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UBIQUITIN-PROTEASOME SYSTEM IN THE HYPOTHALAMUS: IMPLICATIONS FOR THE GENESIS OF OBESITY

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Ao meu primo, irmão, Jorge Luiz.
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..."Naquela mesa 'tá' faltando ele, e a saudade dele 'tá' doendo em mim".
(Nelson Gonçalves)

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"...toda a nossa ciência, comparada à realidade, é primitiva e inocente; e, portanto, é o que temos de mais valioso."

(Albert Einstein)

RESUMO

IGNACIO-SOUZA, L. M. **Sistema Ubiquitina-Proteassoma no hipotálamo: implicações para a gênese da obesidade.** 2013. 99f. Tese (Doutorado) – Faculdade de Ciências Médicas, Universidade Estadual de Campinas, São Paulo, 2013.

Dentre os fatores ambientais que contribuem para o desenvolvimento de obesidade, o consumo de dietas ricas em ácidos graxos saturados desempenha o papel mais importante. Estudos recentes realizados por vários grupos, inclusive o nosso, revelam que ácidos graxos saturados presentes na dieta levam ao desenvolvimento de resistência hipotalâmica à ação dos hormônios leptina e insulina, fenômeno este fundamental para que ocorra a quebra no equilíbrio entre ingestão e gasto calórico. Até o momento caracterizaram-se dois mecanismos moleculares potencialmente envolvidos na iniciação do processo que resulta na disfunção hipotalâmica na obesidade, a ativação de TLR4 e a indução de estresse de retículo endoplasmático, ambos levando a uma resposta inflamatória local e, eventualmente, a apoptose neuronal. Estudos recentes têm revelado que frente a situações que oferecem risco de dano celular, ativa-se um mecanismo de controle de tráfico e degradação protéica chamado sistema ubiquitina-proteassoma (UPS). O acúmulo de agregados protéicos positivos para ubiquitina pode gerar toxicidade celular e regular a plasticidade neuronal. Também a modulação de componentes do UPS pode gerar neurodegeneração hipotalâmica e fenótipo obeso em animais experimentais. Neste estudo aventamos a hipótese que durante períodos prolongados de obesidade a ativação anômala do UPS contribuiria para a perpetuação do quadro de obesidade. De fato, os resultados obtidos revelam que roedores com predisposição para a obesidade induzida por dieta mantém, a princípio, a capacidade de regular adequadamente a UPS no hipotálamo. Com o passar do tempo esta capacidade é perdida resultando numa maior dificuldade para perda de peso frente à redução do aporte calórico. Roedores com mutações que os protegem da inflamação, não apresentam distúrbio funcional do UPS quando expostos a dieta rica em ácidos graxos e, são também protegidos da obesidade. Portanto, o defeito funcional do UPS no hipotálamo no curso de obesidade prolongada, constitui-se num fator importante contribuindo para a refratariedade ao tratamento e perpetuação da doença.

Palavras-chave: obesidade, ubiquitina, inflamação, hipotálamo

ABSTRACT

IGNACIO-SOUZA, L. M. **Ubiquitin Proteasome System in the hypothalamus: implications to the genesis of obesity.** 2012. 99 f. Tese (Doutorado) – Faculty of Medical Sciences, University of Campinas, Sao Paulo, 2012.

The consumption of high-fat diets, especially those rich in saturated fatty acids, plays the most important role in the development of obesity. Recent studies by several groups, including ours, have shown that dietary long-chain saturated fatty acids lead to the development of hypothalamic resistance to leptin and insulin, an important condition contributing for breaking of the balance between caloric intake and energy expenditure. Two molecular mechanisms are currently known to play a triggering role in this process; activation of TLR4 and endoplasmic reticulum stress, both leading to local inflammation and eventually apoptosis of neurons. The ubiquitin-proteasome system (UPS) plays an important role in the control of protein recycling in the cell. The accumulation of ubiquitin-positive protein aggregates can cause cell toxicity and regulate neuronal plasticity. Also the modulation or differential activation of UPS can produce hypothalamic neurodegeneration and obese phenotype in experimental animals. Here, we hypothesized that under prolonged diet-induced obesity, a defect in the UPS in the hypothalamus could contribute for the defective control of energy homeostasis leading to the refractoriness of obesity to caloric restriction. In fact in an obesity-prone rodent strain, prolonged, but not short-term obesity was accompanied by functional abnormality of the UPS in the hypothalamus. In mutants protected from inflammation, resistance to diet-induced obesity was accompanied by stability of the UPS in the hypothalamus. Thus, defect of the UPS in the hypothalamus, during prolonged obesity is an important factor contributing the refractoriness of obesity to caloric restriction.

Key words: obesity, ubiquitin, inflammation, hypothalamus

LISTA DE ABREVIATURAS

AgRP	Peptídeo relacionado ao *agouti*
Akt	Proteína quinase B
a-MSH	Hormônio estimulador de melanócitos ‘alfa’
ANOVA	Análise de variância
BSA	Albumina de soro bovino
CART	Transcrito relacionado à cocaína e à anfetamina
Cd11b	*Cluster* de diferenciação 11b
CRH	Hormônio liberador de corticotrofina
DTT	Ditiotreitol
EDTA	Ácido etileno diamino tetracético
ELISA	Ensaio imunoenzimático
HuR	Proteína neuronal semelhante a ELAV
icv	Intracerebrovenricular
IkB	Inibidor ‘capa’ B
IKK	Quinase do inibidor ‘capa’ B
IL1b	Interleucina 1 ‘beta’
IL6	Interleucina 6
INFg	Interferon ‘gama’
IR	Receptor de insulina
IRS1	Primeiro substrato do receptor de insulina
JNK	Quinase c-Jun N-Terminal
NFkB	Fator nuclear ‘capa’ B

NPY	Neuropeptídeo Y
ObRb	Receptor de leptina de forma longa
PGC1a	Receptor ativado por proliferador de peroxissoma ‘alfa’
PI3	Inositol trifosfato
PMSF	Fluoreto de fenilmetil sulfonila
POMC	Pro-opiomelanocortina
SDS-PAGE	Eletroforese em gel de poliacrilamida com dodecil sulfato de sódio
SE	Erro padrão da média
siRNA	RNA de interferência
SOCS3	Supressor de sinalização de citocina 3
STAT3	Transdutor de sinal e ativador da transcrição 3
TLR4	Receptor *toll-like* 4
TNFa	Fator de necrose tumoral ‘alfa’
TRH	Hormônio liberador de tireotropina
Tris	Tri(hidroximetil)-aminometano
TTBS	Tampão salino tris-tween20
UCP1	Proteína desacopladora 1

SUMÁRIO

INTRODUÇÃO

Definida pela Organização Mundial de Saúde (OMS) como um acúmulo anômalo de massa gordurosa no organismo, resultando no comprometimento da saúde, a obesidade é hoje um dos maiores problemas de saúde pública no mundo. Para a OMS, "sobrepeso", define-se como um índice de massa corporal (IMC) igual ou superior a 25, e "obesidade", como um IMC igual ou superior a 30. Estes pontos de corte proporcionam uma referência para a avaliação individual, mas há indícios de que o risco de doenças crônicas na população aumente progressivamente a partir de um IMC de 21 (WHO 2012).

A partir da década de 70, quando as transições nutricionais começaram a ficar mais claras e o mundo viu uma revolução nos sistema de abastecimento, transporte e produção de alimentos; junto com a melhora da qualidade de vida da população, veio a necessidade de se modificar o contexto médico da obesidade como doença e sua relação com a saúde pública. Foi nessa época, em 1977, que a Organização Mundial de Saúde revisou a Classificação Internacional de Doenças (ICD-9), em Geneva, e referendou a obesidade como uma doença independente de outros distúrbios relacionados à alimentação (Braun, Rybarz et al. 1978). Esse documento pontuou ainda a sua gravidade, possibilitando, a partir daí, ao invés de usar muitos códigos diagnósticos para reportar a sinais e sintomas dos pacientes, declarar a complexidade da circunstância e da patologia.

Nas últimas três décadas, a média de IMC, a métrica mais utilizada para definir sobrepeso e obesidade, aumentou 0,4-0,5 Kg/m^2/ano. Esses dados se tornam cada vez mais assustadores quando olhamos pelo menos para os estudos mais recentes, que mostraram aproximadamente 1,6 bilhões de adultos com algum grau de sobrepeso, pelo menos 400 milhões de adultos obesos e 20

milhões de crianças com idade inferior a 5 anos com excesso de peso, em 2005. E, se o crescimento quantitativo desta doença continuar neste mesmo ritmo, as projeções indicam que cerca de 2,3 bilhões de adultos terão excesso de peso em 2015 e 25% da população mundial será obesa em 2030 (Kopelman 2000, Mokdad, Marks et al. 2004, WHO 2012).

No Brasil, a Pesquisa de Orçamentos Familiares (IBGE, 2009) também observou um aumento acentuado e contínuo do excesso de peso e da obesidade na população adulta desde 1974. Para a população masculina, o excesso de peso quase triplicou, passando de 18,5% em 1974-75 para 50,1% em 2008-09. Para as mulheres, o aumento foi menor: de 28,7% para 48%. No que diz respeito à obesidade, esta já atingiu mais de 25% da população nesse período. Além disso, estudos em populações norte-americanas e canadenses mostram que para qualquer grau de excesso de peso corporal existe uma correlação positiva com a perda da longevidade em adultos jovens, levando a quase 300 mil mortes por ano nos Estados Unidos (Mokdad, Marks et al. 2004).

O fácil acesso a alimentação de alta densidade energética, o desbalanço de macronutrientes e o estilo de vida cada vez mais sedentário são alguns dos fatores que contribuem para o avanço desta doença. Entretanto, a exposição a tais fatores ambientais não garante o desenvolvimento do fenótipo completo. Isso porque, excluindo alguns raros tipos de defeitos monogênicos (Farooqi 2006), a obesidade é o resultado final de um processo de adaptações do organismo ao ambiente em associação à sua carga genética (Galgani and Ravussin 2008). Além disso, a complexidade das adaptações entre os fatores biológicos que operam durante a vida fetal e neonatal e os desbalanços energéticos que se exacerbam ao longo da vida frente à exposição ambiental diversa dificulta cada vez mais o controle das doenças crônicas não transmissíveis (Gluckman, Hanson et al. 2011).

Assim, embora saibamos que a obesidade decorre de um balanço energético positivo e que os dois componentes modificáveis nessa equação são a ingestão alimentar e o gasto energético voluntário, são os sucessivos insultos a esse ponto de equilíbrio do nosso organismo que vão deflagrar o estado obesogênico grave. O panorama atual mostra que, tanto em obesidade humana quanto animal, a progressão dos danos pode levar a um estado em que as modificações ambientais podem não ser suficientes para reverter completamente o quadro (Guo, Jou et al. 2009, Kraschnewski, Boan et al. 2010).

Dessa forma, busca-se com interesse crescente, a elucidação dos mecanismos fisiopatológicos fundamentais que levam à perda da homeostase energética, resultando em adaptações do organismo em longo prazo que fazem com que as intervenções médico-nutricionais sejam cada vez menos eficazes. Isso porque o fino controle sobre o balanço energético entre o que consumimos o que gastamos, e o que estocamos em forma de energia, é um dos sistemas mais complexos e intrigantes da biologia. E, para conectar os sinais periféricos que indicam os níveis de nutrientes no corpo, e os sinais de hormônios metabólicos aos comandos centrais que regulam o estoque e gasto energético, existe uma região especializada do cérebro denominada hipotálamo.

Existem duas subpopulações de neurônios no núcleo arqueado do hipotálamo que atuam como sensores dos estoques de energia corporais (Flier and Maratos-Flier 1998). Elas são caracterizadas pelos neuropeptídeos que produzem, sendo orexigênicos como o NPY e AgRP ou anorexigênicos como POMC (α-MSH) e CART (Schwartz, Woods et al. 2000, Horvath 2005). Projeções axonais são direcionadas do núcleo arqueado para o hipotálamo lateral ou núcleo paraventricular de maneira a controlar outros grupos de neurônios, os de segunda ordem.

Assim, durante períodos de privação nutricional, como jejum ou situações de depleção dos estoques energéticos, os níveis reduzidos de leptina e insulina mantêm seus receptores hipotalâmicos desocupados e ativam os neurônios produtores de NPY/AgRP que emitem projeções inibitórias ao núcleo paraventricular, reduzindo a expressão de TRH (hormônio liberador de tireotropina) e CRH (hormônio liberador de corticotrofina) e estimulando, no hipotálamo lateral, a produção de orexina e MCH (Badman and Flier 2005, Cone 2005). Esse amplo sistema de sinalização se reflete em aumento da fome e diminuição da termogênese.

De modo oposto, após uma refeição ou quando as concentrações de insulina e leptina estão mais altas na circulação sanguínea, a sinalização desses hormônios induz à inibição de neurônios produtores de NPY/AgRP e ativam aqueles produtores de POMC/CART. Isso culmina com a inibição da orexina e do MCH e ativação do CRH e do TRH (Cone 2005, Horvath 2005). Os efeitos macrofuncionais são a diminuição da fome e o aumento da termogênese.

Apesar de se constituir num sistema de controle metabólico fundamental para manutenção da vida, a atividade do hipotálamo na manutenção da homeostase energética do organismo está sujeita a distúrbios que podem resultar na perda do perfeito acoplamento entre consumo e gasto calórico. O fácil acesso a dietas ricas em gorduras e o sedentarismo cria condições especialmente danosas para a função do hipotálamo resultando na indução de uma resposta inflamatória local e consequente perda funcional (Zhang, Wu et al. 2008, Milanski, Degasperi et al. 2009).

Um perfil de expressão gênica hipotalâmico revelou a modulação de cerca de 15% dos genes avaliados após o consumo de dieta rica em gordura por animais experimentais (De Souza, Araujo et al. 2005). Após análise funcional dos genes modulados, observou-se que aqueles relacionados a resposta inflamatória, incluindo TNF-α, IL-1β, IL-6 e INF-γ, eram os mais

afetados. Desta forma, demonstrou-se que, o consumo de dieta rica em ácidos graxos saturados leva a ativação de inflamação no hipotálamo.

Instalado o quadro inflamatório, quatro mecanismos principais atuam como responsáveis pela indução de resistência à insulina e leptina no sistema nervoso central. Tanto o TNF-α quanto o consumo de dietas hiperlipídicas podem induzir a ativação de serinas quinases como a JNK, que tem como alvo, o IRS1. A fosforilação em serina deste substrato do receptor de insulina bloqueia o sinal transdutório desse hormônio por impedir a ativação da PI3-quinase e Akt (De Souza, Araujo et al. 2005).

Ratos alimentados com dieta rica em gordura exibiram ainda o aumento de IKK, uma serina quinase que leva a degradação o IkB e, consequentemente, ativa o NFkB (Zhang, Wu et al. 2008). A ativação de IKK no hipotálamo desses animais promoveu a fosforilação em serina do IRS1, bloqueando o sinal da insulina.

Outro mecanismo proposto envolve a ativação de uma fosfatase de tirosina, a PTP1B. Essa proteína promove a defosforilação do IR e seus substratos, bloqueando ou pelo menos diminuindo o sinal da insulina (Picardi, Calegari et al. 2008).

Além disso, em modelos animais de obesidade induzida por dieta, ocorre a superexpressão de uma proteína responsiva a um grande número de citoquinas, a SOCS3 (Bjorbaek, Elmquist et al. 1998). Uma vez ativada, ela pode interagir com o receptor de leptina ObRb ou com o fator de transcrição STAT3 e bloquear esse sinal. Outra possibilidade envolve ubiquitinação e degradação proteassomal de substratos do receptor de insulina mediados por SOCS3 (Myers, Cowley et al. 2008).

Como a indução por fatores inflamatórios é particularmente interessante, numerosos estudos recentes sugerem que diabetes e obesidade são estados pró-inflamatórios e que a

inflamação atua como um importante papel mediador na resistência à insulina (Shoelson, Lee et al. 2006) e ao dano neuronal hipotalâmico (Milanski, Degasperi et al. 2009, Moraes, Coope et al. 2009).

A imunidade inata é a primeira linha de defesa do organismo contra microorganismos. A presença de um patógeno é detectada por vários tipos de células, imunes ou não-imunes. Essa detecção ocorre por meio de PAMPs (*pathogen-associated molecular patterns*), que são elementos dos patógenos, essenciais para sua sobrevivência e tipicamente distintos das moléculas do hospedeiro. A ligação dos PAMPs aos receptores do hospedeiro, chamados de PRR (*pattern recognition receptors*) ativa vias de sinalização que levam à indução de citocinas e quimiocinas como TNF-α e IFN-γ promovendo morte ou sobrevivência por meio da regulação de NFκB e apoptose (Bhoj and Chen 2009).

Embora amplamente expresso, NFκB é mantido inativo na maioria das células, sendo sequestrado no citoplasma por membros da família de proteínas inibidoras, IκB. Em resposta à estimulação por uma grande variedade de agentes, incluindo muitos derivados de microrganismos, IκBs são rapidamente degradadas pela via ubiquitina-proteossoma, permitindo a entrada do NFκB para o núcleo para modulação de um amplo espectro de genes cujos produtos atuam como mediadores pró-inflamatórios (Haddad 2002).

TNF-α, por meio do seu receptor TNFR1 e ativação de TLR4 são alguns dos responsáveis pela ativação do sistema imune inato, limitando o crescimento de patógenos e recrutando células imunes para o local da infecção. Após estimulação, uma sequência de proteínas promovem o processamento da procaspase-8 e o início do programa apoptótico. No entanto, em níveis normais, essa apoptose induzida por TNF-α ou TLR4 não ocorre, pois o próprio NFκB ativa a produção de proteínas antiapoptóticas, como o c-FLIP, um potente inibidor da caspase-8 além da

ativação de respostas celulares adaptativas como o estresse de retículo endoplasmático (Coope, Milanski et al. 2012).

Dentre os mecanismos que conectam a indução de resposta inflamatória ao desbalanço do controle energético há uma família de receptores de membrana denominados "*toll-like receptors*", TLR. Ácidos graxos saturados, provenientes da dieta ou administrados diretamente no sistema nervoso central, ativam TLR4 e uma cascata inflamatória que envolve ativação e aumento de expressão de JNK e IκB quinase (IKK) que, por sua vez, ativam a sinalização inflamatória via TNF-α e IL-1β e deflagram a resistência à insulina e leptina. É verdade ainda que, a interrupção genética ou farmacológica da sinalização mediada por TLR4 restabelece o ambiente inflamatório hipotalâmico e reverte a sensibilidade a esses hormônios tanto no sistema nervoso central, quanto na periferia, reduzindo adiposidade e esteatose hepática induzida por dieta (Milanski, Degasperi et al. 2009, Milanski, Arruda et al. 2012). Adicionalmente, também o bloqueio da sinalização de TNF-α melhora os sinais inflamatórios e de indução de Estresse de Retículo Endoplasmático além da sensibilidade periférica à insulina e as mudanças no balanço energético trazidas pela obesidade (Romanatto, Roman et al. 2009, Arruda, Milanski et al. 2011).

