L.P., Jacobs, F.M., Burbach, J.P., Hoekman, M.F. and Smidt, M.P., 2005. FoxO6 transcriptional activity is regulated by Thr26 and Serl84, independent of nucleo-cytoplasmic shuttling. Biochem. J. 391, 623–629.

Vizi, E.S., 2000. Role of high affinity receptors and membrane transporters in nonsynaptic communication and drug action in the central nervous system. Pharmacol. Rev. 52, 63–89.

Zhang, G., Bai, H., Zhang, H., Dean, C., Wu, Q., Li, J., Guariglia, S., Meng, Q. and Cai, D., 2011. Neuropeptide exocytosis involving synaptotagmin-4 and oxytocin in hypothalamic programming of body weight and energy balance. Neuron. 69, 523–535.

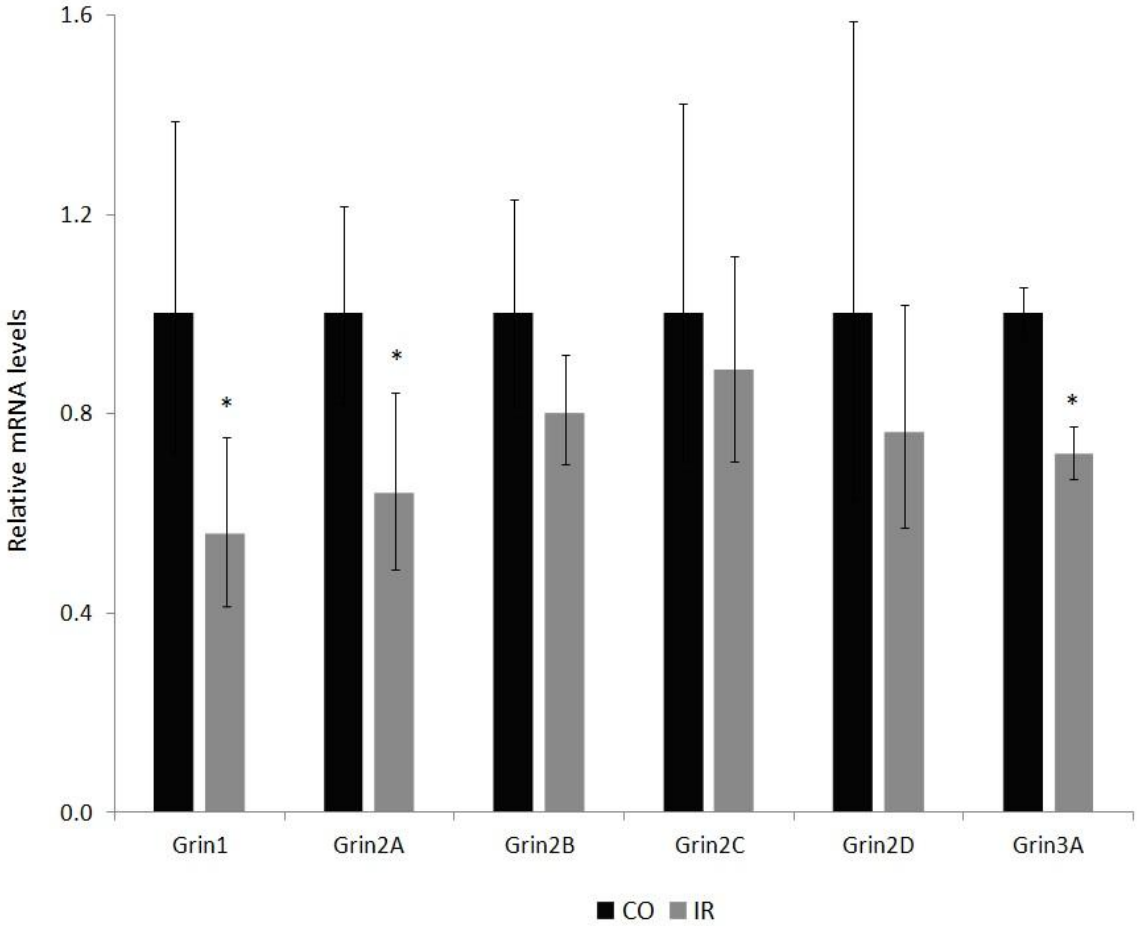

Figure 1 – Relative mRNA levels of NMDARs subunits in cortex. Data are presented as mean ± SD (n = 4 per group) and compared using student t-test. Significant levels (*) were considered when $p < 0.05$.

