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A INFLUÊNCIA DA FLORA INTESTINAL E DA ESPLENECTOMIA NA RESISTÊNCIA À INSULINA INDUZIDA POR OBESIDADE

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Bruno de Melo Carvalho

Tese de doutorado apresentada à pós-graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas para obtenção do título de Doutor em Ciências. Sob orientação do Prof. Dr. Mario José Abdalla Saad

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"O único lugar onde o sucesso vem antes do trabalho, é no dicionário." - Albert Einstein.

LISTA DE ABREVIATURAS

AMPK	*5´AMP-activated protein kinase*	Proteína quinase ativada por AMP
AT1	*Angiotensin receptor 1*	Receptor de Angiotensina 1
AT2	*Angiotensin receptor 2*	Receptor de Angiotensina 2
Atgr1α	*Angiotensin receptor 1 gene*	Gene do receptor de angiotensina 1
CCR2	*chemokine (C-C motif) receptor 2*	Receptor de quimiocinas 2
CD11c	*Integrin, alpha X (complement component 3 receptor 4 subunit)*	Integrina alfa X
CLS	*Crown-Like Structures*	Estruturas semelhantes à coroa
DAMPs	*Damage associated molecular pattern molecules*	Padrões moleculares associados a lesão
DNA	*Deoxyribonucleic acid*	Àcido desoxirribonucléico
F4/80	*EGF-like module-containing mucin-like hormone receptor-like 1*	
Fiaf	*Fasting-induced adipocyte factor*	Fator derivado de adipócito induzido por jejum
FoxO1	*Forkhead box protein O1*	Proteína Forkhead box O1
G6pc	*Glucose-6-phosphatase gene*	Gene da glicose 6-fosfatase
GLUT4	*Glucose transporter type 4*	Transportador de glicose 4
GMF-γ	*Glia Maturation Factor-g*	Fator de maturação da glia-γ
IL-6	*Interleukin-6*	Interleucina-6
IκBα	*Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha*	Inibidor a de NF-κB
IKKβ	*Inhibitor of nuclear factor kappa-B kinase subunit beta*	IκB quinase inhibitor β
iNOS	*Inducible Nitric Oxide Synthase*	Óxido nítrico sintase induzível
IMC		Índice de massa corporal
IR	*Insulin Receptor*	Receptor de insulina
IRAK	*Interleukin-1 Receptor Associated Kinase*	Quinase associada

		ao receptor de interleucina-1
IRS	*Insulin Receptor Substrate*	Substrato do receptor de insulina
JNK	*c-Jun N-terminal kinase*	
LPL	*Lipoprotein Lipase*	Lipase lipoprotéica
LPS	*Lipopolysaccharides*	Lipopolissacarídeos
Ly-6C	*Gr-1 (granulocyte marker)*	Marcador de granulócitos
MCP-1	*Monocyte Chemotactic Protein 1*	Proteína quimioatraente de monócitos 1
MyD88	*Myeloid Differentiation primary response gene 88*	Gene de resposta à diferenciação mielóide 88
NAFLD	*Non Alcoholic Fatty Liver Disease*	Doença hepática gordurosa não alcoólica
NASH	*Non Alcoholic Steatohepatitis*	Esteato-hepatite não alcoólica
NF-κB	*Nuclear factor kappa-light-chain-enhancer of activated B cells*	Fator nuclear κB
PAMPs	*Pathogen associated molecular pattern molecules*	Padrões moleculares associados a patógenos
Pck-1	*Phosphoenolpyruvate carboxykinase 1 gene*	Gene da fosfoenolpiruvato carboxiquinase 1
PI3K	*Phosphatidylinositol 3-kinase*	Fosfatidil-inositol 3 quinase
PGC-1α	*Peroxisome proliferator-activated receptor gamma coactivator 1-alpha*	Co-ativador 1a de PPAR-γ
RNAm	*Messenger Ribonucleic Acid*	Ácido ribonucléico mensageiro
SCFA	*Short Chain Fatty Acids*	Ácidos graxos de cadeia curta
TLR4	*Toll-Like Receptor 4*	Receptor semelhante a Toll 4
TNF-α	*Tumor Necrosis Factor-a*	Fator de necrose tumoral-α
TNFR	*Tumor Necrosis Factor Receptor*	Receptor de TNF
ZO-1	*Zonula Occludens-1*	