Dentro do mesmo contexto, Moraes, Coope et al. (2009) mostraram a modulação de 57% dos genes avaliados em uma varredura de alvos pró-apoptóticos em hipotálamo de animais alimentados com dieta hiperlipídica. A exposição prolongada a ácidos graxos saturados levou a uma perda de neurônios anorexigênicos no núcleo arqueado, por meio de apoptose e, consequentemente, diminuição dos disparos sinápticos no núcleo arqueado e hipotálamo lateral. Além disso, esses efeitos se mostraram dependentes da composição da dieta e não do aumento da ingestão calórica *per se*.

Transpondo esses achados para evidências em humanos, um estudo com pacientes obesos, antes ou após a realização de uma cirurgia bariátrica também mostra indícios de dano neuronal irreversível durante o desenvolvimento da obesidade. Treze pacientes obesos, selecionados para a realização de tratamento cirúrgico, e oito voluntários eutróficos foram avaliados por ressonância magnética funcional para investigar as correlações temporais frente a um estímulo nutricional (solução de glicose por via oral) e a ativação de regiões cerebrais. Em indivíduos magros, o hipotálamo apresentou o mais alto grau de ativação e conectividade com outras regiões cerebrais, ao passo que a ativação dessa região em pacientes obesos antes da intervenção cirúrgica estava diminuída e não foi completamente restabelecida mesmo após a perda de quase 30% do peso corporal total (van de Sande-Lee, Pereira et al. 2011).

Confirmando todas essas evidências, Thaler, Yi et al. (2012), demonstraram por diversos métodos a existência de dano neuronal e inflamação no hipotálamo de animais e humanos obesos. O estudo destacou ainda que, diferentemente da inflamação em tecidos periféricos (Posey, Clegg et al. 2009), o processo que se desenvolve no hipotálamo já está elevado nas primeiras 24 horas de exposição à dieta hiperlipídica. Subsequencialmente, com uma semana de exposição, é possível identificar alterações nas regiões do núcleo arqueado e da eminência média, envolvendo gliose e recrutamento de micróglia e astrócitos.

Tomados em conjunto, esses dados sugerem que a exposição sustentada a fatores ambientais obesogênicos excede a capacidade adaptativa e neuroprotetora do sistema nervoso central contribuindo assim para o dano irreversível de neurônios. Portanto, a identificação e caracterização de mecanismos potencialmente envolvidos no processo que leva ao dano de neurônios hipotalâmicos na obesidade pode contribuir para o desenvolvimento de abordagens terapêuticas mais eficazes para esta doença.

Em células eucarióticas, a imensa diversidade funcional das proteínas e a dinâmica do proteoma são estabilizadas por uma série de processos pós-traducionais de regulação. De forma simples, as proteínas podem ser modificadas pela ligação de pequenas moléculas como grupos fosfato, grupos metil, acetil ou, alternativamente, outros peptídeos (Xu and Peng 2006). A fina regulação do proteoma celular assegura sua viabilidade graças a uma rede de fatores que medeiam a expressão, o processamento e o transporte de proteínas e complexos protéicos, acoplados aos sistemas de degradação de curto e longo prazo (Balch, Morimoto et al. 2008). A degradação de proteínas envelhecidas, malformadas ou mal processadas é a última linha de defesa no controle de qualidade do proteoma celular e é realizada principalmente pelos sistemas ubiquitina-proteassoma (UPS) e pela autofagia (Arias and Cuervo 2011).

A ubiquitinação é um processo de modificação pós-traducional de proteínas. Composta por 76 aminoácidos, a ubiquitina é uma proteína que pode ser conjugada às proteínas-alvo de forma única ou em cadeias de poliubiquitinas (conjugação adicional de ubiquitinas) e essa marcação pode sinalizar para mecanismos proteolíticos ou não proteolíticos. Os primeiros incluem a degradação proteassômica para a eliminação de certos substratos visando à progressão adequada do ciclo celular, regulação transcricional, controle de qualidade das proteínas, correta transdução de sinais e ritmos circadianos (Bhoj and Chen 2009).

Dentre os mecanismos não proteolíticos encontram-se a endocitose de proteínas, tráfego intracelular, regulação da transcrição mediada por cromatina, reparo do DNA e interligação dos complexos de sinalização (Hochstrasser 2009).

O processo de ubiquitinação ocorre basicamente em três etapas: 1), A enzima ativadora de ubiquitina (E1) possui um resíduo cisteína que é ligado ao resíduo C-terminal da ubiquitina, formando uma ligação tioéster. Essa reação necessita de adenilação de um resíduo glicina da

ubiquitina pela ligação de um AMP proveniente da hidrólise do ATP, liberando pirofosfato. 2), Uma vez conjugada à E1, ocorre a transferência da molécula de ubiquitina para uma cisteína da enzima conjugadora (E2), formando outra ligação tioéster; e, 3), finalmente, a enzima ubiquitina-ligase (E3) é capaz de se ligar tanto na E2 como à proteína alvo de maneira a catalisar a ligação da porção C-terminal da molécula de ubiquitina na porção amina de uma lisina presente na proteína alvo (Pickart 2001, Huang, Miller et al. 2004) (Fig.1).

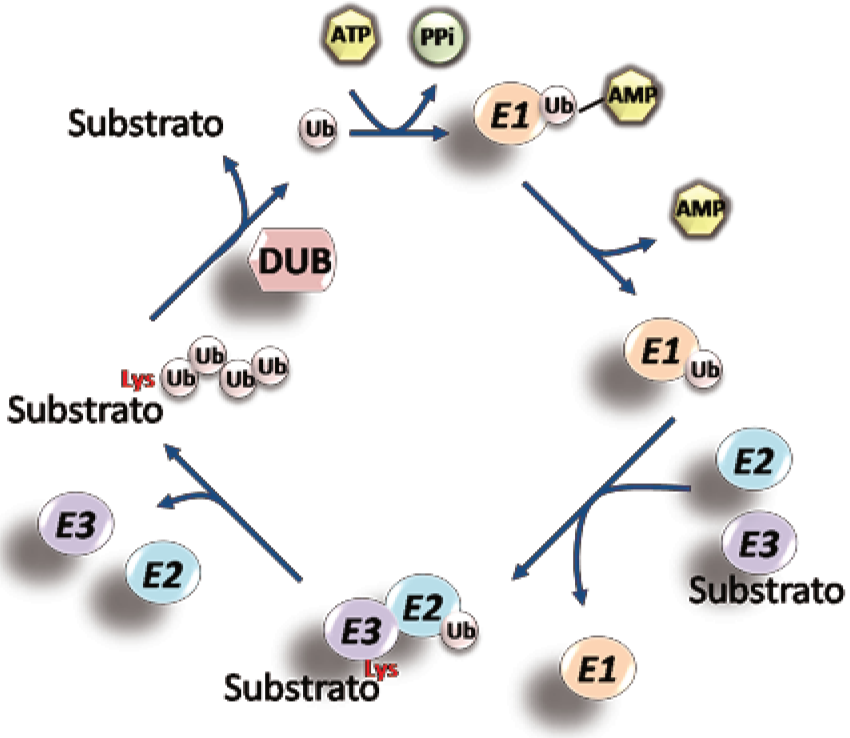

Figura 1. Processo de ubiquitinação de substratos

As células eucarióticas expressam algumas isoenzimas do tipo E1, até várias dezenas de E2 e muitas centenas de E3. Essa hierarquia de especificidade do UPS permite a modificação de muitas proteínas de maneira específica de acordo com padrões temporais e espaciais específicos. Durante a modificação de proteínas, diferentes E3 podem auxiliar na conjugação de moléculas de ubiquitina a proteínas que já foram modificadas por uma ou mais ubiquitinas. Certas E3 são

chamadas, algumas vezes, de E4, especialmente quando elas participam da extensão de cadeias de poliubiquitinas (Amerik and Hochstrasser 2004).

Enzimas conhecidas como deubiquitadoras, deubiquitinases (DUBs) ou isopeptidases podem ainda remover moléculas de ubiquitina ligadas às proteínas. Como resultado das atividades das DUBs, a ubiquitina modifica proteínas de modo transitório. Este processo de modificar dinamicamente as proteínas com ubiquitina cria parâmetros funcionais reversíveis de um substrato, que permite controlar numerosos processos celulares (Amerik and Hochstrasser 2004, Nijman, Luna-Vargas et al. 2005). Adicionalmente, algumas DUBs como a A20, têm dupla função: além de serem capazes de retirar moléculas de ubiquitina (pelo seu domínio N-terminal) ligadas em resíduo Lys63, elas podem, alternativamente, ligar outros monômeros (pelo resíduo C-terminal) em resíduos Lys48 e, dessa forma, controlar não só o destino da proteína modificada na sua via de sinalização, como direcioná-la para a degradação pelo proteassoma. É dessa forma que esse tipo de proteína controla a sinalização inflamatória induzida por TNF-α ou TLR4 modificando os destinos de TRAF6, RIP1 e IKK e bloqueando a citotoxicidade e apoptose induzida por ela (Wertz, O'Rourke et al. 2004).

Nos polímeros de ubiquitina, o resíduo de lisina de uma molécula de ubiquitina da cadeia está ligado à região C-terminal de outra molécula de ubiquitina e assim sucessivamente. Cada ubiquitina possui sete resíduos de lisina, cada um dos quais podem contribuir para essas ligações. Acredita-se que cadeias de poliubiquitinas ligadas alternativamente, como por exemplo, no resíduo 63 (Lys 63) possam executar funções de sinalização independentes da proteólise, enquanto ligadas na posição 48 (Lys 48), marcam proteínas alvo para a degradação num complexo protéico especializado em degradar proteínas, o proteassoma 26S (Chen, 2005). Além disso, cadeias de poliubiquitinas ligadas em Lys 63 têm sido recentemente associadas com a

formação e reconhecimento de agregados protéicos que podem ser encaminhados para a via autofágica.

O proteassoma é formado por dois subcomplexos protéicos, o complexo principal, ou *core* complexo, 20S e o complexo regulatório 19S. O primeiro é composto por subunidades a e b que representam a base da homologia nas diferentes escalas evolutivas. A subunidade 19S é regulatória e pode executar uma série de diferentes funções, primeiro pelo reconhecimento de substratos ubiquitinados, em seguida pela sua atividade isopeptidase que cliva as cadeias de poliubiquitinas em monômeros e, então, recicla as proteínas a ele encaminhadas. É a ligação da subunidade 19S a cada uma das cadeias do complexo 20S que abre o poro das extremidades permitindo a entrada das proteínas para serem desnaturadas e translocadas em pequenos peptídeos dentro dos compartimentos (Baumeister, Walz et al. 1998). Acredita-se que, em determinados tipos de células a regulação do proteassoma possa ser diferente, estando mais ou menos permissivo à degradação de proteínas marcadas com ubiquitina e modificadas adicionalmente, como é o caso de proteínas oxidadas. Também, a atividade desse sistema proteolítico no processamento de antígenos para apresentação ao MHC de classe II revela um papel diferencial dos chamados imunoproteassomas no controle da inflamação e da formação de agregados protéicos (Seifert, Bialy et al. 2010, Pickering and Davies 2012).

Todas as células eucarióticas têm dois sistemas principais para a degradação de componentes intracelulares: o UPS e a autofagia. Autofagia é um termo genérico para a degradação de componentes celulares nos lisossomos, incluindo macromoléculas solúveis e organelas. Durante esse processo, parte do citoplasma é rodeada por uma dupla camada de membrana, presumidamente originária do retículo endoplasmático, para formar um autofagossomo que então, se funde ao lisossomo a fim de degradar o material anteriormente

sequestrado. Isso requer a participação de proteínas relacionadas à autofagia (ATGs) (Levine and Kroemer 2008). Existem pelo menos três tipos de autofagia: a macroautofagia, a autofagia mediada por chaperonas e a microautofagia (Mizushima 2010). Acredita-se que a macroautofagia (daqui por diante referida como autofagia) seja a principal via entre vários subtipos de autofagia e, ao contrário do UPS que responde pela maior parte da degradação protéica intracelular seletiva, a autofagia pode ser menos seletiva.

Dentre os eventos que se sucedem até o estabelecimento do autofagossomo, existe uma diversidade de proteínas que são ligadas umas às outras em um processo "*ubiquitin like*" e, que dependem basicamente do recrutamento e ativação de proteínas Atg e Beclin para o isolamento da membrana (Itakura and Mizushima 2010) seguido da complexação de Atg5-Atg12 durante a elongação do autofagossomo, até a sua ativação e fusão com o lisossomo que se dá pelo reconhecimento e ativação de LC3 (Nakatogawa, Suzuki et al. 2009).

Ambos, UPS e autofagia possuem a capacidade de reconhecer e degradar proteínas ou complexos protéicos conjugados com moléculas de ubiquitina. O exato ponto de intercessão entre esses dois sistemas não está completamente elucidado, mas existem fortes linhas de evidências que sugerem a inibição do proteassoma como suficiente para ativar a autofagia em cardiomiócitos (Zheng, Su et al. 2011) e o aumento de p62 em decorrência tanto da insuficiência funcional do proteassoma, quanto da inibição crônica da autofagia (Komatsu, Waguri et al. 2007).

A p62/SQSTM1 é uma proteína adaptadora multifuncional que está envolvida na sinalização e diferenciação celular por interagir com outras proteínas em sua região N-terminal e, ao mesmo tempo, por possuir uma região C-terminal que se liga a ubiquitina ligadas em Lys48 e Lys63 (Moscat, Diaz-Meco et al. 2007, Wooten, Geetha et al. 2008). Por outro lado, p62 recruta proteínas ubiquitinadas ao autofagossomo por ligação em regiões específicas (domínio LIR) que

promovem o recrutamento e ativação de LC3, na membrana do autofagossomo (Itakura and Mizushima 2011).

Em situações em que existe aumento da produção ou no processamento de proteínas, bem como a formação de proteínas malformadas, o controle de qualidade do proteoma pode ter sua capacidade excedida ou funcionar de maneira inadequada, permitindo um estresse proteotóxico que contribui para a progressão de várias doenças humanas de aparecimento tardio como as doenças neurodegenerativas e, mais recentemente doenças endócrino-metabólicas (Wang and Robbins 2006).

A inabilidade de alguns neurônios em prevenir o acúmulo de proteínas ubiquitinadas em inclusões neuronais pode levar à neurodegeneração (Lowe, Mayer et al. 2001) por meio da indução de resposta inflamatória que pode ser o fator gerador, ou ainda ser desencadeada pela expansão de agregados protéicos anormais no espaço nuclear e citosólico (Leroy, Boyer et al. 1998).

A importância desses sistemas proteostáticos nas mais diversas vias fisiopatológicas começaram a ser caracterizadas na última década, quando os mecanismos de dano celular foram estreitamente conectados com a sobrevida e metabolismo dessa célula. Foi nesse contexto que Ryu, Sinnar et al. (2008) caracterizaram um fenótipo de normofagia, hiperleptinemia e obesidade na idade adulta de animais experimentais que sofreram a deleção do gene Ubb, que codifica as ubiquitinas. O *knockout* provocou perda de 30% de neurônios predominantemente do núcleo arqueado, co-localizando proteínas ubiquitinas e neurônios produtores de NPY, AgRP e POMC, associadas a gliose persistente além de distúrbios no sono.

De maneira muito semelhante do ponto de vista fenotípico, a super-expressão de uma E4 ligase aumentou a formação de agregados protéicos positivos para ubiquitina e p62, levando a

degeneração neural e déficit funcional de subpopulações neuronais hipotalâmicas, resultando no desenvolvimento de obesidade hiperfágica e anormalidades metabólicas associadas a ela como redução de gasto energético total, acúmulo de tecido adiposo em hepatócitos e intolerância à glicose (Susaki, Kaneko-Oshikawa et al. 2010).

Simultaneamente, um estudo destacou a importância do processo autofágico na indução de estresse de retículo endoplasmático e resistência à insulina em tecidos periféricos, como o fígado. A redução da expressão de Atg7 resultou em defeito na sinalização hepática da insulina e estresse de retículo endoplasmático, em contrapartida quando a expressão proteica foi restaurada, as adaptações celulares foram interrompidas e a sensibilidade à insulina restabelecida. Adicionalmente, em ambos os modelos de obesidade, genético ou ambiental, existe defeito na autofagia hepática, particularmente na expressão de Atg7 e LC3 (Yang, Li et al. 2010).

O fenótipo obeso em decorrência de defeito hipotalâmico foi também obtido quando ATG7 foi depletada geneticamente em neurônios POMC. A perda desta subpopulação neuronal, em decorrência de uma atenuação do processo autofágico causou um aumento dramático no peso corporal após o desmame acompanhado de aumento da adiposidade e intolerância à glicose. Além disso, conectando as vias proteolíticas, essas modificações surgiram possivelmente devido a acúmulo de proteínas ubiquitinadas e marcadas com p62 no hipotálamo, levando a perda das projeções neuronais (Coupe, Ishii et al. 2012). Esses acúmulos de agregados protéicos no hipotálamo levaram a uma diminuição da sensibilidade à leptina, hiperfagia, redução de gasto energético e maior susceptibilidade à hiperglicemia após uma dieta hiperlipídica (Quan, Kim et al. 2012).

Uma vez que, durante o estabelecimento da obesidade, concomitante à disfunção de vias de sinalização que regulam a fome e a termogênese e à inflamação subclínica está claro que

existe perda anátomo-funcional no hipotálamo como resultado da apoptose neuronal, a obesidade pode ser considerada uma doença que decorre, pelo menos em parte, de uma disfunção com características neurodegenerativas no cérebro.

Considerando ainda que, na maior parte das doenças neurodegenerativas clássicas, ainda que a ativação anômala do UPS não seja o fator desencadeador, mas que existe o acúmulo de proteínas ubiquitinadas, aventamos a hipótese de que durante o desenvolvimento da obesidade há distúrbios das vias de ubiquitinação no hipotálamo. Isso poderia então contribuir para a lesão hipotalâmica observada bem como para a o estabelecimento do ambiente celular cíclico que alimenta esse dano e as sucessivas perdas no controle da homeostase energética corporal.

OBJETIVOS

Objetivo Geral

Caracterizar o perfil de ativação do sistema ubiquitina-proteassoma em hipotálamo de animais submetidos à dieta hiperlipídica.

Objetivos Específicos

- Caracterizar o fenótipo metabólico e inflamatório de camundongos Swiss submetidos à dieta hiperlipídica durante 16 semanas;
- Avaliar, por varredura de PCR em tempo real, a expressão de genes do sistema ubiquitina-proteassoma (UPS) em hipotálamo de camundongos Swiss submetidos à dieta hiperlipídica durante 16 semanas;
- Avaliar o conteúdo e a distribuição hipotalâmica de proteínas do UPS em camundongos Swiss submetidos à dieta hiperlipídica durante 8 e 16 semanas;
- Avaliar o conteúdo proteico de componentes do UPS em hipotálamo de camundongos geneticamente protegidos da obesidade e submetidos à dieta hiperlipídica durante 16 semanas;
- Avaliar as repercussões fenotípicas da obesidade em camundongos Swiss submetidos a intervenções farmacológicas ou genéticas do UPS no hipotálamo.