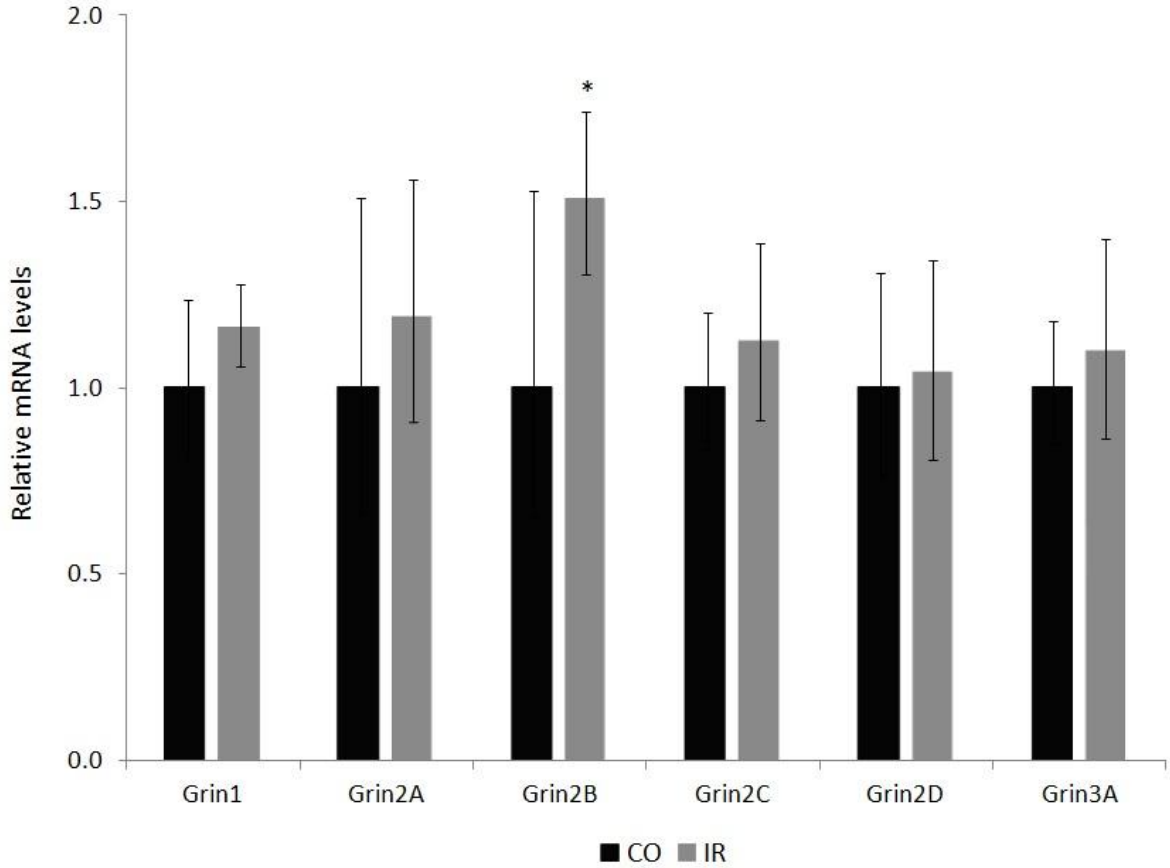

Figure 2 – Relative levels of NMDARs subunits mRNA in hippocampus. Data are presented as mean ± SD (n = 4 per group) and compared using student t-test. Significant levels (*) were considered when $p < 0.05$.

PARTE III

DISCUSSÃO

O SNC desempenha um papel fundamental para equilibrar a equação de energia. Sinais ambientais e internos são integrados nos circuitos do sistema nervoso central. A insulina é transportada a partir da circulação para o cérebro e estimula receptores no hipotálamo e em outras regiões do cérebro para reduzir a ingestão de alimentos (Ryan, 2012). A sinalização da insulina anormal, como no estado de resistência à insulina, tem um papel fundamental na patogênese de uma série de doenças metabólicas, que incluem a DM2, a hipertensão, dislipidemias, aterosclerose e anormalidades reprodutivas (Matsumoto *et al*., 2006). Desde 1971 já existem evidências de que a estimulação do DNA, RNA ou a síntese proteica pela insulina parece atuar rapidamente em processos que conduzem à uma síntese aumentada destes compostos (Wang & Amor, 1971). Em nosso estudo demonstramos que os genes envolvidos na via de sinalização da insulina e resistência à insulina interagem com fatores ambientais (ingestão calórica por meio de dieta hiperpalatável e consequente aumento da gordura), resultando em alterações dos níveis de mRNA, da quantidade de proteínas no SNC e no desenvolvimento da obesidade e resistência à insulina (Kahn *et al*., 2006).

A resistência à insulina decorrente do tratamento com uma dieta hiperpalatável está bem estabelecida e vem sendo utilizada em diversos estudos no nosso grupo de pesquisa (Muller *et al*., 2010; Muller *et al*., 2008; Dietrich *et al*., 2007). No presente estudo, foram avaliados os efeitos da resistência à insulina em parâmetros metabólicos e na expressão gênica e proteica de proteínas envolvidas nos processos de sinalização da insulina em córtex e hipocampo no modelo experimental estabelecido em ratos Wistar.

Em um primeiro momento, o perfil lipídico e a tolerância à glicose nos animais submetidos à dieta hiperpalatável foram avaliados para validar o modelo de resistência à

insulina. Conforme esperado, após 130 dias de dieta os animais se mostraram intolerantes à glicose e seu perfil lipídico revelou níveis de colesterol total e de LDL elevados. Os níveis de triglicerídeos também se mostraram levemente aumentados, mas não significativos estatisticamente (Souza *et al*., 2010).

Após estabelecidos estes primeiros parâmetros metabólicos, partiu-se para a determinação da expressão de alguns genes envolvidos direta ou indiretamente, na rota de sinalização da insulina. Os genes escolhidos foram: Insr, Irs1, Irs2, Akt1, Akt2, Akt3, Pi3kr1, Gsk3b, Creb1, Camk2a, Ppargc1a, Cdk5, Casp3, Foxo1, Fyn, Sod2 e Ide. Com exceção do gene que codifica a enzima degradadora de insulina (Ide), quase todos os genes avaliados no córtex se mostraram menos expressos no grupo de ratos submetidos à dieta hiperpalatável, resistentes à insulina (IR), quando comparados ao grupo controle (CO). A Ide está relacionada com o peptídeo beta-amilóide cujos níveis estão associados com um risco aumentado para a DA. A Ide é uma das várias proteases potencialmente envolvidas na degradação de beta-amilóide, hidrolisando estes peptídeos como demonstrado em um estudo que utilizou modelo animal com deficiência do gene Ide. Há uma correlação inversa entre a atividade de Ide no cérebro e os níveis de peptídeo beta-amilóde, sugerindo, assim, que a modulação da atividade Ide pode alterar o risco para a DA (Miller *et al*., 2003). Em um estudo com ratos Ide nulos, foi demonstrado um aumento da acumulação cerebral de beta-amilóide endógeno, um achado neuropatológico característico da DA, e os animais também apresentaram hiperinsulinemia e intolerância à glicose, características de DM2. A hipofunção de Ide pode ser a base ou contribuir para certas formas de DA e DM2, e de um mecanismo para a associação reconhecida entre hiperinsulinemia, diabetes e DA (Farris *et al*., 2003). Mas a Ide tem uma afinidade preferencial pela insulina de tal modo que a presença de insulina vai inibir a degradação, mediada por Ide, de várias substâncias, incluindo a beta-amilóide (Cook *et al*., 2003). No nosso estudo os níveis de

Ide não se mostraram alterados, e não seria possível fazer uma associação direta entre os efeitos da dieta hiperpalatável, ou seja, resistência à insulina, com um risco aumentado para o desenvolvimento da DA.