LISTA DE FIGURAS

RESUMO

A ingestão de alimentos ricos em gordura leva à obesidade e inflamação crônica sub-clínica, a qual desenvolve papel importante na resistência à insulina. Aumentados níveis circulantes de citocinas pró-inflamatórias, ácidos graxos livres e lipopolissacarídeos ativam o sistema imune inato, que desencadeia inflamação e aumento na expressão de citocinas, levando à resistência à insulina. Dessa forma, nós investigamos o efeito da modulação da flora intestinal, na resistência à insulina e avaliamos o baço como uma nova fonte de inflamação e células do sistema imune, responsáveis pela resistência à insulina induzida por obesidade, cujas funções ainda não estão completamente determinadas. Para investigar os efeitos da modulação da flora intestinal, nós submetemos camundongos a dieta hiperlipídica com antibióticos ou em regime de *pair-feeding* por oito semanas e realizamos análises metagenômicas de amostras de DNA provenientes das fezes dos camundongos. A fim de avaliar a influência do baço no metabolismo, nós realizamos esplenectomia em camundongos e induzimos obesidade com a utilização de dieta rica em gordura, além de fazer abordagens proteômicas para determinar novas moléculas que poderiam estar envolvidas na inflamação e migração de células. Em ambos os experimentos, glicose, insulina e citocinas circulantes foram avaliadas, assim como as vias de sinalização da insulina e inflamatória em fígado, músculo e tecido adiposo, como também avaliamos a infiltração de macrófagos no fígado e no tecido adiposo. A flora intestinal estava extremamente modificada pelo tratamento com antibióticos, reduzindo a prevalência de *Bacteroidetes* e *Firmicutes*, a quantidade de bactérias no intestino e LPS circulante, bem como glicemia, insulinemia e citocinas. Este quadro também apresentou regulação negativa do TLR4 e redução na inflamação, a qual induziu aumento na sensibilidade à insulina, além da notável redução de macrófagos infiltrados nos tecidos dos

camundongos tratados com antibióticos. Em camundongos obesos esplenectomizados, houve grande aumento na sensibilidade à insulina, refletida por redução na glicemia, insulinemia e TNF-α circulante quando comparado com camundongos obesos. A inflamação estava reduzida no fígado, músculo e tecido adiposo dos camundongos obesos esplenectomizados, e como consequência, a via de sinalização de insulina estava mais ativa em comparação aos camundongos obesos que tiveram os baços mantidos. Também houve uma imensa redução na infiltração de macrófagos, no fígado e no tecido adiposo dos camundongos esplenectomizados após a indução de obesidade, quadro semelhante ao encontrado na indução da infiltração de macrófagos pela lipólise. A análise proteômica do baço indicou que GMF-γ está mais expresso nos camundongos obesos em relação aos magros, dado confirmado por *immunoblot*. Concluindo, a modulação da flora intestinal por uma terapia com antibióticos reduziu os níveis circulantes de LPS, inflamação e infiltração de macrófagos, aumentando a sensibilidade à insulina em camundongos alimentados com dieta hiperlipídica. Além disso, a esplenectomia também reduziu a inflamação e promoveu melhora na sensibilidade à insulina, bem como inibiu a infiltração de macrófagos induzida pela obesidade, fenômeno que pode ser coordenado pelo baço e quimiocinas, e possivelmente pelo GMF-γ, uma nova proteína, que estariam envolvidas na regulação da migração celular e estabelecimento de resistência à insulina.

ABSTRACT

A high-fat diet intake induces obesity and chronic subclinical inflammation, which play important roles in insulin resistance. Increased circulating levels of proinflammatory cytokines, free fatty acids and lipopolysaccharides activate innate immune system, which triggers inflammation and cytokine expression, leading to insulin resistance. Thus, we investigated the effect of gut microbiota modulation, on insulin resistance and evaluated the spleen as a novel source of inflammation and immune cells, responsible for the obesity-induced insulin resistance, which roles are not yet fully understood. To investigate microbiota modulation effects, we submitted Swiss mice to a high-fat diet with antibiotics or pair-feeding for eight weeks and performed metagenomic analyses from mice fecal DNA samples. In order to evaluate the spleen influence over the metabolism, we performed splenectomy in Swiss mice and induced obesity with a high-fat diet and performed proteomic approaches to determine novel molecules that could promote inflammation and immune cell migration. In both experiments, blood glucose, serum insulin and cytokines were evaluated, as well as liver, muscle and adipose tissue insulin and inflammatory signaling pathway, and liver and adipose tissue macrophage infiltration. Gut microbiota was greatly modified by the antibiotic treatment, reducing *Bacteroidetes* and *Firmicutes* prevalence, overall bacterial count and circulating LPS, as well as fasting blood glucose and serum insulin and cytokines. It also promoted TLR4 downregulation and reduction in inflammation, which promoted improvement in insulin sensitivity, besides a striking reduction in macrophage infiltration in antibiotic-treated mice. In splenectomized obese mice, a great improvement in insulin sensitivity was seen, reflected by blood glucose, serum insulin and TNF-α levels reduction. Inflammation was reduced in the liver, muscle

and adipose tissue of obese splenectomized mice, in consequence, insulin signaling was improved when compared to obese mice that maintained the spleen. There was an immense reduction in liver and adipose tissue macrophage infiltration in splenectomized mice after obesity induction, which was repeated when we observed lipolysis-induced adipose tissue macrophage infiltration. Spleen proteomic studies indicated that GMF-γ is overexpressed in obese mice compared to lean ones. In conclusion, gut microbiota modulation by antibiotic therapy reduced circulating LPS levels, inflammation and macrophage infiltration, improving insulin sensitivity in mice fed a high-fat diet. In addition, splenectomy also reduced inflammation and promoted insulin sensitization, as well as inhibited obesity-induced macrophage infiltration, which can be ruled by the spleen and chemokines, and possibly by GMF-γ, a novel protein, that could be involved in the regulation of cell migration and insulin resistance settlement.