CAPÍTULO 1

Regular Manuscript

Defective regulation of the ubiquitin/proteasome system in the hypothalamus of obese mice

Abbreviated Title: Ubiquitin/proteasome system in the hypothalamus

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Abstract

In both human and experimental obesity, an inflammatory damage of the hypothalamus plays an important role in the loss of the coordinated control of food intake and energy expenditure. Upon prolonged maintenance of increased body mass, the brain changes the defended set-point of adiposity and returning to normal weight becomes extremely difficult. Here, we show that in prolonged, but not short-term obesity, the ubiquitin/proteasome system in the hypothalamus fails to maintain an adequate rate of protein recycling, leading to the accumulation of ubiquitinated proteins. This is accompanied by increased co-localization of ubiquitin and p62 in the arcuate nucleus and reduced expression of markers of autophagy in the hypothalamus. Genetic protection from obesity is accompanied by normal regulation of the ubiquitin/proteasome system in the hypothalamus, while inhibition of proteasome or p62 results in the acceleration of body mass gain in mice exposed for a short period to a high-fat diet. Thus, the defective regulation of the ubiquitin/proteasome system in the hypothalamus may be an important mechanism involved in the progression and auto-perpetuation of obesity.

Introduction

Neurons of the medial-basal hypothalamus play a central role in whole-body energy homeostasis (Schwartz and Porte 2005, Williams and Elmquist 2012), and a number of interventions aimed at modulating the activity of such neurons can result in obesity (Schwartz and Porte 2005, Velloso and Schwartz 2011, Williams and Elmquist 2012). Feeding on a high-fat diet is commonly used as a method to produce experimental obesity. Work carried out during the last 10 years has shown that, besides increased caloric intake, high-fat diets can induce an inflammatory process in the hypothalamus, leading to neuronal resistance to leptin and eventually, to neuronal apoptosis (De Souza, Araujo et al. 2005, Zhang, Zhang et al. 2008, Milanski, Degasperi et al. 2009, Moraes, Coope et al. 2009, Ozcan, Ergin et al. 2009), providing an anatomical and functional basis for obesity.

It is noteworthy that, although most studies exploring the mechanisms behind hypothalamic dysfunction in obesity have been performed in rodents, two recent studies using neuroimaging to evaluate the hypothalamus of humans have provided strong evidence for dysfunction and neuronal loss associated with obesity (van de Sande-Lee, Pereira et al. 2011, Thaler, Yi et al. 2012). Thus, defining the mechanisms that link dietary components with hypothalamic inflammation, neuronal dysfunction and eventually neuronal loss, may unveil potential targets for a more efficient treatment of obesity.

An important aspect of both experimental and human obesity is that, the longer obesity persists, the harder it is to reestablish correct energy homeostasis (Guo, Jou et al. 2009, Kraschnewski, Boan et al. 2010). This is particularly evident in patients undergoing a number of dieting programs and continuously regaining body mass (Leibel 2008, Kraschnewski, Boan et al. 2010). Although the reasons for the progressive increase in the defended set-point for body adiposity are

unknown, we suspect that diet-induced loss of hypothalamic neurons involved in the regulation of energy homeostasis may play an important role in this process (Moraes, Coope et al. 2009, Li, Tang et al. 2012).

In this study, we hypothesized that a malfunction of the ubiquitin/proteasome system could contribute to the continuous deterioration of the hypothalamic neurons regulating body energy homeostasis. Ubiquitination of proteins plays a broad role in cellular homeostasis. It can, through its canonical function, target old and damaged proteins to proteasomal degradation (Welchman, Gordon et al. 2005); in addition, it targets potentially harmful protein aggregates that cannot be degraded by the 26S proteasome, to autophagic disassembly (Kirkin, McEwan et al. 2009). Ubiquitination can also modulate and be modulated by inflammation (Balch, Morimoto et al. 2008, Vereecke, Beyaert et al. 2009), which is involved in obesity-dependent insulin resistance (Hotamisligil 2006). Defects in any of these functions of the ubiquitin system can potentially lead to uncontrolled inflammation, and eventually to apoptosis. Here, we evaluated the expression, activity and hypothalamic distribution of proteins of the ubiquitin/proteasome system in experimental obesity. We show that prolonged-, but not short-term feeding on a high-fat diet results in the accumulation of ubiquitinated proteins in the hypothalamus, which is accompanied by decreased proteasome expression and formation of protein aggregates.

Materials and Methods

Experimental Animals. Six-week old male Swiss mice, male TNFRp55$^{-/-}$ or TNFRp55$^{+/}$mice (knockout for the TNFα receptor 1 and its respective control) (Romanatto, Roman et al. 2009) and male C3H/HeJ or C3H/HeN mice (loss-of-function mutation for TLR4 and its respective control) (Tsukumo, Carvalho-Filho et al. 2007) were fed on standard rodent chow or on a high-fat

diet (Table 1) for 8 or 16 weeks. In some experiments, Swiss mice fed on a high-fat diet for 8 weeks were stereotaxically instrumented using a Stoelting stereotaxic apparatus, according to a previously described method (Romanatto, Roman et al. 2009). Stereotaxic coordinates were: anteroposterior, 0.34 mm; lateral, 1.0 mm; and depth, 2.2mm to lateral ventricle and anteroposterior, 0.5mm; lateral, 0.2mm; and depth 3.5mm to third ventricle. Cannula efficiency was tested one week after cannulation by the evaluation of the drinking response elicited by the intracerebroventricular injection of angiotensin II (2uL, 10^{-6}M; Sigma, St. Louis, MO, USA). Thereafter, mice were intracerebroventricularly treated with a proteasome inhibitor, lactacystin (2uL, 100uM; Calbiochem, Darmstadt, Germany) for five days or with siRNA to p62, as described below. All experimental procedures were performed in accordance with the guidelines of the Brazilian College for Animal Experimentation and were approved by the ethics committee at the State University of Campinas.

Small interfering RNA (siRNA) treatment. A siRNA targeting p62 and a scrambled siRNA (sc29828 and sc37007, Santa Cruz Biotechnology, CA) were complexed and diluted as previously described (Kinote, Faria et al. 2012). The animals were evaluated for 6 days when two microliters of the mixtures containing either the siRNA to p62 or the scrambled siRNA were injected through the cannula positioned in third ventricle on the first, third, and fifth days.

Blood biochemistry, hormone and cytokine determination. Glucose was determined in blood using a glucometer from Abbott (Opptimum, Abbott Diabetes Care, Inc., Alameda, CA, USA). Insulin, leptin, IL-1β, TNF-α and IL-6 were determined using ELISA kits from Millipore (Billerica, MA, USA).

Hyperinsulinemic–euglycemic clamp. Glucose consumption were assessed after 12h of starvation, when the mice were anesthetized with sodium pentobarbital (50 mg/kg body weight) injected ip,

and catheters were then placed in the left jugular vein (for tracer infusions) and the carotid artery (for blood sampling), as previously described (Prada, Zecchin et al. 2005). A prime continuous insulin infusion at a rate of 3.6 mU/kg body weight/min was performed to raise the plasma insulin concentration to approximately 800–900 pmol/L. Plasmatic glucose concentrations was measured in blood samples at 5-min intervals and 10% unlabeled glucose was then infused at variable rates to maintain plasma glucose at fasting levels. Blood samples were collected before the start and during the glucose infusion period (every 30 min) for measurement of plasma insulin concentrations. All infusions were performed using Harvard infusion pumps.

Determination of oxygen consumption/carbon dioxide production and respiratory exchange ratio. Oxygen consumption/carbon dioxide production and respiratory exchange ratio (RER) were measured during a dark cycle in fasted mice using a computer-controlled, open circuit calorimeter system LE405 Gas Analyzer (Panlab-Harvard Apparatus, Holliston, MA). The air flow within each chamber was monitored by a sensor Air Supply and Switching (Panlab-Harvard Apparatus). Gas sensors were calibrated prior to the onset of experiments with primary gas standards containing known concentrations of O_2, CO_2, and N_2 (Air Liquid, Sao Paulo, Brazil). The data were obtained for 6 min for each chamber during the 12-hour dark cycle and the mean for each 6 min of measurement was used for data analysis. Thus, each mouse was evaluated for 72 min. Outdoor air reference values were sampled after every four measurements. Sample air was sequentially passed through O_2 and CO_2 sensors for determination of O_2 and CO_2 content, from which measurements of oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were estimated. The VO_2 and VCO_2 were calculated by Metabolism 2.2v software based on Withers equation and the RER was calculated using VCO_2/VO_2.

Leptin Tolerance Test. Mice were cannulated in the lateral ventricle and submitted to a 12 h fast. Leptin was administered *icv* (2 uL, 10^{-6}M; Sigma, St. Louis, MO, USA) at 18h and spontaneous food intake was measured for four hours during dark cycle.

Immunoblotting. Hypothalamus was homogenized in a tissue homogenizer (15s) (Polytron-Aggregate, Kinematica, Littau/Luzern, Switzerland) at maximum speed in an anti-protease cocktail (10 mmol/L imidazole, pH 8.0, 4 mmol/L EDTA, 1 mmol/L, aprotinin, 2.5 mg/L leupeptin, 30 mg/L trypsin inhibitor, 200 µmol/L DTT and 200 µmol/L phenylmethylsulfonyl fluoride). After sonication, an aliquot of extract was collected and the total protein content was determined by the dye-binding protein assay kit (Bio-Rad Laboratories, Hercules, CA). Samples containing 50µg of protein from each experimental group were incubated for 5 minutes at 95°C with 4x concentrated Laemmli sample buffer (1 mmol sodium phosphate/L, pH 7.8, 0.1% bromophenol blue, 50% glycerol, 10% SDS, 2% mercaptoethanol) and then separated on 10% polyacrylamide gels for approximately 4h. Electrotransfer of proteins to nitrocellulose membranes (Bio-Rad) was performed in a Trans Blot SD Semi-Dry Transfer Cell (Bio-Rad) for 20min at 25V (constant) in buffer containing methanol and SDS. After checking the efficiency of transfer by staining with Ponceau S, the membranes were blocked with 5% skimmed milk in TTBS (10 mmol Tris/L, 150 mmol NaCl/L, 0.5% Tween 20) overnight at 4°C. Ubiquitin, proteasome, A20, p62, LC3, Beclin and beta-actin were detected in the membranes after overnight incubation at 4°C with primary antibodies (Ubiquitin, ab7780; Proteasome, ab58115; p62, ab56416; Beclin, ab16998; β-actin ab8227; all from AbCam, Cambridge, MA, USA; A20, sc166692, from Santa Cruz Biotechnology, Santa Cruz, CA; and LC3, #2775 from Cell Signaling, Boston, MA, USA) (diluted 1:500 in TTBS containing 3% dry skimmed milk). The membranes were then incubated with a secondary specific IgG antibody (diluted 1:5000 in TTBS

containing 1% dry skimmed milk) for 2h at room temperature. Enhanced chemiluminescence (SuperSignal West Pico, Pierce) after incubation with a horseradish peroxidase-conjugated secondary antibody was used for detection by autoradiography. Band intensities were quantified by optical densitometry (UN-Scan-it Gel 6.1, Orem, Utah, USA).

RNA extraction, Real Time qRT-PCR and PCR Array. The samples were homogenized in TRIzol reagent (Invitrogen, São Paulo, Brasil) in a tissue homogenizer (15s) (Polytron-Agregate, Kinematica, Littau/Luzern, Switzerland) at maximum speed. The total RNA content was then isolated according to the manufacturer´s instructions, quantified and analyzed by spectrophotometry (NanoDrop 8000, Thermo Scientific, Wilmington, DE, USA). The integrity of RNA was assessed by running a denaturing agarose gel. cDNA synthesis was performed in 3ug of total RNA, according the manufacturer´s instructions (High Capacity cDNA Reverse Transcription Kit, Life Technologies, Van Allen Way Carlsbad, CA, USA). The TaqMan System was used in association with real-time PCR to detect hypothalamic TNF-α, IL6, IL-1β and PGC1□, UCP1 in the brown adipose tissue (Mm99999068_m1; Mm00446190_m1; Mm00434228_m1; Mm01208835_m1; Mm01244861_m1, respectively - Life Technologies, Van Allen Way Carlsbad, CA, USA) and the mouse GAPDH gene was used as an endogenous control (#4352339E). The cycle threshold was obtained by analysis with 7500 System SDS Software (Applied Biosystems – Life Technologies). Gene expression was analyzed by Real Time PCR using the PCR Array System (RT² Profiler PCR Array Mouse Ubiquitin Proteasome System #PAMM099Z, QIAGEN Biotecnologia Brasil, Sao Paulo, SP, BR). Global analysis of 84 genes specific to the Ubiquitin Proteasome System was performed using a 7500 Platform and analyzed by PCR Array Data Analysis Software (Excel & Web based).

Immunofluorescence staining. For histological evaluation, hypothalamic tissue samples were fixed in paraformaldehyde (4% final concentration in phosphate-buffered saline [PBS; 50mmol/L of $NaH_2PO_4 \cdot H_2O$; 5mmol/L of KCl; 1.5mmol/L of $MgCl_2 \cdot 6H_2O$; and 80.1 mmol/L of NaCl; pH 7.4]) and processed routinely for embedding in a paraffin block. The samples were submitted to dehydration (alcohol at 70%, 80%, 90%, 95%, and absolute alcohol) being diaphanized by immersion in xylol and embedded in paraffin. Subsequently, the hydrated (alcohol at absolute, 95%, 90%, 80%, and 70% concentrations) 5.0 um paraffin sections were processed for immunofluorescence staining using the Ubiquitin, p62, Cd11b and HuR antibodies (sc20050; sc5261, respectively - Santa Cruz Biotechnology, Santa Cruz, CA) and the secondary antibodies conjugated to FITC or rhodamine (sc2777; sc2092, respectively - Santa Cruz Biotechnology, Santa Cruz, CA). The images were obtained using a Confocal Laser Microscopy (LSM510, Zeiss, New York, NY). Analysis and documentation of results were performed using a Leica Application Suite V3.6 (Switzerland)**.**

Transmission electron microscopy. Mice were submitted to whole-body trans-cardiac perfusion with Karnovsky solution (2.5% glutaraldehyde and 1% paraformaldehyde in phosphate buffer pH 7.4). The brain was dissected and stored overnight in fixative at 4°C. The material was osmicated, dehydrated and embedded in Durcupan (Fluka Sigma-Aldrich, St. Louis, MO). Coronal sections (55-60 nm) from brain were obtained by using a PowerTome X ultramicrotome (RMC Products, Boeckeler Instruments, Tucson, AZ). Ultrathin sections were collected and stained with toluidine blue. The third ventricle area containing neurons of the medium-basal hypothalamus was trimmed and ultrathin sections were collected on formvar-coated copper grids, counterstained with uranyl acetate and lead citrate, and examined in a Tecnai G2 Spirit Twin (FEI, Hillsboro, OR) transmission electron microscope operated at 80 kV. Neurons were

photographed and the digital images were used for ultrastructural analysis. For each condition, eight randomly selected distinct neurons were initially photographed in medium-magnification 10 μm^2 fields. Distinct areas of the selected neurons were thereafter photographed in high-magnification 5 μm^2 fields and autophagosomes and protein aggregates were counted in 4-6 high-magnification fields of each of the eight randomly selected neurons.

Statistical analysis. Results are presented as means ± SE. Levene's test for the homogeneity of variances was initially used to check the fit of data to the assumptions for parametric analysis of variance. When necessary, to correct for variance heterogeneity or non-normality, data were log-transformed (Lundbaek 1962). All results were analyzed by *t-test* or One-way ANOVA and complemented by the Tukey test to determine the significance of individual differences. The level of significance was set at $p<0.05$. The data were analyzed using Statistic for Windows, 7.0 (StatSoft, Inc., Tulsa, OK, USA).

Results

Phenotypic characterization of Swiss mice fed on a high-fat diet. Swiss mice are an outbred strain genetically related to the diabetes-prone AKR mice (Rossmeisl, Rim et al. 2003). When fed on a high-fat diet (obese), Swiss mice present 1.2- and 1.5-fold increases in body mass, as compared to mice fed on chow (lean), at 8 and 16 weeks, respectively (Fig. 1A). This is not accompanied by any significant change in caloric intake (Fig. 1B) at the time of evaluation; i.e., at 8 or 16 weeks on high-fat diet. Increased caloric intake occurs only during the first 2-3 weeks after introduction of high-fat diet, normalizing thereafter (data not shown). At 16 weeks, the serum levels of leptin (Fig. 1C) and insulin (Fig. 1D) are increased, while glucose consumption during a

hyperinsulinemic-euglycemic clamp is reduced (Fig. 1E). The serum levels of TNF-α (Fig. 1F), IL-1β (Fig. 1G) and IL-6 (Fig. 1H), as well as the hypothalamic expression of the mRNAs of TNF-α (Fig. 1I), IL-6 (Fig. 1J) and IL-1β (Fig. 1K) are increased in the obese mice. Finally, at 16 weeks, the obese mice are resistant to the anorexigenic effect of leptin in the hypothalamus (Fig. 1L).

Return to chow results in more pronounced body mass loss in mice fed for 8 weeks on the high-fat diet, as compared to 16 week-fed mice. Both humans and experimental animals with long-lasting, severe obesity are expected to be resistant to body mass loss when undergoing caloric restriction (Kraschnewski, Boan et al. 2010, Bumaschny, Yamashita et al. 2012). To evaluate the impact of diet change in body mass reduction in our experimental model, Swiss mice fed *ad libitum* for 8 or 16 weeks on high-fat diet were transferred to chow *ad libitum* and followed up for 7 days. At the beginning of the experimental period, mice fed on the high-fat diet for 16 weeks had a significantly higher body mass, as compared to mice fed on the high-fat diet for 8 weeks (Fig. 2A). Although total caloric intake was similar between the groups (Fig. 2B), mice fed for 8 weeks on a high-fat diet presented a significantly greater reduction of body mass, as compared to 16 week-fed mice (Fig. 2C).

Modulation of hypothalamic ubiquitin-related proteins in diet-induced obesity. The expressions of 84 ubiquitin-related genes were evaluated, using a Real Time qRT-PCR array, in the hypothalamus of diet-induced obese Swiss mice fed for 16 weeks on a high-fat diet. In general, there was a 15.4% modulation of gene expression, where 7.1% of genes were up-regulated and 8.3% were down-regulated (Fig. 3A). Out of all the regulated genes, those coding for proteins with E3 ligase activity were the most affected, followed by those coding for E2 conjugating activity (Fig. 3B). Genes coding for proteins with E3 ligase activity were predominantly down-

regulated (Fig. 3C). All the genes evaluated in the array are presented as Supplementary Table and the modulated genes are presented in Table 1.

Accumulation of ubiquitinated proteins in the hypothalamus of mice fed on high-fat diet for 16 but not 8 weeks. After 8 weeks on the diet, the amounts of ubiquitinated proteins in the hypothalamic extracts were lower in obese, as compared to lean mice (Fig. 4A, upper left-hand panel). This was accompanied by the increased expression of the A20 deubiquitinase (Fig. 4A, upper right-hand panel) and proteasome (Fig. 4A, lower right-hand panel), while the levels of p62 were similar between lean and obese mice (Fig. 4A, lower left-hand panel). Conversely, at 16 weeks of diet, there was an increased amount of ubiquitinated proteins in the hypothalamus of obese mice (Fig. 4B, upper left-hand panel). This was accompanied by reduction in A20 (Fig. 4B, upper right-hand panel) and proteasome (Fig. 4B, lower right-hand panel), and by an increased expression of p62 (Fig. 3B, lower left-hand panel).