Conforme citado anteriormente, muitos genes se mostraram menos expressos no córtex dos animais IR quando comparados ao grupo CO. A hipótese aventada é de que esta estrutura cerebral seria menos protegida durante uma fase de privação de glicose devido à resistência à insulina. Sendo assim, muitos genes estariam com sua expressão diminuída devido à ineficiente sinalização da insulina que, como já está bem estabelecido, regula a expressão gênica e a síntese proteica (Wang & Amor, 1971; O'Brian *et al*., 2001). Em alguns casos, as alterações não foram estatisticamente significativas, mas em Insr, Irs2, Akt1, Akt3, Sod2 e Casp3 observamos diminuições estatisticamente significativas no grupo IR. A sensibilidade à insulina também é determinada pelo número de RI na membrana plasmática e os níveis de mRNA do RI (Lee & Gorospe, 2010). Assim, níveis mais baixos de mRNA do RI contribuem com o estado de resistência à insulina. Ela também leva à diminuição dos níveis de mRNA de alguns efetores downstream (Irs2, Akt1 e Akt3). O estado de resistência à insulina também afeta a quantidade de mRNA em nível mitocondrial, como demosntrado pelo nível reduzido de mRNA Sod2, um protetor contra o estresse oxidativo, o que corrobora com a nossa hipótese de que o córtex é mais afetado pelo insulto de insulina (sinal de deficiência). Considerando-se os níveis de proteínas, aumento da PI3K, CAMK2A e de CREB foi observado. Levando-se em conta que a insulina, quando não utilizada pelo RI, permanece na fenda extracelular aumentando sua concentração a ponto de ativar IGF-1, liberando o cálcio das vesículas intracelulares que leva à ativação de CREB por CaMK2 (Stears *et al*., 2012; Zuloaga *et al*., 2013), é razoável que o estado de resistência à insulina no córtex está tentando manter funções normais aumentando os níveis de proteínas desta via. Pode não ser devido ao aumento na transcrição de mRNA, mas devido a uma

regulação da tradução por outros fatores, como promotores de tradução, para tentar manter a homeostase da glicose no córtex.

Por outro lado, no hipocampo foi observado um aumento geral nos níveis de mRNA quando comparamos os grupos IR e CO. Mas em apenas três genes observamos níveis significativamente mais altos no grupo IR: Pi3kr1, Creb1, Camk2a. Quando comparamos os níveis proteicos, apenas CREB mostrou níveis alterados. A hipótese, nesse caso, é um efeito compensatório da sinalização prejudicada da insulina na estrutura. A regulação de CREB em níveis de mRNA e proteína é compatível com os resultados de aumento dos níveis de mRNA, uma vez que CREB é conhecido como um regulador fisiológico da transcrição da gliconeogênese. Além disso, CREB não é apenas importante na ativação da transcrição direta de genes gliconeogênicos, mas também responsável pela progressão da resistência hepática à insulina nos modelos induzidos por dieta ou modelos genéticos de obesidade. Ao alterar a atividade transcricional de CREB há uma repressão transcricional de genes gliconeogênicos (Jitrapakdee, 2012). Apesar da importância de CREB em diversos tecidos, os resultados apresentados demonstram que CREB também é importante para o estado de resistência à insulina no SNC, principalmente no hipocampo. Dessa forma, um aumento dos níveis de CREB parece ser essencial para a manutenção dos níveis normais de todas as outras moléculas envolvidas na via de sinalização da insulina para evitar lesões graves no hipocampo. Desta forma, mais estudos são necessários para delinear os diversos papéis de CREB no controle transcricional da homeostase energética em nível de SNC.

Um defeito na via insulina/RI/IRS/PI3K/Akt/FOXO1-CREB pode ser encontrado na maioria dos pacientes com a síndrome metabólica, bem como em pacientes com doenças relacionadas com o RI e pacientes com lipodistrofia. A perda, mesmo parcial, de sinalização através dessa via é suficiente para permitir a ativação constitutiva de FOXO1 e estimula ainda

mais a produção de glicose através da gilcose-6-fosfato e fosfoenolpiruvato carboxicinase 1 (Stears *et al.*, 2012). Uma outra alternativa seria que a resistência à insulina seja mediada pela sinalização através de uma via alternativa, tal como a que envolve IGF1/PI3K/CaMK2/CREB (Zuloaga *et al.*, 2013). Nosso estudo aponta para a ocorrência dessas duas vias de sinalização descritas acima: a via clássica no hipocampo e a via alternativa no córtex. Estas hipóteses devem ser confirmadas por estudos bioquímicos e/ou histológicas em futuras investigações para saber se todas essas alterações moleculares podem ser traduzidas em ganho e perda de função das proteínas.

O presente estudo agrega informações para a elucidação da influência deste estado de resistência à insulina sobre a plasticidade sináptica e a sobrevivência neuronal. Estes dois processos são dependentes de insulina e estão interligados pela via da PI3K. Desta forma, os sinais que atuam na sobrevivência neuronal devem alterar a plasticidade sináptica e os que atuam na atividade sináptica devem alterar a sobrevivência neuronal. A insulina modula a plasticidade sináptica no hipocampo, facilitando a indução da potenciação de longa duração (*long-term potentiation* – LTP) e da depressão de longa duração (*long-term depression* – LTD), de um modo dependente do receptor NMDA (NMDAR). Esta modulação estabelece uma relação mecanicista entre insulina e plasticidade sináptica e explica o funcionamento da insulina como neuromodulador, sugerindo que, tanto o estímulo do NMDAR como a sinalização da insulina, são necessários para que ocorram mudanças na plasticidade sináptica (van der Heide *et al.*, 2006). Assim, a expressão e a funcionalidade do gene de NMDAR parecem estar intimamente correlacionadas com a sinalização da insulina. Mesmo considerando o cérebro como um sistema integrado, diferentes áreas podem ter níveis distintos de expressão e função do gene, bem como diferenças na vulnerabilidade a insultos (Vizi, 2000).