SUMÁRIO

INTRODUÇÃO

A prevalência de obesidade no mundo e a contribuição desta para a ocorrência de doenças no mundo já ultrapassou os perigos representados pela sub-nutrição e as doenças infecciosas para a população. Na prática clínica, é avaliada através do cálculo do Índice de Massa Corporal (IMC), representando relação entre a altura e o peso dos indivíduos, assumindo que a principal variação no peso em pessoas da mesma altura é devido à massa adiposa e classificando os casos de obesidade de acordo com este valor [1].

A obesidade é uma doença em que a prevalência adquiriu proporções epidêmicas em todo o mundo, notadamente em países desenvolvidos, como os Estados Unidos, mas também em países em desenvolvimento como o Brasil, bem como em outros países da América Latina [2]. Em estudo prospectivo realizado com participação de 9 milhões de pessoas, entre os anos de 1980 e 2008, demonstrou-se que a média de crescimento mundial do IMC foi de 1,4 kg/m^2 para os homens e de 1,9 kg/m^2 para as mulheres por década [3].

O aumento do percentual de gordura e a resultante redução na ação da insulina estão associadas com uma gama de problemas de saúde, incluindo destacado aumento no risco de desenvolvimento de diabetes do tipo 2, esteatose e esteato-hepatite não alcoólica, dislipidemias, doenças das vias biliares e da bexiga, doenças cardiovasculares, doenças neuro-degenerativas, asma, apnéia do sono e relação com uma variedade de tipos de câncer [4].

O peso corpóreo é definido pela interação de fatores genéticos, ambientais e psicossociais, que agem através de mediadores da ingestão e do gasto energético. Existem raras síndromes associadas à obesidade, demonstrando intrínseca relação genética na

indução de obesidade, embora esta influência pareça ser exercida principalmente por genes suscetíveis, que aumentam o risco de desenvolvimento da obesidade, mas não o determinam, hipótese sustentada por estudos em gêmeos que foram expostos a períodos de equilíbrio energético positivo ou negativo [5].

Dentre os fatores ambientais, o gasto energético, baseado principalmente na atividade física, influencia de forma determinante no ganho de peso [6]. Alterações no ritmo metabólico que indica o metabolismo basal, ou seja, o gasto energético para a manutenção da homeostase também levam ao desequilíbrio energético, de forma a reduzir ou aumentar o gasto, influenciando no ganho de peso [7]. A facilidade de obtenção e a ingestão de alimentos de alta densidade energética, associada à redução da prática de atividades físicas observadas na atual sociedade, também é um dos fatores ambientais determinantes para o ganho de peso e propagação em níveis epidêmicos da obesidade [8, 9].

A distribuição da obesidade no mundo é avaliada pelo IMC da população, havendo uma significante variação de acordo com a situação econômica e a industrialização de um país (Figura 1). Isso indica uma tendência para uma mudança populacional com o sobrepeso substituindo a magreza seguindo a melhora da condição sócio-econômica. De semelhante importância, as alterações na prevalência de obesidade nos adultos são reflexos do grande aumento no peso das crianças e adolescentes tanto em países industrializados quanto em países em desenvolvimento e esta relação também acarreta aumento da prevalência de doenças relacionadas à obesidade [10, 11]. A expansão da obesidade nos níveis atuais provoca impacto severo nas economias dos países desenvolvidos, com custos estimados entre 2% e 7% das finanças direcionadas à saúde, ao passo que este problema

também é refletido na economia dos países em desenvolvimento e que possuem crescentes níveis de prevalência de obesidade [12].