Increased co-localization of ubiquitin and p62 in the hypothalamus of mice fed on a high-fat diet for 16 weeks. The formation of intracellular aggregates containing ubiquitin and the adaptor protein p62 occurs in neurodegenerative conditions such as Parkinson's and Alzheimer's diseases (Gal, Strom et al. 2007, Komatsu, Waguri et al. 2007) and also in the hypothalamus of obese mice overexpressing the E4 enzyme, E4B (Susaki, Kaneko-Oshikawa et al. 2010). Here, we found ubiquitin and p62 distributed throughout the hypothalamic areas involved in the control of energy homeostasis: arcuate nucleus (Fig. 5A and 5D); paraventricular nucleus (Fig. 5B and 5E); and the lateral hypothalamus (Fig. 5C and 5F), with a clear predominance in cells in the arcuate nucleus. However, the co-localization of ubiquitin and p62 was much more evident in the hypothalamus of mice fed on the high fat diet for 16 weeks, as compared to 8 weeks, particularly in the arcuate nucleus (Fig. 5D).

Ubiquitin and p62 are expressed in neurons and microglia of the hypothalamus. Labeling cells with the neuron and microglia cells specific markers, HuR and CD11b, respectively, provided evidence that both ubiquitin and p62 are expressed in both cell types in the arcuate nucleus of obese mice (Fig 5G-5J). However, co-expression was more evident in neurons than in microglia (Fig 5G-5J).

Increased presence of protein aggregates in hypothalamic neurons of long-term obese mice. Transmission electron microscopy evaluation of neurons of the hypothalamus revealed an increased presence of protein aggregates in the medium-basal region of obese mice (Fig. 6).

Reduced expression of markers of autophagy in the hypothalamus of obese mice fed for 16 weeks on a high-fat diet. Reduced autophagy in hypothalamic neurons results in increased adiposity (Meng and Cai 2011, Coupe, Ishii et al. 2012). Since p62 is involved in connecting the ubiquitin and the autophagy routes, we evaluated the impact of prolonged feeding with a high-fat diet on markers of autophagy in the hypothalamus of Swiss mice. As depicted in Figure 7, 8 weeks of a high-fat diet is insufficient to change the expression of beclin and LC3 (Fig. 7A). Conversely, after 16 weeks on a high-fat diet, the expressions of both beclin and LC3 were significantly reduced (Fig. 7B). This was accompanied by a significant reduction in the number of autophagosomes detected by transmission electron microscopy (Fig. 7C)

Stability of the ubiquitin/proteasome system in the hypothalamus of obesity-resistant mutants. Knockouts for the TNFR1 and TLR4 genes are protected from diet-induced obesity at least in part because they do not present hypothalamic inflammation upon high-fat feeding (Milanski, Degasperi et al. 2009, Romanatto, Roman et al. 2009, Arruda, Milanski et al. 2011). In fact, after 16 weeks on high-fat feeding, TNFR1 (Fig. 8A) and TLR4 (Fig. 8B) knockouts present similar body masses to their respective chow-fed controls. In the hypothalamus of TNFR1 knockout

mice fed on high-fat diet, the amount of ubiquitin (Fig. 8C, upper left-hand panel) was reduced as compared to mice fed on chow, while the expressions of A20 (Fig. 8C, upper right-hand panel), p62 (Fig. 8C, lower left-hand panel) and proteasome (Fig. 8C, lower right -hand panel) were similar to those of chow-fed mice. In the hypothalamus of TLR4 knockout mice fed for 16 weeks on a high-fat diet, the expressions of ubiquitin (Fig. 8D, upper left-hand panel), A20 (Fig. 8D, upper right-hand panel), p62 (Fig. 8D, lower left-hand panel) and proteasome (Fig. 8D, lower right-hand panel) were all similar to those of chow-fed mice.

Chemical inhibition of hypothalamic proteasome results in body mass gain. To test the hypothesis that the stability of the ubiquitin/proteasome system in the hypothalamus of mice fed for 8 weeks on a high-fat diet plays an important role in the maintenance of energy homeostasis, mice were treated via intracerebroventricular injections with the chemical inhibitor of proteasome, lactacystin, for 5 days (Fig. 9A), leading to the increased accumulation of ubiquitin in the hypothalamus (Fig. 9B-C). The lactacystin-treated mice presented a greater increase in body mass (Fig. 9D-E) and increased caloric intake (Fig. 9F), as compared to control. Lactacystin treatment produced no changes in energy expenditure (Fig. 9G-I) and in the expression of markers of thermogenesis in the brown adipose tissue, such as cytochrome-C (Fig. 9J), PGC-1□ (Fig. 9K) and UCP1 (Fig. 9L). In addition, lactacystin treatment was not sufficient to modulate the hypothalamic expression of TNF-□(Fig. 9M), but produced an increased expression of IL-1β (Fig. 9N).

Inhibition of hypothalamic p62 results in body mass gain. The anomalous expression of the adaptor protein p62 is known to be involved in the formation of protein aggregates in neurodegenerative diseases, and changes in its expression can lead to obesity (Gal, Strom et al. 2007, Komatsu, Waguri et al. 2007, Susaki, Kaneko-Oshikawa et al. 2010). The

intracerebroventricular treatment of Swiss mice, fed for 8 weeks on high-fat diet, with a siRNA targeting p62 (Fig. 10A), resulted in a 70% reduction in hypothalamic p62 expression (Fig. 10B – lower, left-hand panel), which was accompanied by increased accumulation of ubiquitin (Fig. 10B – upper, left-hand panel), increased expression of proteasome (Fig. 10B – lower, right-hand panel), and no change in the expression of A20 (Fig. 10B – upper, right-hand panel) in the hypothalamus. The inhibition of hypothalamic expression of p62 resulted in increased body mass and increased caloric intake (Fig. 10C-E).

Discussion

One of the most relevant questions in obesity is understanding the reason why prolonged and severe increase in body mass results in changes in the defended set-point of adiposity, which contributes to the auto-perpetuation of the disease (Kraschnewski, Boan et al. 2010, Bumaschny, Yamashita et al. 2012). A few recent studies have provided some functional and anatomical basis for this phenomenon. In diet-induced obesity, there is selective activation of apoptosis in the neurons of the hypothalamus (Moraes, Coope et al. 2009, Thaler, Yi et al. 2012). Genetic predisposition to obesity favors increased apoptosis of neurons, exerting catabolic functions (Moraes, Coope et al. 2009), while diet-induced activation of IKKβ/NFκB signaling in the hypothalamus leads to neuronal apoptosis and impairment of the neural stem cell-dependent replacement of neuronal loss (Li, Tang et al. 2012). As a whole, these processes can lead to an imbalance in the subpopulations of hypothalamic neurons, promoting orexigenic/anti-thermogenic versus anorexigenic/pro-thermogenic effects (Moraes, Coope et al. 2009, Li, Tang et al. 2012, Thaler, Yi et al. 2012). Thus, diet-induced apoptosis of selected neuronal groups in the hypothalamus may be at least one of the factors contributing to the refractoriness of obesity to

conventional therapy. Remarkably, recent studies using distinct methods of neuroimaging have shown signs of irreversible damage in the hypothalamus of obese humans (van de Sande-Lee, Pereira et al. 2011, Thaler, Yi et al. 2012), providing additional interest to this question.

Further advance in the characterization of the roles played by distinct hypothalamic neuronal subpopulations and prolonged obesity in the auto-perpetuation of the disease was obtained by the generation of a reversible mouse model of obesity in which the expression of the POMC gene in neurons of the hypothalamus was selectively blocked. POMC reactivation during early obesity resulted in a complete reversal of the phenotype, while late reactivation of POMC was insufficient to promote a complete normalization of body weight (Bumaschny, Yamashita et al. 2012).

Although great advance has been obtained in the characterization of the mechanisms linking the consumption of high-fat diets to the activation of inflammation in the hypothalamus (De Souza, Araujo et al. 2005, Zhang, Zhang et al. 2008, Milanski, Degasperi et al. 2009, Ozcan, Ergin et al. 2009), we are yet to understand which mechanisms link prolonged inflammation of the hypothalamus and prolonged obesity to the irreversible damage of the hypothalamic system that controls whole body energy homeostasis.

In the present study, we evaluated the regulation of the ubiquitin/proteasome system in the hypothalamus of mice with short- and long-term diet-induced obesity. The regulation of protein turnover is essential for cellular homeostasis (Bingol and Sheng 2011), and the ubiquitin/proteasome system is the most important mechanism regulating protein degradation in eukaryotic cells, promoting proteolysis of up to 80% of short-lived proteins (Ciechanover 2006, Nath and Shadan 2009). Most of the remaining proteolysis is performed in lysosomes or by autophagy, where a molecular connection between the ubiquitin/proteasome system and

autophagy has been recently identified and shown to be dependent on the activity of p62 (Komatsu, Waguri et al. 2007). In neurons, the correct regulation of protein turnover is crucial for cell viability and particularly for synaptic plasticity (Yi and Ehlers 2007). The impairment of this process leads to the accumulation of protein aggregates into the cells, which is a common feature of neurodegenerative conditions, such as Parkinson´s and Alzheimer´s diseases (Almeida, Takahashi et al. 2006, Bedford, Hay et al. 2008).

In the first part of the study, we show that long-term diet-induced obesity has a substantial impact on the hypothalamic expression of ubiquitin-related genes, as shown by the PCR array. Most of the genes undergoing changes belong to the E3 family, which suggests that the regulation imposed by the diet and prolonged obesity is somewhat specific, as E3 ligases and some E3 enzymes with deubiquitinase activity, are more specific for their targets than proteins with E1 and E2 activities (van Wijk, de Vries et al. 2009). Next, we explored the hypothesis that the activity of the ubiquitin/proteasome system in the hypothalamus differs between mice with short- and prolonged periods of obesity. As in humans with obesity for long periods of life, which are extremely resistant to body mass reduction (Kraschnewski, Boan et al. 2010), prolonged experimental obesity resulted in a less pronounced body mass loss after the transfer from high-fat diet to chow. This was accompanied by increased accumulation of ubiquitin in the hypothalamus and by changes in the expression of proteins exerting distinct functions in the ubiquitin/proteasome system. As a whole, the differences between 8- and 16 weeks of high-fat feeding suggest that the efficiency of the machinery for protein degradation is lost over time, a phenomenon that is not related to ageing, since our lean controls, with the same age as the obese mice, did not display similar alterations.

The impact of the anomalous regulation of ubiquitin expression on body adiposity was evidenced recently in two studies that either removed or increased the expression of this protein in mice (Ryu, Garza et al. 2008, Susaki, Kaneko-Oshikawa et al. 2010). Surprisingly, in both conditions the resulting phenotype was the increase of adiposity, suggesting that the fine tuning of protein degradation is crucial for whole body energy homeostasis. Now, we show that the system can be regulated by a nutritional factor, which is the most important determinant of obesity in human populations (Qi and Cho 2008, Abete, Astrup et al. 2010). Moreover, we show that changes in the regulation of the ubiquitin/proteasome system in the hypothalamus is connected with inflammation, as inflammation-protected, obesity-resistant mutants such as TLR4- and TNFR1-knockout mice (Milanski, Degasperi et al. 2009, Romanatto, Roman et al. 2009) were also protected from diet-induced modulation of the ubiquitin/proteasome system in the hypothalamus.

Interestingly, the overexpression of E4B ligase, which produces an increased ubiquitination of proteins, was shown to result in the formation of protein aggregates in the hypothalamus (Susaki, Kaneko-Oshikawa et al. 2010), leading to obesity. The presence of protein aggregates is evidenced by the physical association between ubiquitin and p62 (Komatsu, Waguri et al. 2007). Here, we detected an increased association of p62 and ubiquitin and also increased presence of protein aggregates, particularly in the arcuate nucleus of obese mice fed for 16 weeks on the high-fat diet. This is an important additional evidence of anatomical and molecular features of neurodegeneration in the hypothalamus of an animal model of diet-induced obesity.

In the final part of the study, we employed two distinct approaches to accelerate the impairment of the ubiquitin/proteasome system in the hypothalamus of mice fed on the high-fat diet for 8 weeks. For this, mice were treated with a chemical inhibitor of proteasome, lactacystin or with a

siRNA targeting p62. In both instances, there was accumulation of ubiquitin in the hypothalamus and increased body mass gain, accompanied by increased caloric intake.

Taking data from our present study together with those of former studies evaluating diet-induced hypothalamic inflammation, we conclude that high-fat dietary content initially induces an inflammatory process in the hypothalamus, which leads to leptin resistance, increased caloric intake and reduced energy expenditure, resulting in the progressive increase of adiposity (De Souza, Araujo et al. 2005, Arruda, Milanski et al. 2011, Thaler, Yi et al. 2012). Until a certain point of progression of the disease, the return to a low-fat diet will result in complete rescue of the obese phenotype [present data and (Bumaschny, Yamashita et al. 2012)]. However, as high-fat feeding persists, hypothalamic neuronal damage will progressively contribute to the irreversibility of the disease. We propose that the anomalous activity of the ubiquitin/proteasome system in the hypothalamus is one of the mechanisms contributing to the deterioration of the system that regulates whole body energy homeostasis. Thus, long-term obesity is accompanied by neuronal changes commonly found in classical neurodegenerative conditions, which can explain not only the refractoriness of obesity, but also its frequent association with Alzheimer's and Parkinson's diseases (Chen, Zhang et al. 2004, Xu, Atti et al. 2011, Bomfim, Forny-Germano et al. 2012, Saragat, Buffa et al. 2012).

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Tables

Table 1. Composition of experimental diets

	Chow Diet [1]	**High Fat Diet**
Net protein (g %)	22,5	26,0
Ether Extract (Fat content) (g %)	4,5	35,0
Carbohydrates (g %)	55,0	26,0
Fibrous matter (g %)	8,0	6,0
Ash matter (g %)	10,0	7,0
Total	100,0	100,0
Kcal/g	3,5	5,2

[1] *(NUVILAB®* Cr-1, Nuvital, *PR - Brasil)*

Table 2. Results of real-time PCR array, depicting only genes modulated by diet.

UniGene	RefSeq	Symbol	Description	Function	Fold Change	*p*
Mm.305925	NM_019927	Arih1	Ariadne ubiquitin-conjugating enzyme E2 binding protein homolog 1 (Drosophila)	E3 ligase E2 conjugating	1,12	0,0564
Mm.21981	NM_177613	Cdc34	Cell division cycle 34 homolog (S. cerevisiae)	E2 cell cycle	0,93	0,0214
Mm.327675	NM_028288	Cul4b	Cullin 4B	E3 cell cycle	1,12	0,0088
Mm.218910	NM_027807	Cul5	Cullin 5	E3 cell cycle, apoptosis	0,77	0,0163
Mm.234191	NM_134099	Fbxo4	F-box protein 4	E3 ligase	0,57	0,0069
Mm.374815	NM_026791	Fbxw9	F-box and WD-40 domain protein 9	E3 ligase	0,69	0,0006
Mm.222	NM_011640	Trp53	Transformation related protein 53	E2 e E3 transcription E2 apoptosis, cell cycle	1,54	0,0267
Mm.1104	NM_009457	Uba1	Ubiquitin-like modifier activating enzyme 1	E1 activating	1,30	0,0046
Mm.270530	NM_145420	Ube2d1	Ubiquitin-conjugating enzyme E2D 1, UBC4/5 homolog (yeast)	E2 conjugating	1,52	0,0465
Mm.371673	NM_001039157	Ube2j2	Ubiquitin-conjugating enzyme E2, J2 homolog (yeast)	E2 conjugating	0,53	0,0358
Mm.41438	NM_027315	Ube2q1	Ubiquitin-conjugating enzyme E2Q (putative) 1	E2 conjugating	1,14	0,0013
Mm.29407	NM_009507	Vhl	Von Hippel-Lindau syndrome	E3 ligase apoptosis, cell cycle, transcription	0,68	0,0481
Mm.78312	NM_177327	Wwp1	WW domain containing E3 ubiquitin protein ligase 1	E3 ligase transcription	0,59	0,0176

Legends for the figures

Figure 1. Six-week old, male Swiss mice were randomly assigned to standard rodent chow (lean) or high-fat diet (obese) *ad libitum* for 16 weeks. Body mass was determined every fourth week (A). Mean daily caloric intake was determined at 8 and 16 weeks (B). Serum concentrations of leptin (C) and insulin (D) were determined by ELISA at 16 weeks. The glucose consumption during a hyperinsulinemic-euglycemic clamp (E) was determined at 16 weeks. The serum levels of tumor necrosis factor-□ (TNF-□) (F), interleukin-1β (IL-1 β) (G) and interleukin-6 (IL-6) (H) were determined by ELISA at 16 weeks after the introduction of diets. The mRNA expressions of genes encoding tumor necrosis factor-α (TNF-α) (I), interleukin-6 (IL-6) (J) and interleukin-1β (IL-1β) (K) were determined by real-time PCR 16 weeks after the introduction of the diets. Fasting, icv cannulated mice fed on chow (lean) or high-fat diet (obese) for 16 weeks were injected icv with a single dose (2.0 μL, 10^{-6}M) of leptin or similar volume of saline and spontaneous food intake was determined over a period of four hours (L). For all experiments, n = 6. In A-K, *p<0.05 *vs.* lean. In L, *p<0.05 *vs.* lean-saline.

Figure 2. Six-week old, male Swiss mice were assigned to a high-fat diet, *ad libitum*, for 8 or 16 weeks. At the end of the experimental period, body mass was determined (A) and mice were transferred to chow, *ad libitum*. Cumulative intake (B) and body mass change (C) were evaluated over a period of seven days. In all experiments, n = 6; *p<0.05 *vs.* obese 8 weeks.

Figure 3. Graphic representation of the impact of high-fat diet consumption on the hypothalamic expression of genes of the ubiquitin system in male Swiss mice, as determined by a real-time PCR array. The detailed views of the results of the array are presented in Table 2 and Supplementary 1. The global impact of the high-fat diet on the expression of the genes analyzed

is presented in A. In B, graphic representation of the proportion of all genes coding for proteins with E1, E2, E3 and E2-E3 activities, which have been modulated by the high-fat diet. In C, the genes modulated by the high-fat diet were split into those that were up-regulated (graph in green tonalities) and down-regulated (graph in red tonalities). The results of the array were obtained by analyzing the pools of mRNAs obtained from three animals in each group, mice fed on chow *vs.* mice fed on high-fat diet. The details for fold variation and statistical analysis are presented in Table 2.

Figure 4. Six-week old, male Swiss mice were randomly assigned to standard rodent chow (lean) or high-fat diet (obese), *ad libitum,* for 8 (A) or 16 (B) weeks. At the end of the respective experimental periods, hypothalamic protein extracts were obtained and separated by SDS-PAGE, transferred to nitrocellulose membranes and submitted to immunoblotting determination of the expressions of ubiquitin (Ubi), A20, p62 and proteasome (Prot). All membranes were stripped and reblotted with anti-β-actin antibody. The bar-graphs on the left-hand side of the figure present the means±standard error of the means of the arbitrary scanning units obtained from the densitometric determination of the respective bands in the blots depicted on the right-hand side of the figure. In all experiments, n = 6; *$p<0.05$ *vs.* lean.