Em uma grande variedade de doenças neurológicas, a lesão neuronal está associada com a excitotoxicidade induzida por superativação de NMDAR como uma consequência da libertação de níveis elevados de glutamato (Glu) para o espaço extracelular (Lipton & Rosenberg, 1994; Nishizawa, 2001). Apesar de sua semelhança estrutural, há diferenças funcionais entre subunidades do NMDAR. A ativação de NMDAR produz tanto a sobrevivência neuronal e plasticidade sináptica como pode promover a morte celeular e esta dupla ação é mediada pela composição das subunidades do NMDAR (Liu *et al*., 2007).

Em nosso estudo, apenas a expressão Grin2B está aumentada no hipocampo de ratos IR. Nós acreditamos que este fato envolve CaMKII, que também está aumentada. A entrada de cálcio através do NMDAR ativa CaMKII que pode então se ligar em NMDAR2B. Esta associação é considerada um requisito importante para a manutenção da LTP, visto que ao romper esta interação termina a potenciação (Sanhueza *et al*., 2011).

Em geral, a expressão Grin2B diminui com a idade. Mas, nesse estudo, foi mostrado que um estado anormal, como estado de resistência à insulina, modula a expressão no sentido oposto, aumentando os níveis de mRNA de Grin2B no hipocampo (Flint *et al*., 1997; Quinlan *et al*., 1999).

No córtex, níveis mais baixos de mRNA em Grin1, Grin2A e Grin3A no grupo IR foram observados. A expressão reduzida de mRNA das subunidades NMDAR no córtex indicam que a sinalização da insulina pode afetar de forma mais severa esta estrutura do que o hipocampo. Estudos adicionais se fazem necessários para a melhor compreensão da correlação da via de sinalização da insulina e ativação de NMDAR no SNC, principalmente a nível proteico.

Considerando a grande variedade de ações e consequências da sinalização de insulina no SNC também decidiu-se investigar se as proteínas 14-3-3 não seriam o ponto chave desta

diversidade. Até o momento, existem poucos estudos que exploram a interação de proteínas 14-3-3 com resistência à insulina no cérebro e muitos deles são com pacientes com doença de Alzheimer (Foote & Zhou, 2012).

Aqui demonstramos que isoformas de 14-3-3 são diferencialmente expressas no SNC de ratos com a resistência à insulina. O nível de mRNA de 14-3-3 isoforma η demonstrou ser significativamente menor no córtex grupo IR . Esta isoforma está relacionada com LTP (Simsek - Duran *et al*., 2004). Também podemos ver uma correlação negativa dos níveis de glicose no sangue e os níveis de mRNA desta isoforma. Assim, acreditamos que esta isoforma pode estar envolvida em uma regulação fina na via da insulina levando em conta que a variação no córtex só foi encontrada em níveis de mRNA, enquanto nenhuma alteração significativa foi encontrada no hipocampo. Outra isoforma que tem a expressão de RNA negativamente correlacionada com AUC dos níveis de glicose no sangue é a isoforma θ. O nível de proteína 14-3-3 θ detectada por *Western Blot* (WB) foi significativamente maior no grupo IR em hipocampo. Esta isoforma está envolvida na via de sinalização de fatores de crescimento, como a insulina *per se*, ao estimular o sinal de PI3K - Akt (Kakinuma *et al*., 2008). Assim, acreditamos que o estado de resistência à insulina pode danificar os neurônios e que estas mudanças são devido a uma resposta na tentativa de proteger o hipocampo, aumentando os níveis de proteína de 14-3-3 θ e seu efeito protetor. Estes dados sugerem que esta isoforma pode desempenhar um papel chave na via de sinalização de insulina. Além disso, as proteínas 14-3-3 são muito importantes em várias funções neurológicas normais, bem como em desordens neurológicas. As alterações nas conexões das interações 14-3-3/ligantes e doenças neurais sugerem um papel patológico para as proteínas 14-3-3 e isoformas específicas podem estar intimamente relacionados a algumas doenças ou estados patológicos.

Uma alteração da via insulina/IR/IRS/PI3K/AKT/FOXO1-CREB está presente na maioria dos pacientes com a síndrome metabólica, bem como em pacientes com lipodistrofia. Mas, a resistência à insulina também pode ser regulada pela sinalização através de uma via alternativa, que envolve a IGF/PI3K/CAMK2/CREB (Zuloaga et al., 2013). O nosso estudo corrobora, a nível molecular no SNC, com estas duas vias de sinalização, a via clássica, no hipocampo e a via alternativa, no córtex. Estas hipóteses devem ser confirmadas por estudos bioquímicos e/ou histológicos em futuras investigações para saber se todas essas alterações moleculares podem ser traduzidas em ganho ou perda de função normal das proteínas envolvidas. Há que se investigar também o papel da família de proteínas 14-3-3 como possível alvo terapêutico, bem como a interação destas vias de sinalização com os receptores NMDA e as implicações na memória e plasticidade sináptica de pacientes com doenças neurológicas com a DA.

CONCLUSÃO

O presente trabalho avaliou os efeitos da resistência à insulina em parâmetros metabólicos e na expressão gênica e proteica de proteínas neurais envolvidas nos processos de sinalização da insulina em um modelo experimental de resistência à insulina em ratos submetidos a uma dieta hiperpalatável**.**

A dieta hiperpalatável alterou o perfil lipídico dos animais aumentando os níveis de colesterol total e de HDL. A curva de tolerância à glicose também mostrou-se aumentada, caracterizando, assim, o modelo animal de resistência à insulina proposto.