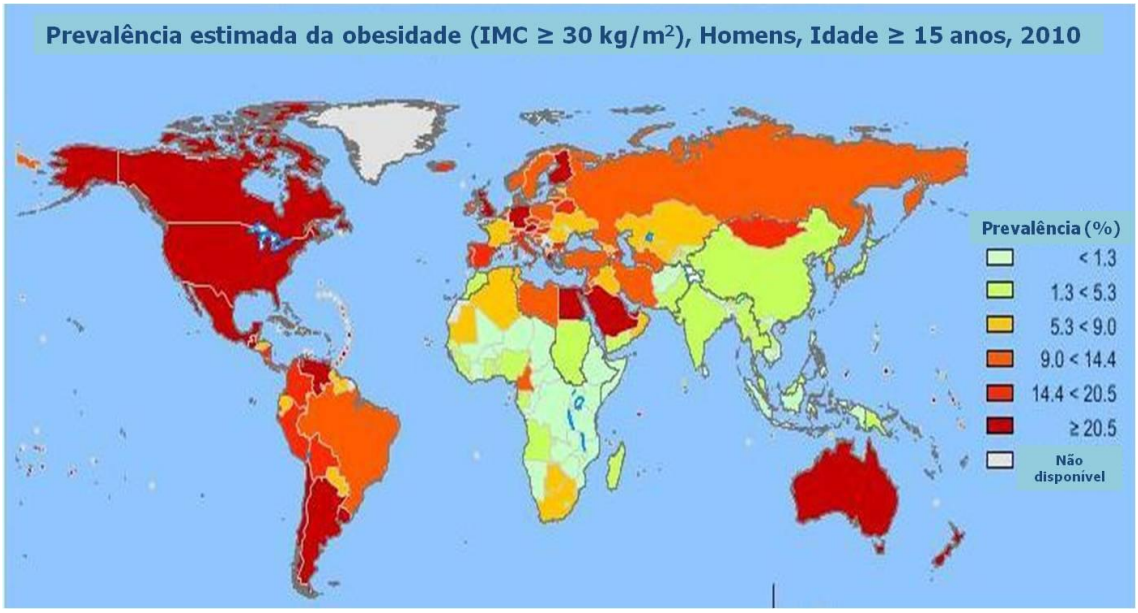

Figura 1: Prevalência mundial de obesidade (adaptado Organização Mundial da Saúde - OMS, 2010).

A obesidade é descrita como uma condição inflamatória sub-clínica de curso crônico, caracterizada pela ocorrência de resistência à insulina, fenômeno pelo qual o hormônio insulina não consegue exercer suas funções anabólicas e é reconhecidamente o elo entre a obesidade e doenças correlatas, como diabetes do tipo 2, esteato-hepatite não-alcoólica e disfunções cardiovasculares [13].

A via de sinalização da insulina é desencadeada a partir da ligação do hormônio ao seu receptor específico (IR), uma estrutura heterotetramérica transmembrana que possui a habilidade de se autofosforilar em resíduos de tirosina, indicando a propagação do sinal [14]. Devido à sua atividade tirosina quinase, o receptor de insulina fosforila uma família de proteínas denominada substratos do receptor de insulina (IRS 1-6) também em resíduos de tirosina, que por sua vez ativa a PI3K [15]. A ativação da PI3K leva à fosforilação da

proteína Akt, que então promove ações inerentes à função da insulina, dentre elas a translocação de GLUT4 para a membrana plasmática de células de músculo e de tecido adiposo [16], a síntese de glicogênio [17], à lipogênese [18], à redução da produção hepática de glicose [19], bem como um maior controle geral dos padrões de expressão gênica [20] (Figura 2).

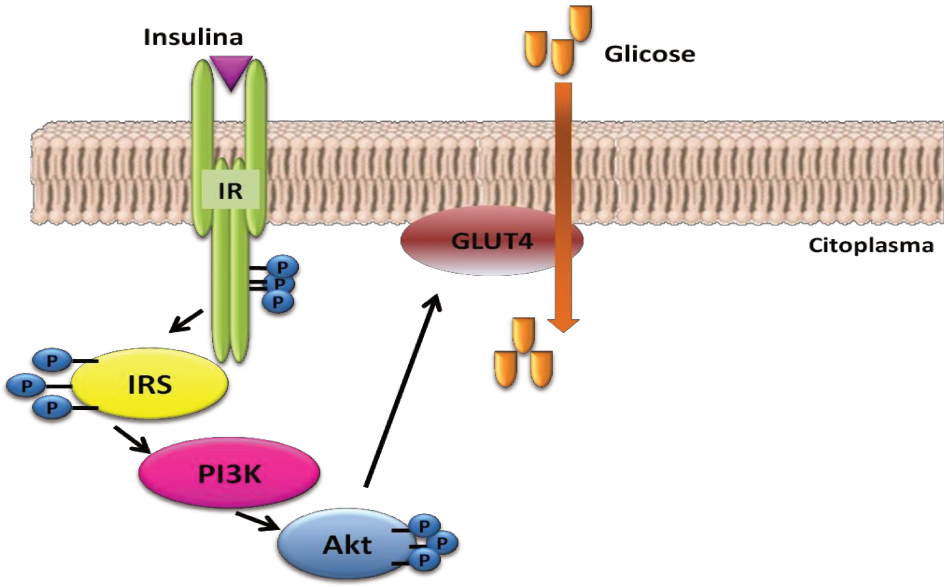

Figura 2: Esquema da ativação da via de sinalização da insulina.