Figure 5. Six-week old, male Swiss mice were randomly assigned to standard rodent chow (lean) or high-fat diet (obese), *ad libitum,* for 8 (A-C) or 16 (D-J) weeks. At the end of the respective experimental periods, hypothalami were obtained for the immunofluorescence study of the expression, distribution and co-localization of ubiquitin (Ubi) and p62 (A-F); Ubi and the neuron marker HuR (G); p62 and HuR (H); Ubi and the microglia marker CD11b (I); and, p62 and CD11b (J). The microphotographs are representative of four independent experiments. Arc, arcuate nucleous; PVN, paraventricular nucleous; LH, lateral hypothalamus.

Figure 6. Six-week old, male Swiss mice were randomly assigned to standard rodent chow (lean) or high-fat diet (obese), *ad libitum,* for 16 weeks. At the end of the respective experimental periods, hypothalami were obtained for transmission electron microscopy studies. Protein aggregates (arrows – shown in caption) were identified and counted in 4-6 high-magnification fields of eight distinct neurons selected randomly. Bar-graph depicts mean protein aggregates per μm^2. The micrographs are representative of 32-48 distinct acquisitions; *$p<0.05$ *vs.* lean.

Figure 7. Six-week old, male Swiss mice were randomly assigned to standard rodent chow (lean) or high-fat diet (obese), *ad libitum,* for 8 (A) or 16 (B) weeks. At the end of the respective experimental periods, hypothalamic protein extracts were obtained and separated by SDS-PAGE, transferred to nitrocellulose membranes and submitted to immunoblotting determination of the expression of beclin and LC3. All membranes were stripped and reblotted with anti-β-actin antibody. The bar-graphs in the upper part of the figure present the means±standard error of the means of the arbitrary scanning units obtained from the densitometric determination of beclin (upper graphs) and the lower LC3 bands (lower graphs) in the blots depicted in the lower part of the figure. In C, six-week old, male Swiss mice were randomly assigned to standard rodent chow (lean) or high-fat diet (obese), *ad libitum,* for 16 weeks. At the end of the respective experimental periods, hypothalami were obtained for transmission electron microscopy studies. Autophagosomes (arrows – shown in caption) were identified and counted in 4-6 high-magnification fields of eight distinct neurons selected randomly. Bar-graph depicts mean autophagomes per μm^2. In A and B, $n = 6$; *$p<0.05$ *vs.* lean. In C, micrographs are representative of 32-48 distinct acquisitions; *$p<0.05$ *vs.* lean.

Figure 8. Male TNFRp55-/- mice and the control C57BL/6J (A and C), and, male C3H/HeJ mice and the control C3H/HeN were fed on chow or high-fat diet (HFD) (B and D) for 16 weeks and

employed in experiments to evaluate body mass gain (A-B) and for immunoblot evaluation of protein expression (C-D). At the end of the experimental period, hypothalamic protein extracts were obtained and separated by SDS-PAGE, transferred to nitrocellulose membranes and submitted to immunoblotting determination of the expressions of ubiquitin (Ubi), A20, p62 and proteasome (Prot). All membranes were stripped and reblotted with anti-β-actin antibody. The bar-graphs on the left-hand side of the figure present the means±standard error of the means of the arbitrary scanning units obtained from the densitometric determination of the respective bands in the blots depicted on the right-hand side of the figure. In all experiments, n = 6; *$p<0.05$ *vs.* chow.

Figure 9. Male, 14-week old Swiss mice fed on a high-fat diet for 8 weeks were stereotaxically cannulated, tested for cannula patency with angiotensin II and then treated with lactacystin (2 □L, 100uM; intracerebroventricular) for five days (A). At the end of the experimental period, hypothalamic protein extracts were obtained and separated by SDS-PAGE, transferred to nitrocellulose membranes and submitted to immunoblotting determination of the expression of ubiquitin (Ubi) (B). All membranes were stripped and reblotted with anti- β-actin antibody (B). The bar-graphs (C) present the means±standard error of the means of the arbitrary scanning units obtained from the densitometric determination of the respective bands in the blots depicted in B. Body mass during the experimental period is presented as a relative variation as compared to vehicle-treated mice (D) and as the cumulative absolute change during the experimental period (E). Daily food intake is presented as the ratio of the food intake on the first day of experiment (F). Determination of O_2 consumption (G), CO_2 production (H) and respiratory quotient (RQ) (I) was performed at the end of the experimental period. The expressions of cytochrome C (Cyt C) (J), PGC1□ (K) and UCP1 (L) were determined by real-time PCR, in the brown adipose tissue at

the end of the experimental period. The expressions of genes encoding TNF-α (M) and IL-1β (N) were determined by real-time PCR, in the hypothalamus, at the end of the experimental period. In all experiments, n = 6; *p<0.05 *vs.* vehicle.

Figure 10. Male, 14-week old Swiss mice fed on a high-fat diet for 8 weeks were stereotaxically cannulated, tested for cannula patency with angiotensin II and then treated with one dose of siRNA targeting p62 (sip62) or an equivalent dose of a scramble siRNA (siSCRAMBLE) on days 1, 3 and 5 (A). At the end of the experimental period, hypothalamic protein extracts were obtained and separated by SDS-PAGE, transferred to nitrocellulose membranes and submitted to immunoblotting determination of the expressions of ubiquitin (Ubi), A20, p62 and proteasome (Prot) (B). All membranes were stripped and reblotted with anti- β-actin antibody. The bar-graphs on the left-hand side of the figure present the means±standard error of the means of the arbitrary scanning units, obtained from the densitometric determination of the respective bands in the blots depicted on the right-hand side of the figure. Body mass during the experimental period is presented as a relative variation, as compared to vehicle treated mice (C) and as the cumulative absolute change during the experimental period (D). Daily food intake is presented as the ratio of the food intake on the first day of the experiment (E). In all experiments n = 6; *p<0.05 *vs.* siSCRAMBLE

Figures

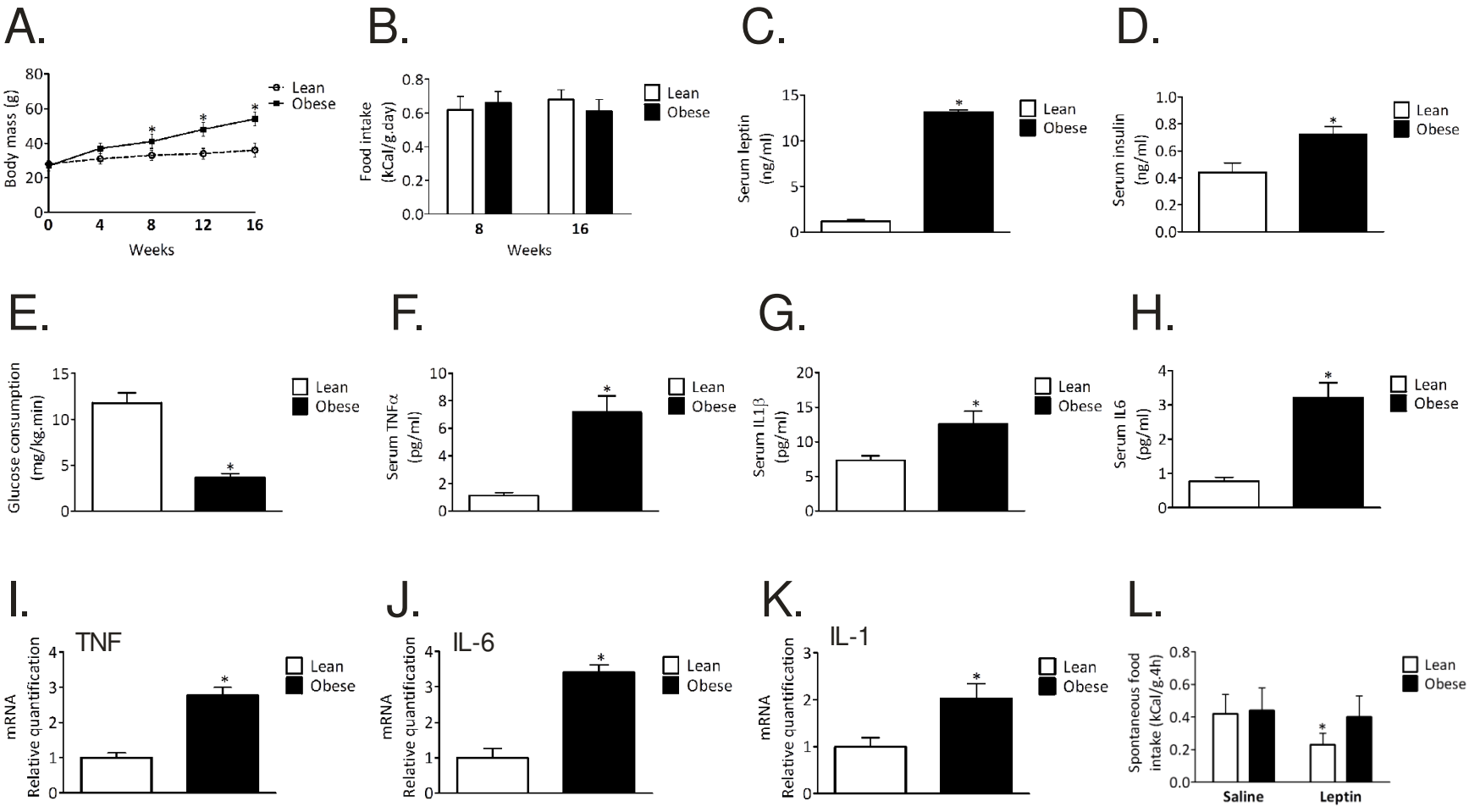

Figure 1

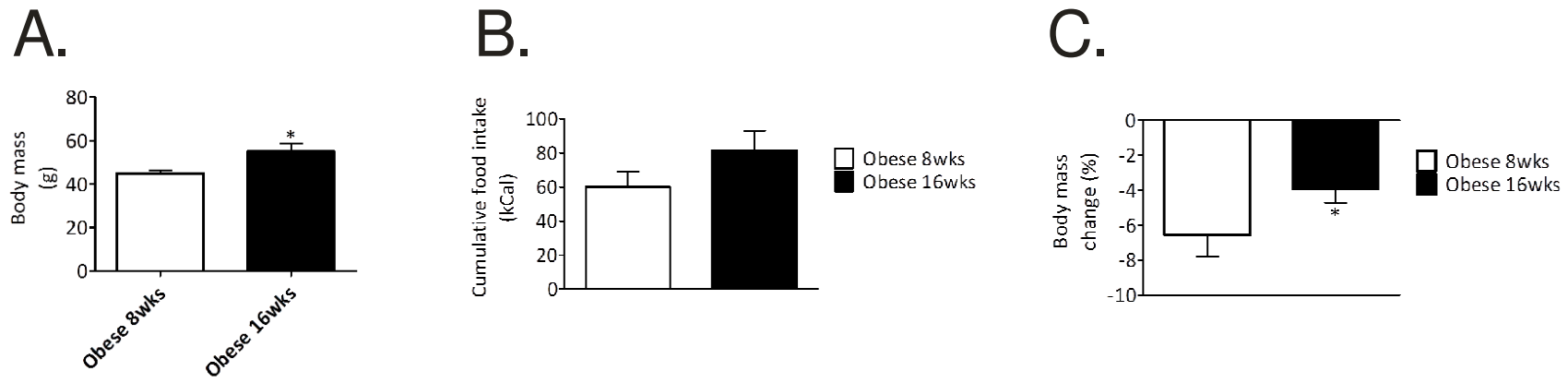
A.
B.
C.
Body mass (g)
80
60
40
20
0
*
Obese 8wks
Obese 16wks
Cumulative food intake (kCal)
100
80
60
40
20
0
Obese 8wks
Obese 16wks
Body mass change (%)
0
-2
-4
-6
-8
-10
*
Obese 8wks
Obese 16wks

Figure 2

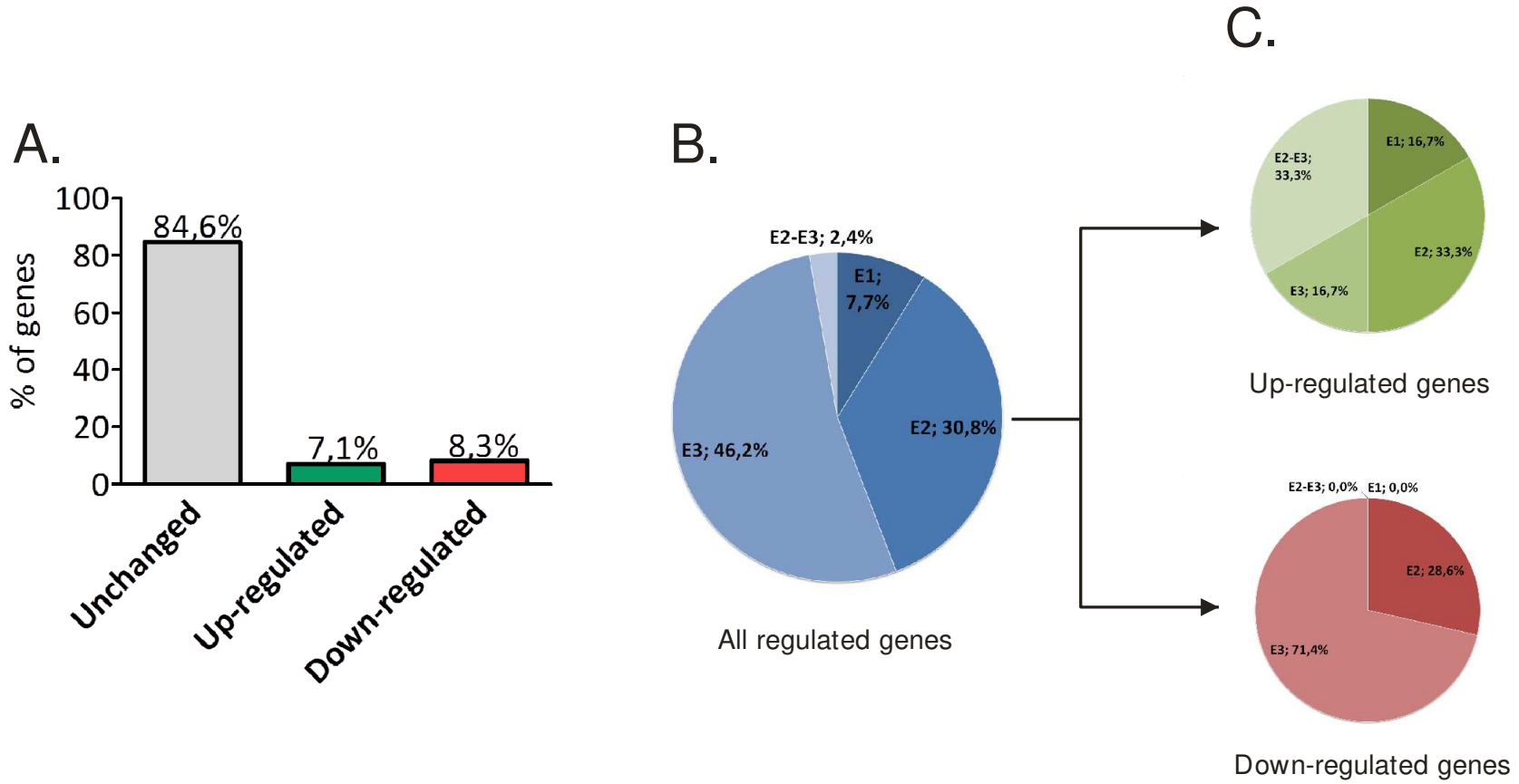
A.
100
80
60
40
20
0
% of genes
84,6%
7,1%
8,3%
Unchanged
Up-regulated
Down-regulated
B.
E2-E3; 2,4%
E1; 7,7%
E2; 30,8%
E3; 46,2%
All regulated genes
C.
E1; 16,7%
E2-E3; 33,3%
E2; 33,3%
E3; 16,7%
Up-regulated genes
E2-E3; 0,0%
E1; 0,0%
E2; 28,6%
E3; 71,4%
Down-regulated genes

Figure 3

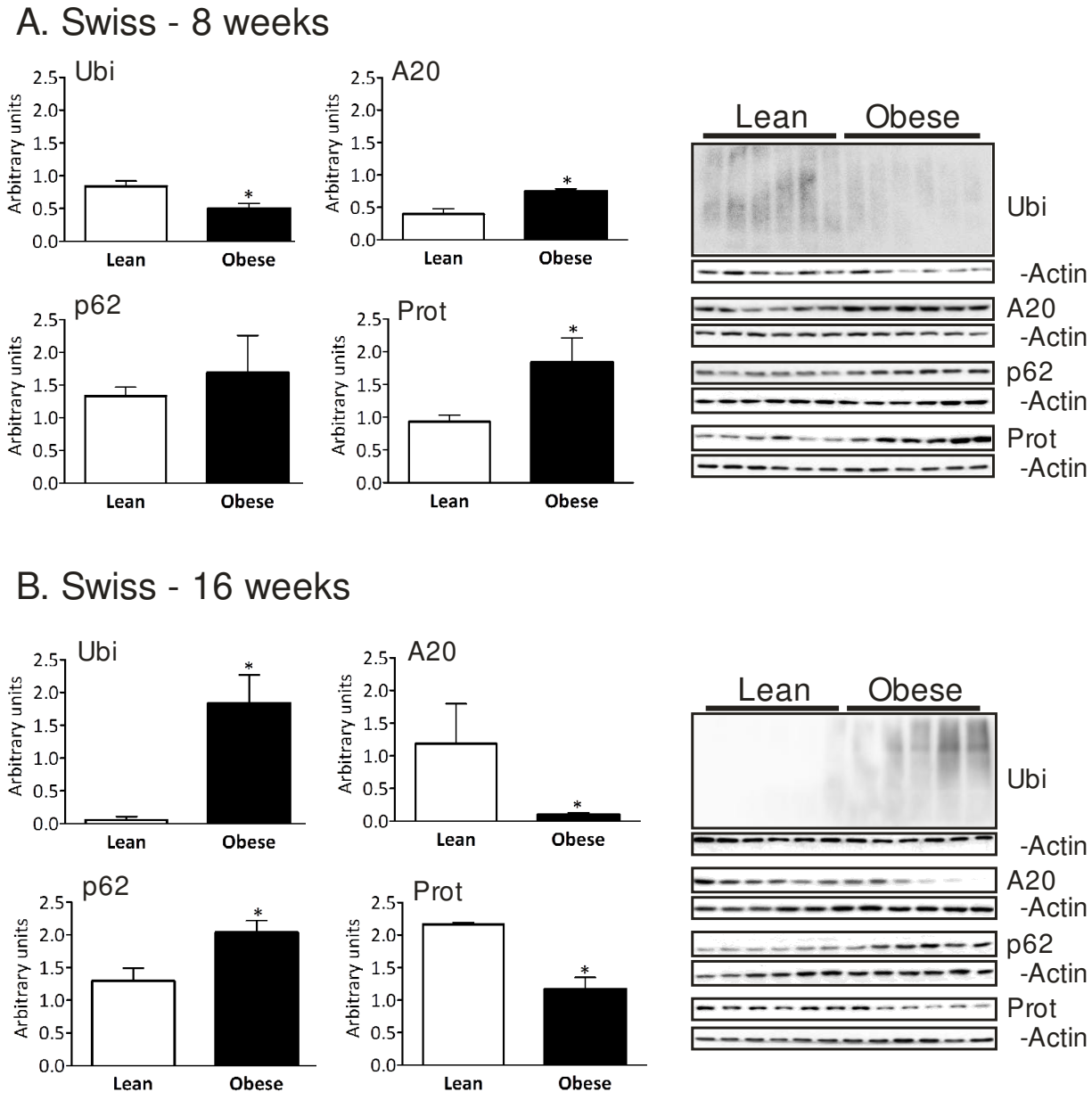
A. Swiss - 8 weeks
Ubi
A20
p62
Prot
Arbitrary units
Lean
Obese
B. Swiss - 16 weeks
Lean
Obese
Ubi
-Actin
A20
-Actin
p62
-Actin
Prot
-Actin