Na avaliação dos níveis de mRNA das isoformas da proteína 14-3-3, observamos uma diminuição das isoformas 14-3-3 η e 14-3-3 θ no córtex, enquanto que a análise proteica mostrou aumento da isoforma 14-3-3 θ no hipocampo.

Quando analisados os genes envolvidos direta ou indiretamente na rota de sinalização da insulina, diminuição nos níveis de mRNA de Insr, Irs-2, Akt1, Akt3, Casp3 e Sod2 foi observada no córtex e aumento de Pi3kr1, Camk2a e Creb1 no hipocampo. Na avaliação proteica, aumento de CREB e PI3K foram identificados no hipocampo e aumento de CaMKII no córtex. Na análise dos níveis de mRNA das subunidades do receptor N-metil-D-aspartato (NMDA) foi evidenciada diminuição de Grin1, Grin2A e Grin3A no córtex e aumento de Grin2B no hipocampo.

Estudos moleculares podem gerar informações de grande interesse para a maioria das doenças cerebrais, pois os mecanismos relacionados às suas causas são pouco conhecidos e multifatoriais. O presente estudo contribui com informações novas e inéditas para a melhor compreensão dos mecanismos fisiopatológicos associados com a resistência encefálica à insulina.

REFERÊNCIAS BIBLIOGRÁFICAS

Accili D, Drago J, Lee EJ, Johnson MD, Cool MH, Salvatore P, Asico LD, José PA, Taylor SI, Westphal H. Early neonatal death in mice homozygous for a null allele of the insulin receptor gene. Nat Genet. 1996;12:106-109.

Agarwal-Mawal A, Qureshi HY, Cafferty PW, Yuan Z, Han D, Lin R, Paudel HK. 14-3-3 connects glycogen synthase kinase-3 beta to tau within a brain microtubule-associated tau phosphorylation complex. J Biol Chem. 2003;278(15):12722-8.

Ashcroft FM, Rorsman P. Diabetes mellitus and the β cell: the last ten years. Cell. 2012;148(6):1160-1171.

Babri S, Badie HG, Khamenei S, Seyedlar MO. Intrahippocampal insulin improves memory in a passive-avoidance task in male wistar rats. Brain Cogn. 2007;64(1):86-91.

Berthoud HR, LenardNR, Shin AC. Food reward, hyperphagia, and obesity. Am J Physiol Regul Integr Comp Physiol. 2011;300(6):R1266–1277.

Boucher J, Tseng YH, Kahn CR. Insulin and insulin-like growth factor-1 receptors act as ligand-specific amplitude modulators of a common pathway regulating gene transcription. J Biol Chem. 2010;285(22):17235–17245.

Boura-Halfon S, Zick Y. Phosphorylation of IRS proteins, insulin action, and insulin resistance. Am J Physiol Endocrinol Metab. 2009;296:E581–591.

Brüning JC, Gautam D, Burks DJ, Gillette J, Schubert M, Orban PC, Klein R, Krone W, Müller-Wieland D, Kahn CR. Role of brain insulin receptor in control of body weight and reproduction. Science. 2000;289(5487):2122–2125.

Caballero B. The global epidemic of obesity: an overview. Epidemiol Rev. 2007;29, 1–5.

Chen S, Synowsky S, Tinti M, MacKintosh C. The capture of phosphoproteins by 14-3-3 proteins mediates actions of insulin. Trends Endocrinol Metab. 2011;22(11):429-436.

Cho H, Thorvaldsen JL, Chu Q, Feng F, Birnbaum MJ. Akt1/PKBalpha is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. J Biol Chem. 2001;276:38349-38352.

Cook DG, Leverenz JB, McMillan PJ, Kulstad JJ, Ericksen S, Roth RA, Schellenberg GD, Jin LW, Kovacina KS, Craft S. Reduced hippocampal insulin-degrading enzyme in late-ons*et al*zheimer's disease is associated with the apolipoprotein E-epsilon-4 allele. Am J Path. 2003;162: 313-319.

Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 1995;378:785–789.

Cross DA, Watt PW, Shaw M, van der Kaay J, Downes CP, Holder JC, Cohen P. Insulin activates protein kinase B, inhibits glycogen synthase kinase-3 and activates glycogen synthase by rapamycin-insensitive pathways in skeletal muscle and adipose tissue. FEBS Lett. 1997;406:211–215.

Cukierman T, Gerstein HC, Williamson JD. Cognitive decline and dementia in diabetes—systematic overview of prospective observational studies. Diabetologia 2005;48:2460–9.

Dietrich MO, Muller A, Bolos M, Carro E, Perry ML, Portela LV, Souza DO, Torres-Aleman I. Western style diet impairs entrance of blood-borne insulin-like growth factor-1 into the brain. Neuromolecular Med. 2007;9(4):324-330.

Dubois F, Vandermoere F, Gernez A, Murphy J, Toth R, Chen S, Geraghty KM, Morrice NA, MacKintosh C. Differential 14-3-3 affinity capture reveals new downstream targets of phosphatidylinositol 3-kinase signaling. Mol Cell Proteomics. 2009;8(11): 2487–2499.

Farris W, Mansourian S, Chang Y, Lindsley L, Eckman EA, Frosch MP, Eckman CB, Tanzi RE, Selkoe DJ, Guenette S. Insulin-degrading enzyme regulates the levels of insulin, amyloid beta-protein, and the beta-amyloid precursor protein intracellular domain in vivo. Proc Nat Acad Sci. 2003;100: 4162-4167.

Field AE, Coakley EH, Must A, Spadano JL, Laird N, Dietz WH, Rimm E, Colditz GA. Impact of overweight on the risk of developing common chronic diseases during a 10-year period. Arch Intern Med. 2001;161(13):1581-1586.

Filippi BM, Abraham MA, Yue JT, Lam TK. Insulin and glucagon signaling in the central nervous system. Rev Endocr Metab Disord. 2013;14(4):365-375.