Através de experimentos genéticos em *C. elegans*, ficou conhecido o mecanismo pelo qual a insulina regula a expressão de genes no fígado, onde um fator de transcrição da família *forkhead* denominado Daf16 foi identificado como efetor chave da sinalização da insulina [21]. Em mamíferos, identificou-se como ortólogo do Daf16, um fator de transcrição denominado FoxO1 (*FOrkhead boX-containing gene, O subfamily*) que possui ação negativa sobre a sinalização da insulina e é substrato da Akt [19]. Na ausência de insulina a Foxo1 permanece desfosforilada e localizada no núcleo, onde se liga ao PGC-1α [19] para promover a transcrição dos genes Pck1 e G6pc. Já sob estímulo da insulina,

através da via da PI3K, a Akt catalisa a fosforilação da Foxo1, resultando na saída desse fator do núcleo e conseqüente redução da produção hepática de glicose.

Foi demonstrado que a resistência à insulina está vinculada à modulação das proteínas da via de sinalização deste hormônio, como o receptor e seus substratos, em diversos modelos de resistência à insulina [22, 23]. Nestes modelos, a fosforilação em tirosina se encontra reduzida, atenuando a resposta ao hormônio, causada pela fosforilação do IR e IRS-1 em resíduos de serina, o que promove mudanças conformacionais nestas proteínas, impedindo a interação do IRS-1 com o IR e o seguimento da atividade da via de sinalização da insulina [24] e consequente permanência do fator de transcrição FoxO1 no núcleo, o que acarreta impedimento da translocação de GLUT4 para a membrana das células e captação de glicose, além da continuação da produção hepática de glicose, respectivamente, mantendo o quadro de hiperglicemia característico destes modelos de resistência à insulina. Foi demonstrado que, em roedores, a serina 307 (Ser 307), dentre os resíduos de serina presentes na estrutura de IRS-1, é a responsável pela inibição da interação do IR com o IRS-1, tornando-se um marcador molecular de resistência à insulina [25].

Estudos demonstraram que o fator de necrose tumoral-α (TNF-α), clássica citocina pró-inflamatória, tem sua expressão aumentada no tecido adiposo de camundongos obesos, introduzindo a inflamação como componente importante da obesidade e diabetes [26], efeito confirmado quando observada a proteção à resistência à insulina induzida por dieta rica em gordura de camundongos deficientes de TNF-α [27] e de seu receptor [28].

Além de citocinas pró-inflamatórias, a ativação de receptores como o Toll-Like Receptor 4 (TLR4) a partir de lipopolissacarídeos (LPS) e ácidos graxos saturados, moléculas que possuem níveis circulantes aumentados na obesidade, promovem a ativação de cascatas inflamatórias intracelulares, como a JNK (c-Jun N-terminal kinase) e o IKKβ (Inhibitor of IκB kinase), que culminam na indução de resistência à insulina mediada por inflamação [29-31].

A atividade da proteína JNK está aumentada em situações de inflamação e obesidade, acarretando na indução da fosforilação do IRS-1 em Ser 307, inibindo sua fosforilação em tirosina e, impedindo assim, a propagação do sinal da insulina [32]. Camundongos que não possuíam a isoforma JNK1 estavam protegidos da resistência à insulina induzida por obesidade pela inibição da fosforilação em serina 307 de IRS-1, demonstrando a importância da influência desta proteína na indução de resistência à insulina [33, 34]. Além dos animais deficientes da proteína JNK, estudos utilizando inibidores específicos da atividade da JNK demonstraram melhora na sensibilidade à insulina e tolerância à glicose em animais com resistência à insulina [35, 36].

O complexo IKK é formado por proteínas que induzem a degradação de IκB (*Inhibitor of κB*), proteínas responsáveis por manter o fator de transcrição NF-κB (*Nuclear Factor κB*) no citoplasma [37]. Sob estímulos inflamatórios, o IκB é fosforilado e posteriormente ubiquitinado e degradado, liberando o NF-κB para o núcleo da célula, onde realiza suas funções de transcrição de elementos inflamatórios [37]. Além de alimentar o ciclo de inflamação corrente em situações de obesidade e diabetes, o IKKβ tem a

capacidade de induzir fosforilação do IRS-1 em Ser 307, contribuindo para a indução e manutenção da resistência à insulina [38] (Figura 3).