Figure 4

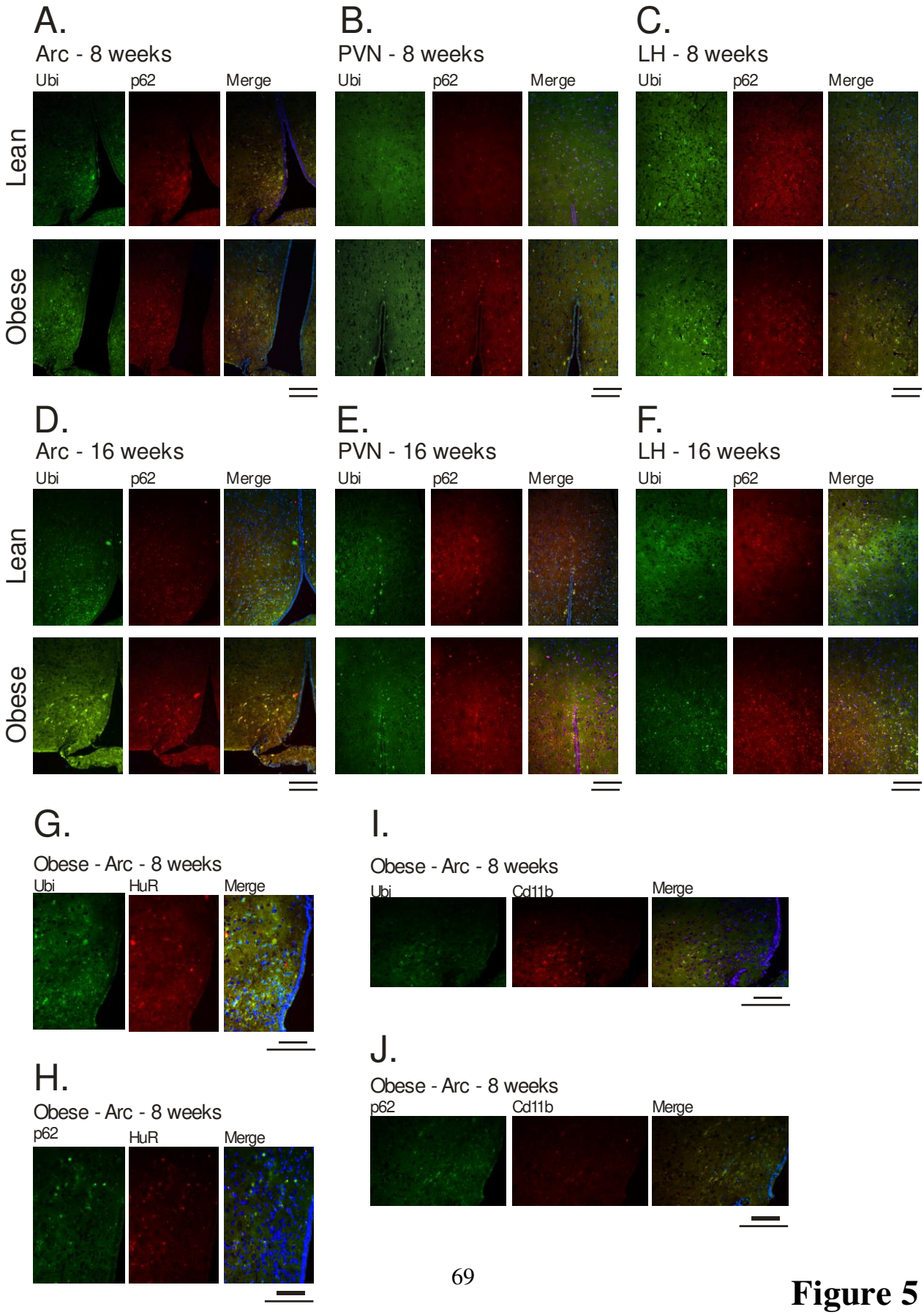
A.
Arc - 8 weeks
Ubi
p62
Merge
Lean
Obese
B.
PVN - 8 weeks
Ubi
p62
Merge
C.
LH - 8 weeks
Ubi
p62
Merge
D.
Arc - 16 weeks
Ubi
p62
Merge
Lean
Obese
E.
PVN - 16 weeks
Ubi
p62
Merge
F.
LH - 16 weeks
Ubi
p62
Merge
G.
Obese - Arc - 8 weeks
Ubi
HuR
Merge
H.
Obese - Arc - 8 weeks
p62
HuR
Merge
I.
Obese - Arc - 8 weeks
Ubi
Cd11b
Merge
J.
Obese - Arc - 8 weeks
p62
Cd11b
Merge

Figure 5

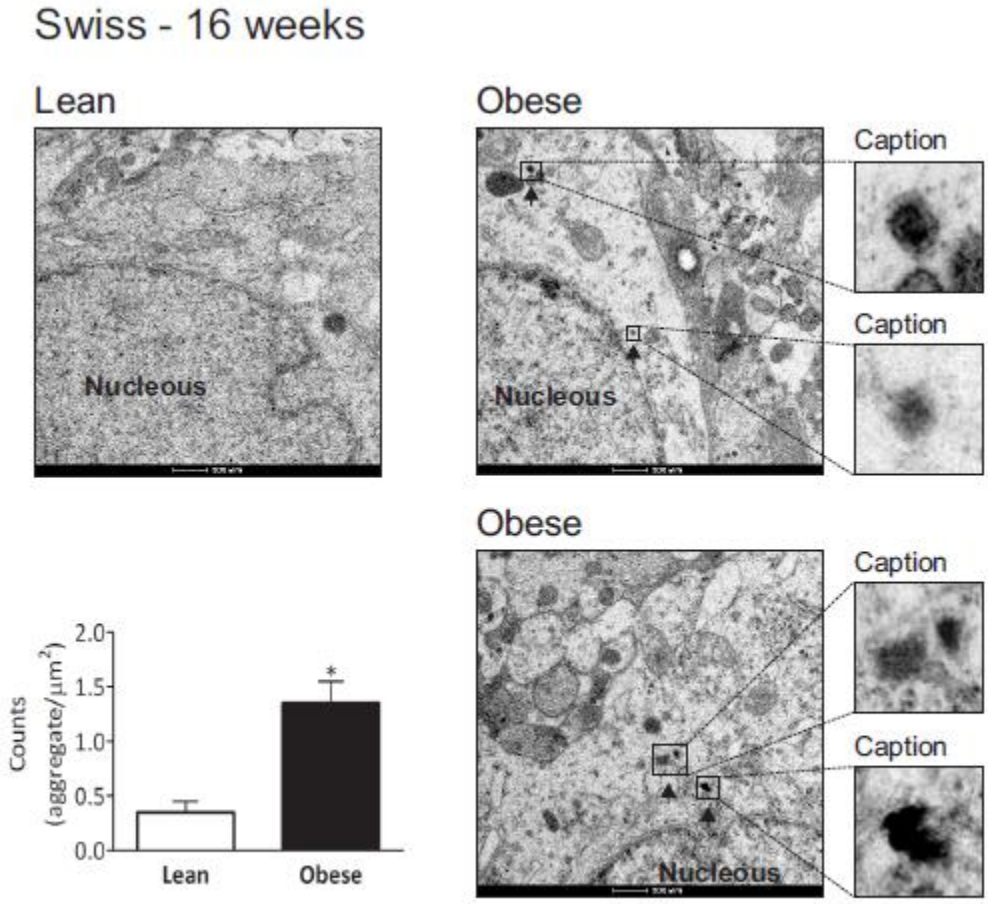
Swiss - 16 weeks
Lean
Nucleous
Obese
Caption
Caption
Nucleous
Obese
Caption
Caption
Nucleous
Counts (aggregate/µm²)
2.0
1.5
1.0
0.5
0.0
*
Lean
Obese

Figure 6

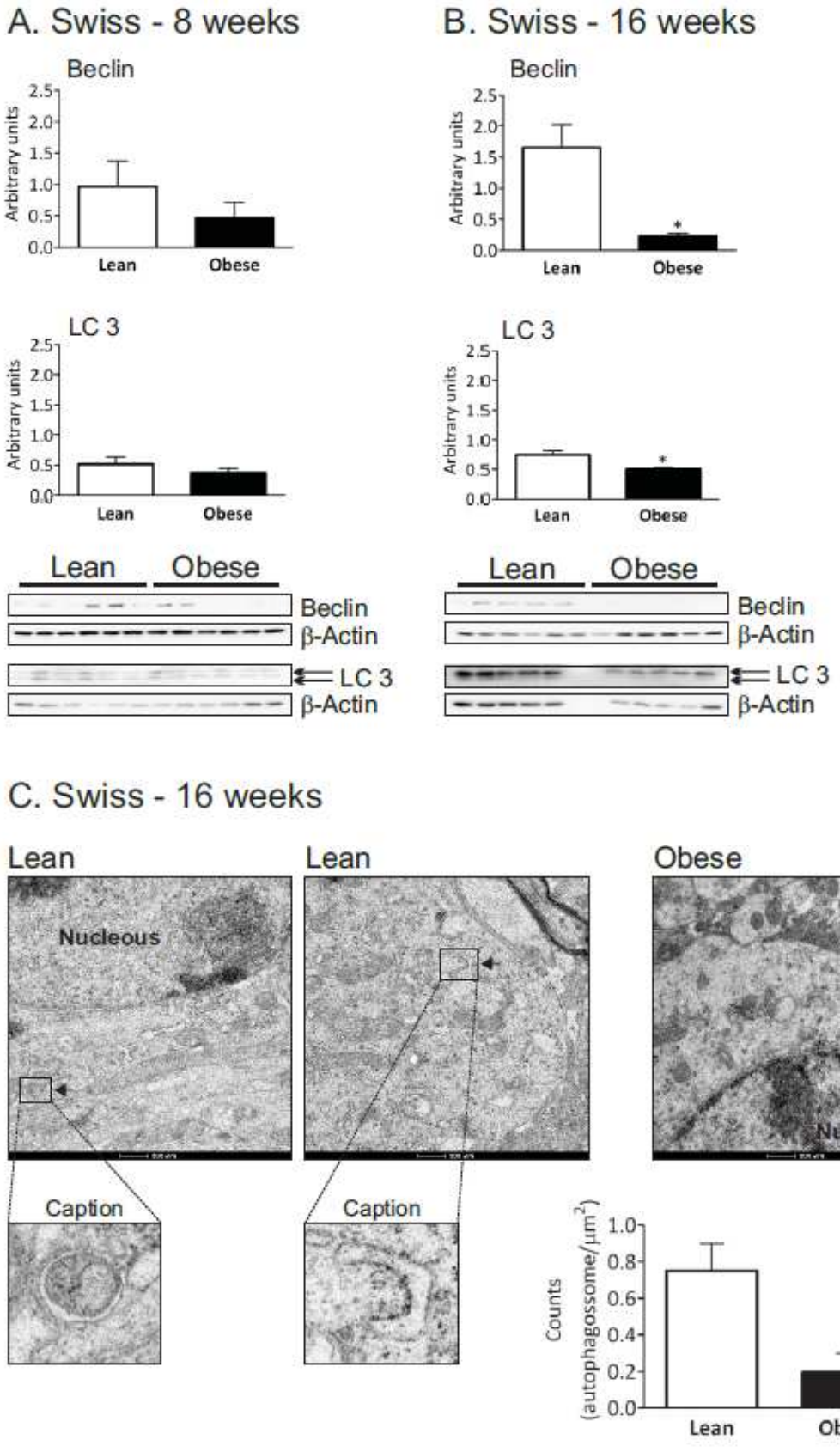
A. Swiss - 8 weeks
B. Swiss - 16 weeks
Beclin
LC 3
Arbitrary units
Lean
Obese
Beclin
β-Actin
LC 3
β-Actin
C. Swiss - 16 weeks
Lean
Lean
Obese
Nucleous
Caption
Caption
Nucleous
Counts (autophagossome/μm²)

Figure 7

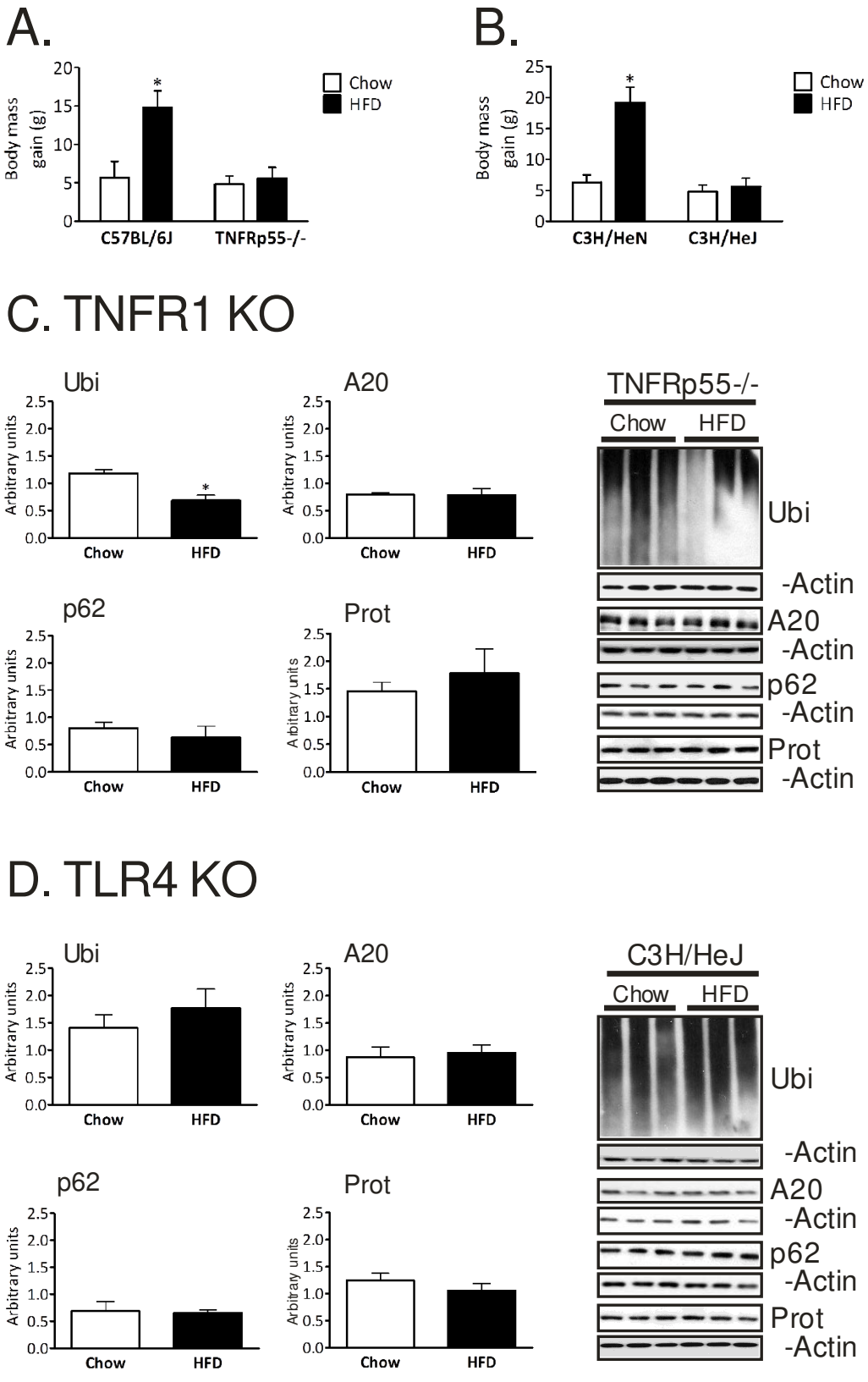
A.
Body mass gain (g)
Chow
HFD
C57BL/6J
TNFRp55-/-
B.
Body mass gain (g)
Chow
HFD
C3H/HeN
C3H/HeJ
C. TNFR1 KO
Ubi
A20
p62
Prot
Arbitrary units
Chow
HFD
TNFRp55-/-
Chow
HFD
Ubi
-Actin
A20
-Actin
p62
-Actin
Prot
-Actin
D. TLR4 KO
Ubi
A20
p62
Prot
Arbitrary units
Chow
HFD
C3H/HeJ
Chow
HFD
Ubi
-Actin
A20
-Actin
p62
-Actin
Prot
-Actin

Figure 8

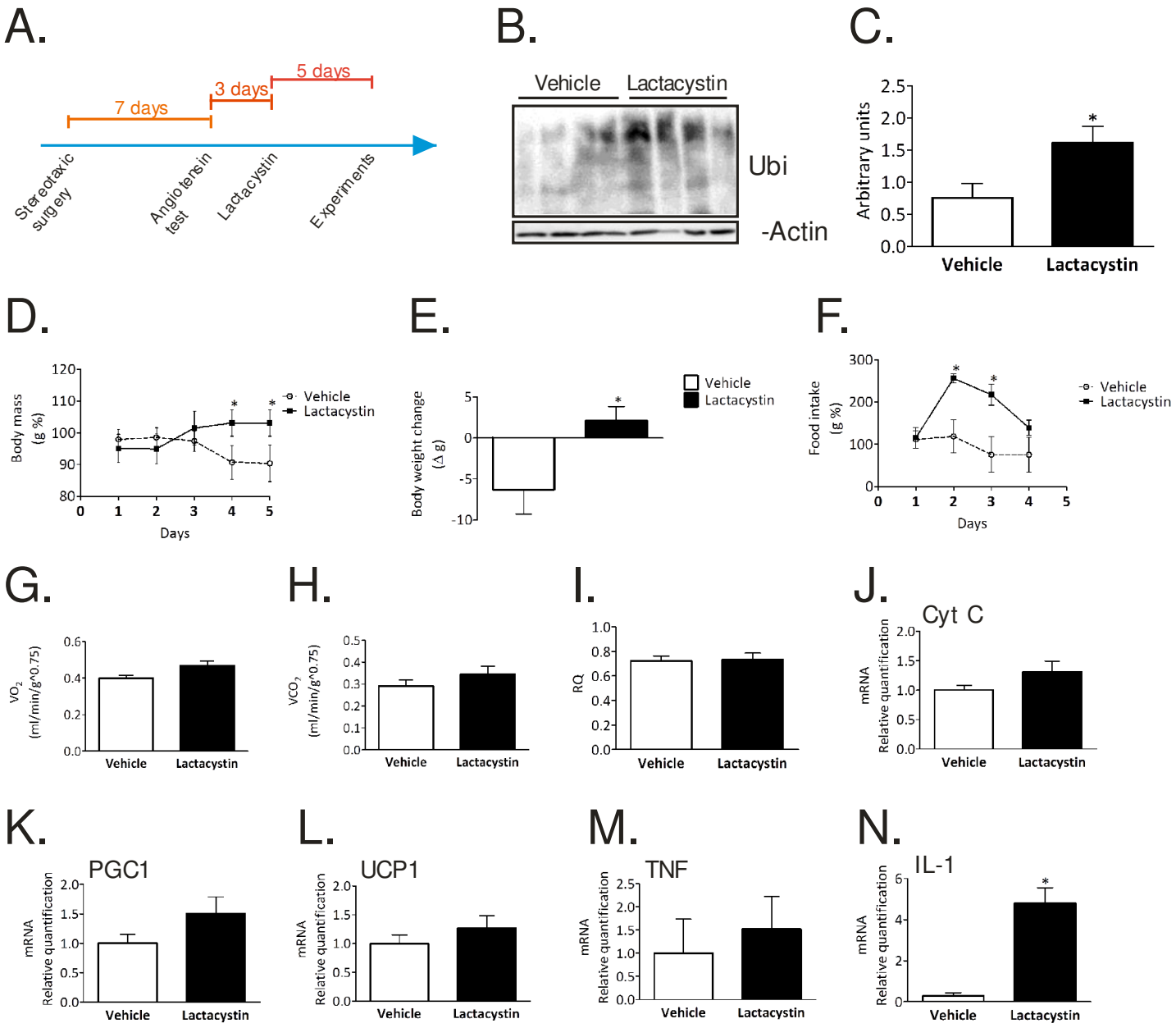
A.
5 days
3 days
7 days
Stereotaxic surgery
Angiotensin test
Lactacystin
Experments
B.
Vehicle
Lactacystin
Ubi
-Actin
C.
Arbitrary units
Vehicle
Lactacystin
D.
Body mass (g %)
Days
Vehicle
Lactacystin
E.
Body weight change (Δ g)
Vehicle
Lactacystin
F.
Food intake (g %)
Days
Vehicle
Lactacystin
G.
VO2 (ml/min/g^0.75)
H.
VCO2 (ml/min/g^0.75)
I.
RQ
J.
Cyt C
mRNA Relative quantification
K.
PGC1
L.
UCP1
M.
TNF
N.
IL-1