Flint AC, Maisch US, Weishaupt JH, Kriegstein AR, Monyer H. NR2A subunit expression shortens NMDA receptor synaptic currents in developing neocortex. J Neurosci. 1997;17:2469–2476.

Foote M, Zhou Y. 14-3-3 proteins in neurological disorders. Int J Biochem Mol Biol. 2012; 3(2):152-164.

Gerozissis K. Brain insulin, energy and glucose homeostasis; genes, environment and metabolic pathologies. Eur J Pharmacol. 2008;585(1):38-49.

Grunblatt E, Salkovic-Petrisic M, Osmanovic J, Riederer P, Hoyer S. Brain insulin system dysfunction in streptozotocin intracerebroventricularly treated rats generates hyperphosphorylated tau protein. J Neurochem. 2007;101: 757-770.

Guh DP, Zhang W, Bansback N, Amarsi Z, Birmingham CL, Anis AH. The incidence of co-morbidities related to obesity and overweight: a systematic review and meta-analysis. BMC Public Health. 2009;9:88.

He Q, Gao Z, Yin J, Zhang J, Yun Z, Ye J. Regulation of HIF-1α activity in adipose tissue by obesity-associated factors: adipogenesis, insulin, and hypoxia. Am J Physiol Endocrinol Metab 2011;300(5):E877-885.

Heni M, Schöpfer P, Peter A, Sartorius T, Fritsche A, Synofzik M, Häring HU, Maetzler W, Hennige AM. Evidence for altered transport of insulin across the blood-brain barrier in insulin-resistant humans. Acta Diabetol. 2014;51(4):679-81.

Hill J, Lesniak M, Pert C, Roth J. Autoradiographic localization of insulin receptors in rat brain: prominence in olfactory and limbic areas. Neurosc. 1986;17(4):1127–1138

Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science. 1993;259:87-91.

Hoyer S, Lannert H. Long-term abnormalities in brain glucose/energy metabolism after inhibition of the neuronal insulin receptor: implication of tau protein. J Neural Transm Suppl. 2007;(72):195-202.

Jitrapakdee S. Transcription factors and coactivators controlling nutrient and hormonal regulation of hepatic gluconeogenesis. Int J Biochem Cell Biol. 2012;44(1):33-45.

Kadowaki T, Ueki K, Yamauchi T, Kubota N. SnapShot: Insulin signaling pathways. Cell. 2012;148(3):624, 624.e1.

Kahn SE, Hull RL, Utzschneider KM. Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature. 2006;444(7121):840-846.

Kaidanovich-Beilin O, Woodgett JR. GSK-3: Functional Insights from Cell Biology and Animal Models. Front Mol Neurosci. 2011;4:40.

Kakinuma N, Roy BC, Zhu Y, Wang Y, Kiyama R. Kank regulates RhoA-dependent formation of actin stress fibers and cell migration via 14-3-3 in PI3K-Akt signaling. J Cell Biol. 2008;181(3):537-549.

Karra E, Yousseif A, Batterham RL. Mechanisms facilitating weight loss and resolution of type 2 diabetes following bariatric surgery. Trends Endocrinol Metab. 2010;21(6):337-344.

Kelly T,Yang W, Chen CS, Reynolds K, He J. Global burden of obesity in 2005 and projections to 2030. Int J Obes (Lond). 2008;32(9):1431-1437.

Ketterer C, Tschritter O, Preissl H, Heni M, Häring HU, Fritsche A. Insulin sensitivity of the human brain. Diabetes Res Clin Pract. 2011;93 Suppl 1:S47-51.

Khardori R. Changing paradigms in type 2 diabetes mellitus. Indian J Endocrinol Metab. 2013;17(Suppl1):S68-S71.

Kim JA, Wei Y, Sowers JR. Role of mitochondrial dysfunction in insulin resistance. Circ Res. 2008;102:401-414.

Kleppe R, Martinez A, Døskeland SO, Haavik J. The 14-3-3 proteins in regulation of cellular metabolism. Semin Cell Dev Biol. 2011;22:713-719.

Kubota N, Tobe K, Terauchi Y, Eto K, Yamauchi T, Suzuki R, Tsubamoto Y, Komeda K, Nakano R, Miki H, Satoh S, Sekihara H, Sciacchitano S, Lesniak M, Aizawa S, Nagai R, Kimura S, Akanuma Y, Taylor SI, Kadowaki T. Disruption of insulin receptor substrate 2 causes type 2 diabetes because of liver insulin resistance and lack of compensatory beta-cell hyperplasia. Diabetes. 2000;49:1880-1889.

Lanner JT, Katz A, Tavi P, Sandström ME, Zhang SJ, Wretman C, James S, Fauconnier J, Lännergren J, Bruton JD, Westerblad H. The role of Ca2+ influx for insulin-mediated glucose uptake in skeletal muscle. Diabetes. 2006;55(7):2077-2083.

Lee EK, Gorospe M. Minireview: posttranscriptional regulation of the insulin and insulin-like growth factor systems. Endocrinology. 2010;151(4):1403-1408.

Leibowitz SF, Wortley KE. Hypothalamic control of energy balance: different peptides, different functions. Peptides. 2004;25, 473–504.

Lim EL, Hollingsworth KG, Aribisala BS, Chen MJ, Mathers JC, Taylor R. Reversal of type 2 diabetes: normalisation of beta cell function in association with decreased pancreas and liver triacylglycerol. Diabetologia. 2011;54, 2506–2514.

Lipton SA, Rosenberg PA. Excitatory amino acids as a final common pathway for neurologic disorders. N Engl J Med. 1994;330:613-622.

Liu Y, Wong TP, Aarts M, Rooyakkers A, Liu L, Lai TW, Wu DC, Lu J, Tymianski M, Craig AM, Wang YT. NMDA receptor subunits have differential roles in mediating excitotoxic neuronal death both in vitro and in vivo. J Neurosci. 2007;27:2846–2857.