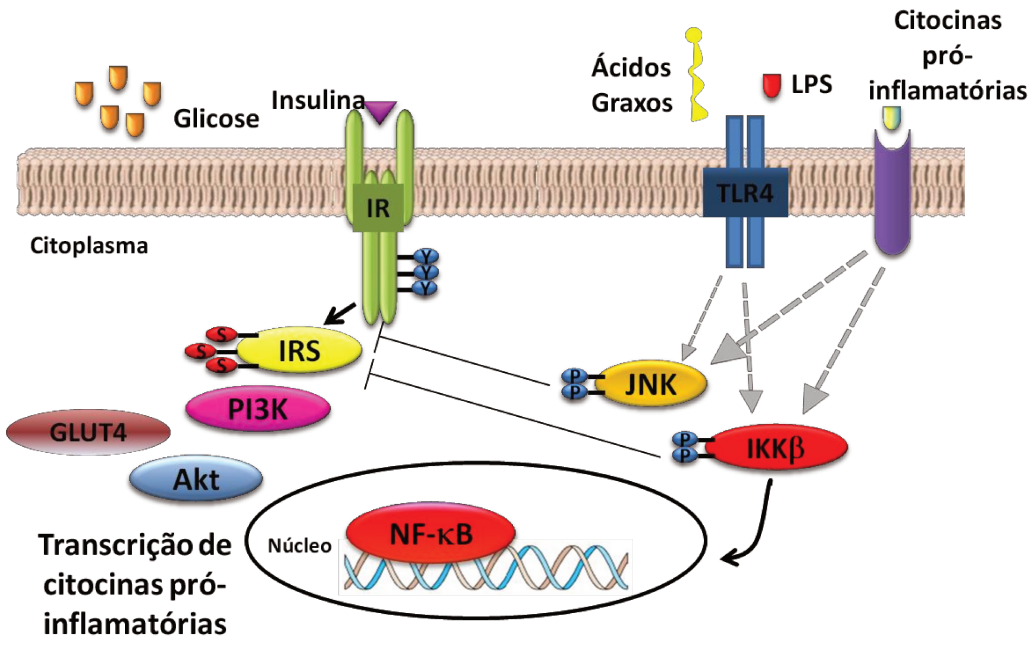

Figura 3: Inflamação e resistência à insulina. Esquema dos principais mecanismos moleculares de indução de resistência à insulina.

Ratos que receberam infusão de lipídeos e desenvolveram resistência à insulina foram tratados com salicilatos, onde se demonstrou que a inibição de IKKβ induzida por esta droga, era responsável pelo aumento da sensibilidade à insulina determinada pelo clamp hiperinsulinêmico-euglicêmico [39]. Ratos obesos tratados com o ácido acetilsalicílico também apresentaram inibição da atividade de IKKβ no músculo e consequente aumento na sensibilidade à insulina [40].

Camundongos obesos apresentaram aumento na atividade de IKKβ no fígado, resultando em resistência à insulina tanto local como sistêmica. Camundongos transgênicos que possuíam uma forma constitutivamente ativa da proteína IKKβ no fígado apresentaram

aspectos de resistência à insulina, como níveis séricos de insulina aumentados, dando indícios de haver uma resposta compensatória para manutenção de glicemia normal, e intolerância à glicose, os quais foram atenuados após tratamento com salicilatos [41]. Contudo, ao avaliar camundongos com deleção específica do IKKβ em hepatócitos, observou-se aumento da sensibilidade à insulina no fígado, embora este efeito não tenha sido propagado para o músculo e tecido adiposo [42].

Além de todos os elementos produzidos pelas células na promoção da inflamação mediante altos níveis circulantes de ácidos graxos e citocinas pró-inflamatórias, característicos da obesidade, há outros participantes neste ciclo inflamatório e de indução de resistência à insulina. Foi demonstrado que no tecido adiposo em expansão, a infiltração de células pertencentes ao sistema imune inato, denominadas de macrófagos, é um importante fenômeno fisiológico, indicando um papel fundamental destas células no metabolismo e estabelecendo um processo inflamatório orquestrado por macrófagos que coexistem com a resistência à insulina induzida pela obesidade [43, 44].

Os macrófagos, que são numericamente e funcionalmente dominantes dentre as células do sistema imune que infiltram o tecido adiposo obeso [45, 46] são identificados pela marcação da proteína de superfície celular F4/80, que demonstrou que em camundongos magros, há cerca de 15% de macrófagos, e em animais obesos, essa prevalência sobe para cerca de 60% das células, alterando a razão entre o número de macrófagos e adipócitos [43]. Esta abundância de macrófagos e aumento de ácidos graxos no tecido adiposo obeso acaba acarretando o aumento da secreção de citocinas pró-inflamatórias e da atividade do receptor TLR4 que induzem resistência à insulina através da fosforilação do IRS-1 em Ser307 mediada pela ativação de JNK e IKKβ [29, 30, 47-49]

(Figura 4). De maneira interessante, estratégias para redução no número de macrófagos infiltrados no tecido adiposo foram associadas com redução na inflamação e aumento da sensibilidade à insulina [49-52].

Uma abordagem temporal da obesidade no tecido adiposo revelou que o recrutamento dos macrófagos coincide com o aparecimento de adipócitos necróticos e ao início de resistência à insulina local, demonstrando que os adipócitos em hipertrofia, devido ao estímulo de crescimento promovido pela obesidade, estão em constante situação de estresse celular que podem levar a uma série de eventos que incluem hipóxia, morte de adipócitos, intensa secreção de quimiocinas, alterações nos fluxos de ácidos graxos e aumentada produção de espécies reativas de oxigênio [53, 54]. Este ambiente leva à formação das *Crown-Like Structures* (CLS), que são macrófagos inflamatórios, que expressam marcadores de células dendríticas CD11c concomitantemente, que circundam os adipócitos em processo de morte com o intuito de fagocitar os componentes celulares e os lipídeos liberados [55, 56]. A importância destes macrófagos na indução de inflamação, resistência à insulina e formação das CLS se evidencia quando após a remoção destas células, a inflamação é reduzida com consequente aumento da sensibilidade à insulina [57].