Figure 9

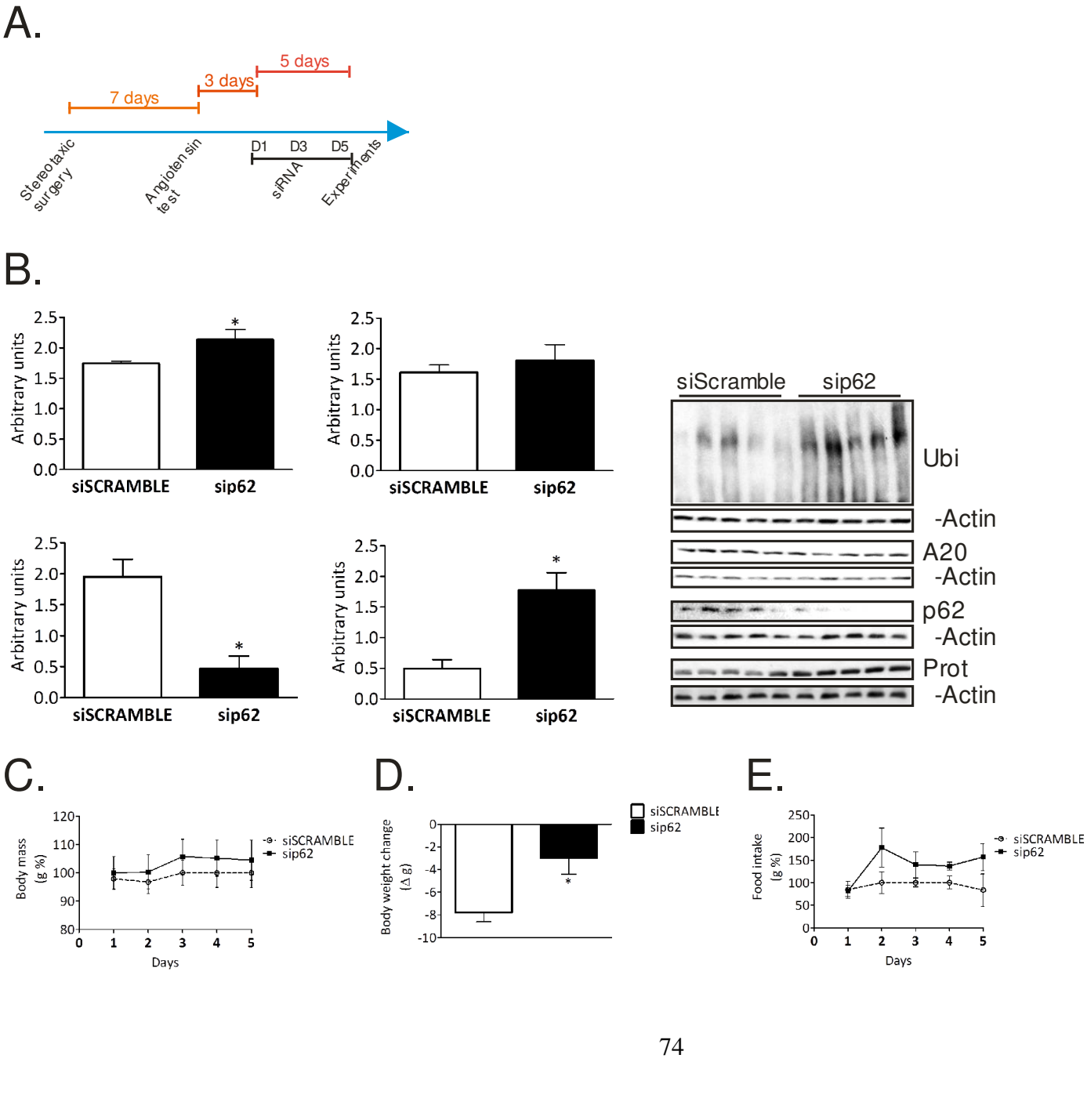
A.
5 days
3 days
7 days
D1 D3 D5
Stereotaxic surgery
Angiotensin test
siRNA
Experiments
B.
Arbitrary units
siSCRAMBLE
sip62
siScramble
sip62
Ubi
-Actin
A20
-Actin
p62
-Actin
Prot
-Actin
C.
Body mass (g %)
Days
siSCRAMBLE
sip62
D.
Body weight change (Δ g)
siSCRAMBLE
sip62
E.
Food intake (g %)
Days
siSCRAMBLE
sip62

Figure 10

CAPÍTULO 2

Co-autoria de outros artigos científicos relacionados ao tema desta tese

1. Coope, A., Milanski, M., Arruda, A.P., Ignacio-Souza, L.M., Saad, M.J., Anhe, G.F., and Velloso, L.A. 2012. Chaperone insufficiency links TLR4 protein signaling to endoplasmic reticulum stress. *J Biol Chem* 287:15580-15589.

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APÊNDICE

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Chaperone Insufficiency Links TLR4 Protein Signaling to Endoplasmic Reticulum Stress*[S]

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Background: Activation of TLR4 leads to endoplasmic reticulum stress. However, the mechanisms involved in this phenomenon are unknown.
Results: In TLR4 signaling, insufficient GRP94 and GRP78 mediate the activation of endoplasmic reticulum stress.
Conclusion: The insufficiency of chaperone expression links TLR4 signaling to endoplasmic reticulum stress.
Significance: This study may improve our understanding about the inflammatory response in metabolic and infectious disease.

Inflammation plays an important pathogenic role in a number of metabolic diseases such as obesity, type 2 diabetes, and atherosclerosis. The activation of inflammation in these diseases depends at least in part on the combined actions of TLR4 signaling and endoplasmic reticulum stress, which by acting in concert can boost the inflammatory response. Defining the mechanisms involved in this phenomenon may unveil potential targets for the treatment of metabolic/inflammatory diseases. Here we used LPS to induce endoplasmic reticulum stress in the human monocyte cell-line, THP-1. The unfolded protein response, produced after LPS, was dependent on CD14 activity but not on RNA-dependent protein kinase and could be inhibited by an exogenous chemical chaperone. The induction of the endoplasmic reticulum resident chaperones, GRP94 and GRP78, by LPS was of a much lower magnitude than the effect of LPS on TLR4 and MD-2 expression. In face of this apparent insufficiency of chaperone expression, we induced the expression of GRP94 and GRP78 by glucose deprivation. This approach completely reverted endoplasmic reticulum stress. The inhibition of either GRP94 or GRP78 with siRNA was sufficient to rescue the protective effect of glucose deprivation on LPS-induced endoplasmic reticulum stress. Thus, insufficient LPS-induced chaperone expression links TLR4 signaling to endoplasmic reticulum stress.

The anomalous activation of macrophages plays an important role in metabolic diseases such as atherosclerosis, obesity, and type 2 diabetes mellitus (1–3). Pattern recognition receptors (PPRs),[2] particularly TLR4 (4), and endoplasmic reticulum stress (ERS) (5) have been independently identified as important inducers of dysfunctional macrophage activation in these diseases, and both genetic and pharmacological targeting of the TLRs and ERS can attenuate or revert the metabolic phenotypes (4, 6–8). Recent data have revealed that signals generated by the activation of TLRs or the induction of ERS can integrate with each other and, thus, boost the pathological inflammatory response, eventually leading to macrophage apoptosis, which in the case of atherosclerosis is a well known complicating event (8–10). However, the mechanisms involved in the integration of TLR signaling with ERS are virtually unknown.

The expression of TLR2 and TLR4 on the cell surface is necessary for pattern recognition and the activation of signal transduction (11). To reach the cell membrane, the nascent TLRs must be correctly folded, which depends on their binding to the ER resident chaperone GRP94 (also known as gp96) (12). Whole body deficiency of GRP94 is lethal at embryonic day 5 (12, 13). However, when only macrophages are depleted of GRP94, rodents are viable, presenting a defective response to *Listeria monocytogenes*, which results from an almost complete absence of cell surface TLRs expression (14).

In macrophages, lipopolysaccharide (LPS) stimulation produces ERS, a phenomenon believed to play an important role in eliciting the cytokine response required for optimal bacterial destruction (15). However, upon LPS stimulation, cells undergo a rapid reduction in the surface expression of TLR2 and TLR4, which is promptly accompanied by an increase in TLRs mRNA expression (16). Because replacement of surface TLR depends upon adequate chaperoning in the ER, we suspected that under LPS stimulation insufficient chaperone expression could mediate TLR4-induced ERS. Here, we show that, upon LPS stimulation, both GRP94 and GRP78 undergo only a discrete increase in protein expression level. After glucose deprivation, the substantial increase in the expression of these chaperones completely inhibits LPS-induced ERS, an effect that can be rescued by targeting either GRP94 or GRP78 with interference RNA.

MATERIALS AND METHODS

Antibodies, Chemicals, and Buffers—Antibodies against eIF2α (sc11386, rabbit polyclonal), RNA-dependent protein

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[S] This article contains supplemental Fig. 1.
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[2] The abbreviations used are: PPR, pattern recognition receptor; PKR, RNA-dependent protein kinase; ERS, endoplasmic reticulum stress; PBA, 5-phenylbutyric acid; UPR, unfolded protein response; PERK, protein kinase-like endoplasmic reticulum kinase.

ORIGINAL ARTICLE

Inhibition of Hypothalamic Inflammation Reverses Diet-Induced Insulin Resistance in the Liver

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Defective liver gluconeogenesis is the main mechanism leading to fasting hyperglycemia in type 2 diabetes, and, in concert with steatosis, it is the hallmark of hepatic insulin resistance. Experimental obesity results, at least in part, from hypothalamic inflammation, which leads to leptin resistance and defective regulation of energy homeostasis. Pharmacological or genetic disruption of hypothalamic inflammation restores leptin sensitivity and reduces adiposity. Here, we evaluate the effect of a hypothalamic anti-inflammatory approach to regulating hepatic responsiveness to insulin. Obese rodents were treated by intracerebroventricular injections, with immunoneutralizing antibodies against Toll-like receptor (TLR) 4 or tumor necrosis factor (TNF)α, and insulin signal transduction, hepatic steatosis, and gluconeogenesis were evaluated. The inhibition of either TLR4 or TNFα reduced hypothalamic inflammation, which was accompanied by the reduction of hypothalamic resistance to leptin and improved insulin signal transduction in the liver. This was accompanied by reduced liver steatosis and reduced hepatic expression of markers of steatosis. Furthermore, the inhibition of hypothalamic inflammation restored defective liver glucose production. All these beneficial effects were abrogated by vagotomy. Thus, the inhibition of hypothalamic inflammation in obesity results in improved hepatic insulin signal transduction, leading to reduced steatosis and reduced gluconeogenesis. All these effects are mediated by parasympathetic signals delivered by the vagus nerve. ***Diabetes*** **61:1455–1462, 2012**

Defective liver gluconeogenesis is regarded as the main mechanism leading to fasting hyperglycemia in type 2 diabetes and, in concert with steatosis, is the hallmark of hepatic insulin resistance (1,2). Both clinical and experimental data support an early link between obesity, hepatic insulin resistance, and hyperglycemia (3), which places this specific defect in a strategic position as a target for the treatment of type 2 diabetes (4,5). The relevance of tackling gluconeogenesis for the treatment of type 2 diabetes can be illustrated by the therapeutic success of metformin, a drug widely used for >50 years, whose molecular mechanism of action recently has been described (6).

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M.M. and A.P.A. contributed equally to this article.

See accompanying commentary, p. 1350.

A number of recent studies have shown that experimental obesity results from the installation of an inflammatory process in the hypothalamus, which leads to resistance to the anorexigenic hormones leptin and insulin and finally to the defective regulation of food intake and energy expenditure (7–12). Although the development of increased adiposity is taken as the main outcome of hypothalamic dysfunction, connecting obesity to type 2 diabetes, other peripheral functions can be controlled by the hypothalamus and play an important role in the development or progression of the hyperglycemic phenotype (13). One such example is the neural control of gluconeogenesis that depends on the adequate functionality of the insulin and AMP-activated protein kinase (AMPK) signaling pathways in hypothalamic neurons (14), which can be disturbed by drug-induced endoplasmic reticulum stress and inflammation, generating a sympathetic signal that triggers hepatic insulin resistance (15). Both the genetic and pharmacological approaches used to modulate both these pathways in the hypothalamus were able to affect liver gluconeogenesis (16).

Here, we explore the hypothesis that hepatic gluconeogenesis and hepatic insulin resistance can be corrected by reducing diet-induced hypothalamic inflammation. Our results show that the inhibition of either TLR4 or TNFα signaling in the hypothalamus improves insulin signal transduction in the liver and reduces hepatic glucose production.

RESEARCH DESIGN AND METHODS

The experimental procedures involving rats and mice were performed in accordance with the guidelines of the Brazilian College for Animal Experimentation and were approved by the ethics committee at the State University of Campinas. Male Wistar rats, male TNFRp55$^{-/-}$ or TNFRp55$^{+/}$ mice (knockout for the TNFα receptor 1 and its respective control) (17), male C3H/HeJ or C3H/HeN mice (loss-of-function mutation for TLR4 and its respective control) (18), and male LDLr-KO mice (knockout for the LDL receptor) (19) were fed standard rodent chow or a high-fat diet (see composition in Supplementary Table 1) for 8 weeks and then stereotaxically instrumented using a Stoelting stereotaxic apparatus, according to a previously described method (20). Cannula efficiency was tested 1 week after cannulation by the evaluation of the drinking response elicited by intracerebroventricular angiotensin II. Stereotaxic coordinates were, for rats, anteroposterior, 0.2 mm lateral, 1.5 mm depth, and 4.0 mm and for mice, anteroposterior, 0.34 mm lateral, 1.0 mm depth, and 2.2 mm. Thereafter, rats or mice were intracerebroventricularly treated with an anti-TLR4 antibody (50 ng twice a day TLR4 sc13591; Santa Cruz Biotechnology, Santa Cruz, CA) or the anti-TNFα monoclonal antibody, infliximab (0.3 μg twice a day), for 7 days. During the experimental period, the experimental animals had access to their respective diets and to water ad libitum and were housed at 22°C with a 12-h light/dark cycle. In some experiments, lean TNFRp55$^{-/-}$, TNFRp55$^{+/}$, C3H/HeJ, or C3H/HeN mice were intracerebroventricularly treated with 2 μL stearic acid (90 μmol/L) twice a day for 5 days. Fatty acid salt solution was added to medium containing fatty acid–free BSA (Sigma) for 1 h of conjugation at 37°C with continuous agitation to avoid precipitation. The fatty acid–to–BSA molar ratio was 3 to 1.

Diet composition. The chow and high-fat diet were evaluated for the content of fatty acids by chromatography, as previously described (21).

Fructose-Induced Hypothalamic AMPK Activation Stimulates Hepatic PEPCK and Gluconeogenesis due to Increased Corticosterone Levels

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Fructose consumption causes insulin resistance and favors hepatic gluconeogenesis through mechanisms that are not completely understood. Recent studies demonstrated that the activation of hypothalamic 5′-AMP-activated protein kinase (AMPK) controls dynamic fluctuations in hepatic glucose production. Thus, the present study was designed to investigate whether hypothalamic AMPK activation by fructose would mediate increased gluconeogenesis. Both ip and intracerebroventricular (icv) fructose treatment stimulated hypothalamic AMPK and acetyl-CoA carboxylase phosphorylation, in parallel with increased hepatic phosphoenolpyruvate carboxy kinase (PEPCK) and gluconeogenesis. An increase in AMPK phosphorylation by icv fructose was observed in the lateral hypothalamus as well as in the paraventricular nucleus and the arcuate nucleus. These effects were mimicked by icv 5-amino-imidazole-4-carboxamide-1-β-D-ribofuranoside treatment. Hypothalamic AMPK inhibition with icv injection of compound C or with injection of a small interfering RNA targeted to AMPKα2 in the mediobasal hypothalamus (MBH) suppressed the hepatic effects of ip fructose. We also found that fructose increased corticosterone levels through a mechanism that is dependent on hypothalamic AMPK activation. Concomitantly, fructose-stimulated gluconeogenesis, hepatic PEPCK expression, and glucocorticoid receptor binding to the *PEPCK* gene were suppressed by pharmacological glucocorticoid receptor blockage. Altogether the data presented herein support the hypothesis that fructose-induced hypothalamic AMPK activation stimulates hepatic gluconeogenesis by increasing corticosterone levels. (***Endocrinology*** **153: 0000–0000, 2012)**

Fructose is a naturally occurring monosaccharide and is rarely present in the human diet as a single nutrient. Human exposure to dietary fructose results mainly from the consumption of sucrose (fructose-glucose) and high-fructose corn syrup. High-fructose corn syrup currently represents the most popular sweetener in the United States, and its consumption, along with the incidence of obesity and diabetes, is continuously increasing (1). In nonobese humans, fructose consumption was described as reducing insulin sensitivity and increasing fasting hepatic glucose output (2). In rodents, fructose-enriched diets have been demonstrated to cause hepatic insulin resistance (3) and to increase gluconeogenesis (4).