MacKnight C, Rockwood K, Awalt E, McDowell I. Diabetes mellitus and the risk of dementia, Alzheimer's disease and vascular cognitive impairment in the Canadian Study of Health and Aging. Dement Geriatr Cogn Disord 2002;14:77–83.

Marra M, Scalfi L, Contaldo F, Pasanisi F. Fasting respiratory quotient as a predictor of long-term weight changes in non-obese women. Ann Nutr Metab. 2004;48(3):189-192

Marx, J. Alzheimer's disease. Play and exercise protect mouse brain from amyloid buildup. Science. 2005;307(5715):1547.

Matsumoto M, Han S, Kitamura T, Accili D. Dual role of transcription factor FoxO1 in controlling hepatic insulin sensitivity and lipid metabolism. J Clin Invest. 2006;116(9):2464-2472.

Mauvais-Jarvis F, Kulkarni RN, Kahn CR. Knockout models are useful tools to dissect the pathophysiology and genetics of insulin resistance. Clin Endocrinol (Oxf). 2002;57:1-9.

Miller BC, Eckman EA, Sambamurti K, Dobbs N, Chow KM, Eckman CB, Hersh LB, Thiele DL. Amyloid-beta peptide levels in brain are inversely correlated with insulysin activity levels in vivo. Proc Nat Acad Sci. 2003;100: 6221-6226.

Miyawaki T, Ofengeim D, Noh KM, Latuszek-Barrantes A, Hemmings BA, Follenzi A, Zukin RS. The endogenous inhibitor of Akt, CTMP, is critical to ischemia-induced neuronal death. Nat Neurosci. 2009;12: 618-626.

Moore BW, Perez VJ. Specific acidic proteins of the nervous system. In: Carlson FD, editor. Physiological and biochemical aspects of nervous integration. Woods Hole MA: Prentice-Hall, Inc., The Marine Biological Laboratory; 1967: 343–59.

Muller AP, Cammarota M, Dietrich MO, Rotta LN, Portela LV, Souza DO, Izquierdo I, Bevilaqua LR, Perry ML. Different effect of high fat diet and physical exercise in the hippocampal signaling. Neurochem Res. 2008;33(5):880-885.

Muller AP, Tort AH, Gnoatto J, Moreira JD, Vinadé ER, Perry ML, Souza DO, Lara DR, Portela LV. Metabolic and behavioral effects of chronic olanzapine treatment and cafeteria diet in rats. Behav Pharmacol. 2010;21(7):668-675.

Neukamm SS, Ott J, Dammeier S, Lehmann R, Häring HU, Schleicher E, Weigert C. Phosphorylation of serine 1137/1138 of mouse insulin receptor substrate (IRS) 2 regulates cAMP-dependent binding to 14-3-3 proteins and IRS2 protein degradation. J Biol Chem. 2013;288(23):16403-16415.

Neumann KF, Rojo L, Navarrete LP, Farias G, Reyes P, Maccioni RB. Insulin resistance and Alzheimer's disease: molecular links & clinical implications. Curr Alzheimer Res. 2008;5(5):438-447.

Nishizawa Y. Glutamate release and neuronal damage in ischemia. Life Sci. 2001;69:369-381.

O'Brien RM, Streeper RS, Ayala JE, Stadelmaier BT, Hornbuckle LA. Insulin-regulated gene expression. Biochem Soc Trans. 2001;29(Pt 4):552-558.

Ogihara T, Isobe T, Ichimura T, Taoka M, Funaki M, Sakoda H, Onishi Y, Inukai K, Anai M, Fukushima Y, Kikuchi M, Yazaki Y, Oka Y, Asano T. 14-3-3 protein binds to insulin receptor substrate-1, one of the binding sites of which is in the phosphotyrosine binding domain. J Biol Chem 1997;272: 25267-25274.

Oh KJ, Han HS, Kim MJ, Koo SH. CREB and FoxO1: two transcription factors for the regulation of hepatic gluconeogenesis. BMB Rep. 2013;46(12):567-574.

Ohara T, Doi Y, Ninomiya T, Hirakawa Y, Hata J, Iwaki T, Kanba S, Kiyohara Y. Glucose tolerance status and risk of dementia in the community: the Hisayama Study. Neurology 2011;77:1126–34.

Organização Mundial de Saúde (OMS). 2014; Fact sheet N°311. Disponível em http://www.who.int/mediacentre/factsheets/fs311/en/. Acesso em 08 nov 2014.

Organização Mundial de Saúde (OMS). 2014; Overweight and Obesity: Data by country Disponível em http://www.who.int/gho/ncd/risk_factors/overweight/en/. Acesso em 08 nov 2014.

Ott A, Stolk RP, Hofman A, van Harskamp F, Grobbee DE, Breteler MMB. Association of diabetes mellitus and dementia: The Rotterdam study. Diabetologia. 1996;39, 1392–1397.

Ott BR, Lafleche G, Whelihan WM, Buongiorno GW, Albert MS, Fogel BS. Impaired awareness of deficits in Alzheimer disease. Alzheimer Dis Assoc Disord. 1996b;10(2):68-76.

Park CR. Cognitive effects of insulin in the central nervous system. Neurosci Biobehav Rev. 2001;25(4):311-323.

Pozuelo Rubio M, Geraghty KM, Wong BH, Wood NT, Campbell DG, Morrice N, Mackintosh C. 14-3-3-Affinity purification of over 200 human phosphoproteins reveals new links to regulation of cellular metabolism, proliferation and trafficking. Biochem J. 2004;379:395–408.

Quinlan EM, Philpot BD, Huganir RL, Bear MF. Rapid, experience-dependent expression of synaptic NMDA receptors in visual cortex in vivo. Nat Neurosci. 1999;2:352–357.

Rhodes CJ. Type 2 diabetes - a matter of β-cell life and death? Science. 2005;307:380–384.