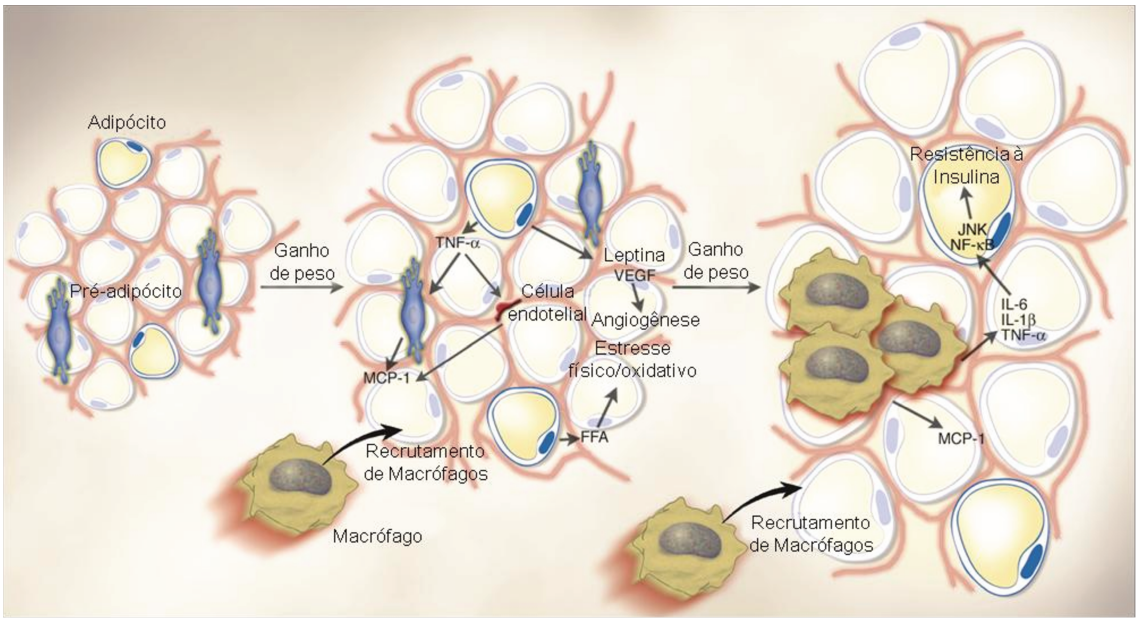

Figura 4: Esquema do processo de infiltração de macrófagos no tecido adiposo induzido por obesidade. (Adaptado de Wellen, K.; Hotamisligil, G., J Clin Invest, v. 112, p. 1786, 2003).

No fígado, há uma população de macrófagos residentes, denominada células de Küpffer, que apresentam enorme plasticidade em seus programas de ativação, variando de um estado clássico inflamatório (M1) a um estado alternativo anti-inflamatório (M2) [58, 59]. Na obesidade e esteato-hepatite não alcoólica, doenças caracterizadas pela inflamação crônica, as células de Küpffer são polarizadas para uma ativação clássica e se tornam as principais produtoras de citocinas pró-inflamatórias no fígado [60]. Já a deleção de IKKβ específica em células mielóides, entre elas os macrófagos, apresentou significativa redução da inflamação e consequente aumento da sensibilidade à insulina em camundongos submetidos à dieta rica em gordura por 20 semanas, conferindo grande importância à ação da inflamação mediada por estas células [42]. Conseguinte, estudos recentes demonstraram que após depleção seletiva das células de Küpffer em camundongos obesos, os níveis de esteatose e inflamação hepática foram reduzidos, culminando em aumento da tolerância à glicose e sensibilidade à insulina [61].

Avançando sobre novos mecanismos na indução de resistência à insulina, estudos recentes demonstraram uma importante atividade da flora intestinal na fisiopatologia da obesidade e suas morbidades relacionadas. Em camundongos geneticamente obesos ou em humanos com IMC maior do que 30 Kg/m^2 observou-se uma diferença na proporcionalidade entre as duas divisões bacterianas dominantes [62].

Além do TNF-α e da interleucina-6 (IL-6), o LPS é um importante ativador da JNK e do IKKβ. Lipopolissacarídeos são glicolipídeos encontrados em abundância na membrana externa de bactérias Gram negativas e possuem a capacidade de incitarem uma resposta inflamatória vigorosa [60]. Alguns estudos sugerem que a endotoxina e citocinas pró-inflamatórias induzidas por esta, podem estar entre os cofatores necessários para provocar NAFLD (*Non alcoholic fatty liver disease)* [63]. Wigg e colaboradores [64] relataram uma maior prevalência de crescimento bacteriano no intestino delgado e níveis circulantes aumentados de TNF-α em pacientes com NAFLD.