Gluconeogenesis is an important component of the increased rate of hepatic glucose production, acting together with peripheral insulin resistance to promote hyperglyce-

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Abbreviations: ACC, Acetyl-CoA carboxylase; AICAR, 5-amino-imidazole-4-carboxamide-1-β-D-ribofuranoside; AMPK, AMP-activated protein kinase; ARC, arcuate nucleus; AUC, area under the curve; ChIP, chromatin immunoprecipitation; CNS, central nervous system; CPT1, carnitine palmitoyltransferase-1; CTL, control; G6Pase, glucose-6-phosphatase; GR, glucocorticoid receptor; Icv, intracerebroventricular; LH, lateral hypothalamus; MBH, mediobasal hypothalamus; p, phosphorylated; PEPCK, phosphoenolpyruvate carboxy kinase; PTT, pyruvate tolerance test; PVN, paraventricular nucleus; siRNA, small interfering RNA.

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ORIGINAL ARTICLE

Taurine enhances the anorexigenic effects of insulin in the hypothalamus of rats

Carina S. Solon · Daniel Franci · Letícia M. Ignacio-Souza · Talita Romanatto · Erika A. Roman · Ana P. Arruda · Joseane Morari · Adriana S. Torsoni · Everardo M. Carneiro · Licio A. Velloso

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Abstract Taurine is known to modulate a number of metabolic parameters such as insulin secretion and action and blood cholesterol levels. Recent data have suggested that taurine can also reduce body adiposity in *C. elegans* and in rodents. Since body adiposity is mostly regulated by insulin-responsive hypothalamic neurons involved in the control of feeding and thermogenesis, we hypothesized that some of the activity of taurine in the control of body fat would be exerted through a direct action in the hypothalamus. Here, we show that the intracerebroventricular injection of an acute dose of taurine reduces food intake and locomotor activity, and activates signal transduction through the Akt/FOXO1, JAK2/STAT3 and mTOR/AMPK/ACC signaling pathways. These effects are accompanied by the modulation of expression of NPY. In addition, taurine can enhance the anorexigenic action of insulin. Thus, the aminoacid, taurine, exerts a potent anorexigenic action in the hypothalamus and enhances the effect of insulin on the control of food intake.

Keywords Obesity · Leptin · Neurotransmitter

Abbreviations

ACC	Acetyl CoA carboxylase
AgRP	Agouti-related peptide
Akt	Protein kinase B
AMPK	Adenosine monophosphate activated kinase
CART	Cocaine and amphetamine related transcript
FOXO1	Forkhead box protein O1
JAK2	Janus kinase 2
mTOR	Mammalian target of rapamycin
NPY	Neuropeptide Y
PAGE	Polyacrylamide gel electrophoresis
PI3K	Phosphatidylinositol 3 kinase
PMSF	Phenylmethylsulfonyl fluoride
POMC	Proopiomelanocortin
SDS	Sodium dodecyl sulphate
STAT3	Signal transducer and activator of transcription 3

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Introduction

Taurine dietary supplementation is reported to exert a number of beneficial effects in diseases such as diabetes, hypercholesterolemia, ischemia and neuronal damage (Brosnan and Brosnan 2006; Lombardini and Militante 2006; Wu 2009). The mechanisms involved on its actions range from increasing insulin secretion (Ribeiro et al. 2009) and action (Carneiro et al. 2009), in diabetes; reduction of hepatic cholesterol production, in hypercholesterolemia (Murakami et al. 2010); and reduction of caspase-8 and -9, in ischemic neuronal apoptosis (Taranukhin et al. 2008). Some of these mechanisms depend, at least in part, on the antioxidant actions of taurine (Penttila 1990; Xiao et al. 2008).

In type 2 diabetes, both experimental and clinical evidence suggest that taurine can reduce insulin resistance, leading to increased insulin-dependent glucose uptake

ARTICLE
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Journal of Cellular Biochemistry

Hypothalamic Action of Glutamate Leads to Body Mass Reduction Through a Mechanism Partially Dependent on JAK2

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ABSTRACT

Glutamate acts in the hypothalamus promoting region-, and cell-dependent effects on feeding. Part of these effects are mediated by NMDA receptors, which are up regulated in conditions known to promote increased food intake and thermogenesis, such as exposure to cold and consumption of highly caloric diets. Here, we hypothesized that at least part of the effect of glutamate on hypothalamic control of energy homeostasis would depend on the control of neurotransmitter expression and JAK2 signaling. The expression of NMDA receptors was co-localized to NPY/AgRP, POMC, CRH, and MCH but not to TRH and orexin neurons of the hypothalamus. The acute intracerebroventricular injection of glutamate promoted a dose-dependent increase in JAK2 tyrosine phosphorylation. In obese rats, 5 days intracerebroventricular treatment with glutamate resulted in the reduction of food intake, accompanied by a reduction of spontaneous motility and reduction of body mass, without affecting oxygen consumption. The reduction of food intake and body mass were partially restrained by the inhibition of JAK2. In addition, glutamate produced an increased hypothalamic expression of NPY, POMC, CART, MCH, orexin, CRH, and TRH, and the reduction of AgRP. All these effects on neurotransmitters were hindered by the inhibition of JAK2. Thus, the intracerebroventricular injection of glutamate results in the reduction of body mass through a mechanism, at least in part, dependent on JAK2, and on the broad regulation of neurotransmitter expression. These effects are not impaired by obesity, which suggest that glutamate actions in the hypothalamus may be pharmacologically explored to treat this disease. J. Cell. Biochem. 113: 1182–1189, 2012.

KEY WORDS: LEPTIN; OBESITY; NEUROTRANSMITTER; FEEDING

Glutamate elicits orexigenic and/or anorexigenic responses when injected directly in the hypothalamus. These effects depend on the site of injection and other variables such as time-course and dose [Guard et al., 2009; Stanley et al., 2011]. All three ionotropic glutamate receptor subtypes, NMDA, AMPA, and KA are known to play a role in these responses and defining the mechanisms behind this control may provide valuable information for the development of drugs for the treatment of obesity and related disorders [Doane et al., 2007; Stanley et al., 2011].

Recently we reported that rats submitted to two different pro-thermogenic conditions, that is, cold exposure and feeding on a high-fat diet, would selectively and coincidently modulate only a minority of hypothalamic genes, suggesting that the products of such genes would play important roles in energy homeostasis [De Souza et al., 2008]. One of the genes is NMDA2B, which codes for a subunit of the NMDA receptor. Upon both cold exposure and high-fat feeding the expression of NMDA2B increases by approximately threefold [De Souza et al., 2008].

A number of studies have evaluated the mechanisms by which NMDA plays a role in the control of food intake [Zhang and Fogel, 2002; Guard et al., 2009; Stanley et al., 2011]. At the cellular level, the activation of NMDA leads to increased excitability and neurotransmitter release [Zhang and Fogel, 2002; Guard et al., 2009; Stanley et al., 2011]. Depending on the neuron type responding to glutamate, orexigenic, or anorexigenic neurotransmitter can be released [Zhang and Fogel, 2002; Guard et al., 2009; Stanley et al., 2011]. In neurons of the arcuate nucleus, insulin, and leptin act in concert to provide the most robust anorexigenic signals [Figlewicz and Benoit, 2009]. These signals control both neurotransmitter expression and release, and the activation of JAK2 provides the cross-talk between the signaling systems of these hormones [Villanueva and Myers, 2008]. Here we tested the hypothesis that

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Research Paper

Enhanced insulin secretion and glucose tolerance in rats exhibiting low plasma free fatty acid levels and hypertriglyceridaemia due to congenital albumin deficiency

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Congenitally analbuminaemic individuals and rats (NARs) exhibit several metabolic abnormalities, including hypertriglyceridaemia and plasma free fatty acid deficiency. Our aim was to study glucose homeostasis and insulin secretion in NARs. Plasma concentrations of lipids, glucose and insulin and secretion of insulin from the pancreatic islets were measured in female NARs and control animals (Sprague–Dawley rats; SDRs). Glucose homeostasis tests were also performed. Plasma glucose levels were similar between NARs and SDRs, irrespective of feeding status. However, fed insulinaemia was ~37% higher ($P \leq 0.05$) in NARs than in SDRs. The NARs displayed a markedly increased glucose tolerance, i.e. the integrated glycaemic response was one-third that of the control animals. Enhanced glucose tolerance was associated with threefold higher insulinaemia at peak glycaemia after a glucose load than in the control animals. Similar peripheral insulin sensitivity was observed between groups. Isolated pancreatic islets from NARs secreted significantly more insulin than islets from SDRs in response to a wide range of glucose concentrations (2.8–33.3 mM). Despite having similar liver glycogen contents in the fully fed state, NARs had ~40% ($P \leq 0.05$) lower glycogen contents than SDRs after 6 h fasting. The injection of a gluconeogenic substrate, pyruvate, elicited a faster rise in glycaemia in NARs compared with SDRs. Overall, NARs displayed enhanced glucose tolerance, insulin secretion and gluconeogenic flux. The higher glucose tolerance in NARs compared with SDRs is attributed to enhanced islet responsiveness to secretagogues, while peripheral insulin sensitivity seems not to be involved in this alteration. We propose that the enhanced glucose metabolism is a chronic compensatory adaptation to decreased free fatty acid availability in NARs.

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Congenital analbuminaemia is a rare autosomal recessive disorder characterized by very low levels of plasma albumin (<1 mg ml^{-1}) in the absence of renal and intestinal protein loss (Weinstock *et al.* 1979). This abnormality results from negligible hepatic albumin production due to mutations in the albumin gene (Minchiotti *et al.* 2008). The first case of human analbuminaemia was reported in 1954 (Kallee, 1996), and several additional cases of human analbuminaemia have been identified. By selectively breeding spontaneously hyperlipidaemic Sprague–Dawley rats (SDRs), Nagase *et al.* (1979) established a colony of rats, Nagase analbuminaemic rats (NARs), that were confirmed to be analbuminaemic. These rats model many features of human familial analbuminaemia (Baldo-Enzi *et al.* 1987).

* T.R.F. and R.A.R. contributed equally to this work.

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Protein restriction in early life is associated with changes in insulin sensitivity and pancreatic β-cell function during pregnancy

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Abstract

Malnutrition in early life impairs glucose-stimulated insulin secretion in adulthood. Conversely, pregnancy is associated with a significant increase in glucose-stimulated insulin secretion under conditions of normoglycaemia. A failure in β-cell adaptive changes may contribute to the onset of diabetes. Thus, glucose homeostasis and β-cell function were evaluated in control-fed pregnant (CP) and non-pregnant (CNP) or protein-restricted pregnant (LPP) and non-pregnant (LPNP) rats, from fetal to adult life, and in protein-restricted rats that were recovered after weaning (RP and RNP). The typical insulin resistance of pregnancy was not observed in the RP rats, nor did pregnancy increase the insulin content/islet in the LPP group. The glucose dose–response curves from pregnant rats were shifted to the left in relation to the non-pregnant rats, except in the recovered group. Glucose utilisation but not oxidation in islets from the RP and LPP groups was reduced at a concentration of 8·3 mM-glucose compared with islets from the CP group. Cyclic AMP content and the potentiation of glucose-stimulated insulin secretion by isobutylmethylxanthine at a concentration of 2·8 mM-glucose indicated increased adenylyl cyclase 3 activity but reduced protein kinase A-α activity in islets from the RP and LPP rats. Protein kinase C (PKC)-α but not phospholipase C (PLC)-β1 expression was reduced in islets from the RP group. Phorbol-12-myristate 13-acetate produced a less potent stimulation of glucose-stimulated insulin secretion in the RP group. Thus, the alterations exhibited by islets from the LPP group appeared to be due to reduced islet mass and/or insulin biosynthesis. In the RP group the loss of the adaptive capacity apparently resulted from uncoupling between glucose metabolism and the amplifying signals of the secretory process, as well as a severe attenuation of the PLC/PKC pathway.

Key words: Glucose homeostasis: Insulin secretion: Malnutrition: Nutritional recovery: Pregnancy

The basic mechanism of insulin secretion involves the coupling of glucose metabolism with secondary signals, which maintains insulin release for the duration of elevated blood glucose levels[1].

In rodents, malnutrition during the critical stages of development causes a permanent loss of glucose sensitivity and secretory capacity in pancreatic islets[2], which is probably the result of alterations to the coupling of stimuli with insulin secretion. In pancreatic islets from rats fed a low-protein (LP) diet during intra-uterine life and/or lactation, reductions in glucokinase (Gck) and hexokinase (Hxk) activity and content[3], decreases in Ca^{2+} uptake and/or Ca^{2+} efflux[4], and impairments in insulin

Abbreviations: ΔG, total area under the glucose curve; ΔI, total area under the insulin curve; AC3, adenylyl cyclase 3; C, control; cAMP, cyclic AMP; CNP, control non-pregnant; CP, control pregnant; Gck, glucokinase; GLUT2, glucose transporter 2; Hxk, hexokinase; IBMX, isobutylmethylxanthine; K_{itt}, constant rate for plasma glucose disappearance; LP, low protein; LPNP, low-protein non-pregnant; LPP, low-protein pregnant; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol-2-myristate 13-acetate; R, recovered; RNP, recovered non-pregnant; RP, recovered pregnant.

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A low-protein diet during pregnancy alters glucose metabolism and insulin secretion

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In pancreatic islets, glucose metabolism is a key process for insulin secretion, and pregnancy requires an increase in insulin secretion to compensate for the typical insulin resistance at the end of this period. Because a low-protein diet decreases insulin secretion, this type of diet could impair glucose homeostasis, leading to gestational diabetes. In pancreatic islets, we investigated GLUT2, glucokinase and hexokinase expression patterns as well as glucose uptake, utilization and oxidation rates. Adult control non-pregnant (CNP) and control pregnant (CP) rats were fed a normal protein diet (17%), whereas low-protein non-pregnant (LPNP) and low-protein pregnant (LPP) rats were fed a low-protein diet (6%) from days 1 to 15 of pregnancy. The insulin secretion in 2.8 mmol l^{-1} of glucose was higher in islets from LPP rats than that in islets from CP, CNP and LPNP rats. Maximal insulin release was obtained at 8.3 and 16.7 mmol l^{-1} of glucose in LPP and CP groups, respectively. The glucose dose–response curve from LPNP group was shifted to the right in relation to the CNP group. In the CP group, the concentration–response curve to glucose was shifted to the left compared with the CNP group. The LPP groups exhibited an "inverted U-shape" dose–response curve. The alterations in the GLUT2, glucokinase and hexokinase expression patterns neither impaired glucose metabolism nor correlated with glucose islet sensitivity, suggesting that β-cell sensitivity to glucose requires secondary events other than the observed metabolic/molecular events. Copyright © 2011 John Wiley & Sons, Ltd.

KEY WORDS—low-protein diet; pregnancy; glucose metabolism; pancreatic islets; insulin secretion

INTRODUCTION

Insulin secretion by pancreatic β-cells is modulated by nutrients, neurotransmitters and hormones.[1] Glucose, which is the major physiologic stimulator of insulin secretion, is transported into the β-cell through GLUT2,[2] is phosphorylated by glucokinase and then undergoes glycolysis and oxidation.[3] This glucose metabolism leads to an increase in the cytosolic adenosine triphosphate (ATP)/adenosine diphosphate ratio, which blocks the ATP-sensitive K^+ channels that are located at the plasma membrane[4] and leads to the opening of voltage-dependent Ca^{2+} channels; the latter results in an influx and subsequent elevation of cytosolic calcium $[Ca^{2+}]_i$.[5] The increase in $[Ca^{2+}]_i$ triggers the exocytosis of insulin-containing granules.[6] In the β-cells, the rate-limiting step in glucose metabolism is the phosphorylation of glucose by the enzyme glucokinase, and even small changes in glucokinase expression or activity have very substantial effects on insulin secretion and, in particular, on the threshold of glucose-stimulated insulin secretion.[7–9]

Pregnancy is a condition that requires a very large increase in insulin secretion at normal glucose levels, and to accommodate the increased demand for insulin, the pancreatic islets of Langerhans undergo functional changes.[10,11] The most important change is the lowering of the threshold for glucose-stimulated insulin secretion,[10] and the primary factors that are responsible for this adaptive change are lactogenic hormones, including placental lactogen and/or prolactin.[12,13] It has been demonstrated that the islets from pregnant rats and those cultured with prolactin have elevated glucose utilization and oxidation rates that correlate with increased GLUT2 and glucokinase expressions and activities.[14–16]

Protein restriction impairs insulin secretion, and this alteration is represented by a shift to the right in the glucose dose–response curves.[17–19] Impaired insulin secretion in malnutrition has been related to alterations at different steps in the secretion mechanism, such as reduced activity of pancreatic glucokinase[20] and β-cell mitochondrial dysfunction,[21] that could lead to impaired glucose metabolism.

Considering the contrary effects of pregnancy and protein restriction on glucose metabolism and insulin secretion, we investigated the biochemical and molecular adaptations that are responsible for these alterations in pregnant rats that have been submitted to protein restriction. For this purpose,

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A soyabean diet does not modify the activity of brown adipose tissue but alters the rate of lipolysis in the retroperitoneal white adipose tissue of male rats recovering from early-life malnutrition

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Abstract

Nutritional recovery with a soyabean diet decreases body and fat weights when compared with a casein diet. We investigated whether the reduced adiposity observed in rats recovering from early-life malnutrition with a soyabean diet results from alterations in lipid metabolism in white adipose tissue (WAT) and/or brown adipose tissue (BAT). Male rats from mothers fed either 17 or 6% protein during pregnancy and lactation were maintained on 17% casein (CC and LC groups), 17% soyabean (CS and LS groups) or 6% casein (LL group) diets over 60 d. The rats maintained on a soyabean diet had similar relative food intakes, but lower body and retroperitoneal WAT weights and a reduced lipid content in the retroperitoneal WAT. The insulin levels were lower in the recovered rats and were elevated in those fed a soyabean diet. Serum T3 concentration and uncoupling protein 1 content in the BAT were decreased in the recovered rats. The thermogenic capacity of the BAT was not affected by the soyabean diet. The lipogenesis rate in the retroperitoneal WAT was similar in all of the groups except for the LL group, which had exacerbated lipogenesis. The enhancement of the lipolysis rate by isoproterenol was decreased in white adipocytes from the soyabean-recovered rats and was elevated in adipocytes from the soyabean-control rats. Thus, in animals maintained on a soyabean diet, the proportions of fat deposits are determined by the lipolysis rate, which differs depending on the previous nutritional status.

Key words: **Nutritional recovery: Soyabean diet: Adipose tissue: Rats**

Soya-containing diets have been shown to alter several parameters involved in maintaining body homeostasis, energy expenditure and feeding behaviour[1–3]. Soya protein and isoflavone have been reported to reduce fat-pad weight by increasing uncoupling protein 1 (UCP-1) expression in brown adipose tissue (BAT)[4]. UCP-1 is a molecule that uncouples mitochondrial oxidative phosphorylation by bypassing the electrochemical gradient across the inner membrane from

Abbreviations: BAT, brown adipose tissue; C, control; CC, offspring born to and suckled by mothers fed a control diet that were fed a control diet after weaning; CS, offspring born to and suckled by mothers fed a control diet that were fed a soyabean diet with 17% protein after weaning; HOMA-IR, homeostasis model assessment of insulin resistance; LC, offspring of mothers fed a low-protein diet that were fed a control diet after weaning; LL, offspring of mothers fed a low-protein diet that were fed a low-protein diet after weaning; LP, low-protein; LS, offspring of mothers fed a low-protein diet that were fed a soyabean diet containing 17% protein after weaning; RWAT, retroperitoneal white adipose tissue; Tris, 2-amino-2-hydroxymethyl-propane-1,3-diol; UCP-1, uncoupling protein 1; WAT, white adipose tissue.

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