Rizzuto R, Pozzan T. Microdomains of intracellular Ca2+: molecular determinants and functional consequences. Physiol Rev. 2006;86:369-408.

Ros Pérez M, Medina-Gómez G. Obesidad, adipogénesis y resistencia a la insulina. Endocrinol Nutr. 2011;58:360-369.

Ryan KK, Woods SC, Seeley RJ. Central nervous system mechanisms linking the consumption of palatable high-fat diets to the defense of greater adiposity. Cell Metab. 2012;15(2):137-149.

Saltiel AR, Kahn CR. Insulin signalling and the regulation of glucose and lipid metabolism. Nature. 2001;414(6865):799-806.

Sanhueza M, Fernandez-Villalobos G, Stein IS, Kasumova G, Zhang P, Bayer KU, Otmakhov N, Hell JW, Lisman J. Role of the CaMKII/NMDA receptor complex in the maintenance of synaptic strength. J Neurosci. 2011;31(25):9170–9178.

Schwartz MW, Porte D, Jr. Diabetes, obesity, and the brain. Science. 2005;307: 375-379.

Semple RK, Savage DB, Cochran EK, Gorden P, O'Rahilly S. Genetic syndromes of severe insulin resistance. Endocr Rev. 2011;32(4):498-514.

Siddle K. Signalling by insulin and IGF receptors: supporting acts and new players. J Mol Endocrinol. 2011;47:R1–10.

Simsek-Duran F, Linden DJ, Lonart G. Adapter protein 14-3-3 is required for a presynaptic form of LTP in the cerebellum. Nature Neurosci 2004;7:1296-1298.

Solomon TP, Malin SK, Karstoft K, Knudsen SH, Haus JM, Laye MJ, Pedersen M, Pedersen BK, Kirwan JP. Determining pancreatic β-cell compensation for changing insulin sensitivity using an oral glucose tolerance test. Am J Physiol Endocrinol Metab. 2014;307(9):E822-9.

Souza CG, Böhmer AE, Müller AP, Oses JP, Viola GG, Lesczinski DN, Souza DG, Knorr L, Moreira JD, Lhullier F, Souza DO, Perry ML. Effects of a highly palatable diet on lipid and glucose parameters, nitric oxide, and ectonucleotidases activity. Appl Physiol Nutr Metab. 2010;35(5):591-597.

Stears A, O'Rahilly S, Semple RK, Savage DB. Metabolic insights from extreme human insulin resistance phenotypes. Best Pract Res Clin Endocrinol Metab. 2012;26(2):145-157.

Steen E, Terry BM, Rivera EJ, Cannon JL, Neely TR, Tavares R, Xu XJ, Wands JR, de la Monte SM. Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease--is this type 3 diabetes? J Alzheimers Dis. 2005;7(1):63-80.

Stenbit AE, Tsao TS, Li J, Burcelin R, Geenen DL, Factor SM, Houseknecht K, Katz EB, Charron MJ. GLUT4 heterozygous knockout mice develop muscle insulin resistance and diabetes. Nat Med. 1997;3:1096-1101.

Steinacker P, Aitken A, Otto M. 14-3-3 proteins in neurodegeneration. Semin Cell Dev Biol. 2011;22(7):696-704.

Tamemoto H, Kadowaki T, Tobe K, Yagi T, Sakura H, Hayakawa T, Terauchi Y, Ueki K, Kaburagi Y, Satoh S, *et al*. Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. Nature. 1994;372:182-186.

Taniguchi CM, Emanuelli B, Kahn CR. Critical nodes in signalling pathways: insights into insulin action.Nat Rev Mol Cell Biol. 2006;7(2):85-96.

Ueki K, Yballe CM, Brachmann SM, Vicent D, Watt JM, Kahn CR, Cantley LC. Increased insulin sensitivity in mice lacking p85beta subunit of phosphoinositide 3-kinase. Proc Natl Acad Sci USA. 2002;99:419-424.

van der Heide, L.P., Jacobs, F.M., Burbach, J.P., Hoekman, M.F. and Smidt, M.P. FoxO6 transcriptional activity is regulated by Thr26 and Serl84, independent of nucleo-cytoplasmic shuttling. Biochem. J. 2005;391:623–629.

van der Heide LP, Ramakers GM, Smidt MP. Insulin signaling in the central nervous system: learning to survive. Prog Neurobiol. 2006;79:205-221.

Vizi ES. Role of high affinity receptors and membrane transporters in nonsynaptic communication and drug action in the central nervous system. Pharmacol Rev. 2000;52:63–89.

Wang DY, Amor V. A study on the effect of insulin on DNA, RNA and protein synthesis in mouse mammary gland tissue in organ culture. J Endocrinol. 1971;50:2:241-249.

Wang J, Obici S, Morgan K, Barzilai N, Feng Z, Rossetti L. Overfeeding rapidly induces leptin and insulin resistance. Diabetes. 2001;50:2786–2791.

Wong VW, Wong GL, Yeung DK, Abrigo JM, Kong AP, Chan RS, Chim AM, Shen J, Ho CS, Woo J, Chu WC, Chan HL. Fatty pancreas, insulin resistance, and β-cell function: a population study using fat-water magnetic resonance imaging. Am J Gastroenterol. 2014;109(4):589-97.

Woods SC, Seeley RJ, Baskin DG, Schwartz MW. Insulin and the blood-brain barrier. Curr Pharm Des. 2003;9:795–800.

Wright DC, Hucker KA, Holloszy JO, Han DH. Ca2+ and AMPK both mediate stimulation of glucose transport by muscle contractions. Diabetes. 2004;53:330-335.

Ye J. Mechanisms of insulin resistance in obesity. Front Med. 2013;7(1):14-24.

Zuloaga R, Fuentes EN, Molina A, Valdés JA. The cAMP response element binding protein (CREB) is activated by insulin-like growth factor-1 (IGF-1) and regulates myostatin gene expression in skeletal myoblast. Biochem Biophys Res Commun. 2013;440(2):258-264.