A relação entre flora intestinal e a obesidade começou a ser detalhada a partir de experimentos que realizaram a transferência da microbiota intestinal de um camundongo normal para um animal criado em ambiente livre de microrganismos (*germ-free*) promovendo um aumento de 60% no conteúdo de gordura corpórea, além de resistência à insulina, mesmo com reduzida ingestão alimentar e maior gasto energético. O aumento do tecido adiposo ocorre pela promoção, por parte da microbiota, de armazenamento de triglicerídeos em adipócitos através da supressão de um inibidor de lipase lipoprotéica (LPL) produzida no intestino, denominada *fasting-induced adipocyte factor* (Fiaf), sugerindo que este seja um mediador da regulação do armazenamento energético com as bactérias que povoam o intestino, indicando que a microbiota dos indivíduos obesos

conseguem extrair mais energia dos alimentos em relação às bactérias mais prevalentes dos indivíduos magros [62, 65]. Outro mecanismo pelo qual a flora intestinal pode influenciar no metabolismo animal, é através da proteína *AMP-activated protein kinase* (AMPK), já que a atividade desta é bastante reduzida quando os camundongos *germ-free* sofrem o contato com a microbiota de um animal convencional [66].

As principais bactérias existentes na microbiota intestinal de mamíferos fazem parte do grupo de *Firmicutes* e *Bacteroidetes*, onde vivem sob uma relação de simbiose, em que conseguem nutrientes e concomitantemente, degradam alimentos não digeríveis pelos mamíferos [67].

Em estudos recentes, foi demonstrado que a abundância relativa desses tipos de bactérias é diferente entre camundongos magros e geneticamente obesos (*ob/ob*), com uma prevalência 50% menor de *Bacteroidetes* e aumento proporcional de *Firmicutes* [62, 68]. Uma das hipóteses para essa diferença, é que a microbiota destes camundongos *ob/ob* é mais eficiente na liberação de calorias do alimento durante a digestão, comparada com a de animais magros [62]. A relação de prevalência dos filos bacterianos e a obesidade ainda não é completamente definida, visto que há relatos que as alterações metagenômicas da flora intestinal são diferentes das previamente demonstradas, com aumento da prevalência de *Bacteroidetes* e redução de *Firmicutes* [69-71].

Altos níveis circulantes de citocinas inflamatórias, usualmente observados em obesidade, podem causar dano na função da barreira intestinal, alterando sua estrutura [72]. Esse dano na função da mucosa foi observado em dois modelos de obesidade, camundongos *ob/ob* e *db/db*, propiciando um extravasamento de endotoxina no sistema

porta, expondo o fígado ao LPS, promovendo o estabelecimento do fenótipo ativado de células de Küpffer e células estreladas hepáticas, que contribuem de forma relevante com a inflamação hepática [73], acarretando em resistência à insulina e contribuição para as morbidades relacionadas à obesidade, como o diabetes tipo 2 e a doença hepática gordurosa não alcoólica.

Recentemente, estudos demonstraram que os inflamassomas, complexos protéicos que atuam como sensores de padrões moleculares associados a patógenos (PAMPs) tanto endógenos como exógenos ou padrões moleculares associados a lesão (DAMPs), são outra classe de proteínas que estariam envolvidas na relação entre a flora intestinal e o metabolismo de carboidratos e lipídeos, regulando atividade de citocinas pró-inflamatórias. Experimentos demonstraram que camundongos deficientes de inflamassoma foram suscetíveis a eventos inflamatórios, consequente resistência à insulina e maior susceptibilidade a desenvolverem esteato-hepatite não alcoólica (NASH), com aumento na prevalência de bactérias que possuem associação com componentes da síndrome metabólica tanto em humanos como em roedores [69, 74, 75].

Além disso, as bactérias presentes no intestino são responsáveis pela digestão de certos carboidratos não digeridos pelas células de mamíferos, e realizam fermentação destes, culminando na produção de ácidos graxos de cadeia curta. A energia proveniente destes ácidos orgânicos suprem energeticamente alguns tipos celulares, são utilizados pelo fígado como precursor de reações metabólicas e entram na circulação para serem metabolizados por tecidos periféricos [76-79]. Estudos demonstraram que o acetato, um dos principais ácidos graxos de cadeia curta, aumentou a ativação da AMPK em ratos diabéticos, reduzindo a hiperglicemia e a intolerância à glicose [80]. Dessa maneira, os

produtos derivados da microbiota, como os ácidos graxos de cadeia curta e as moléculas que induzem inflamação e regulação metabólica são responsáveis pela interação da flora intestinal e do metabolismo.

É perceptível que os novos mecanismos de indução de resistência à insulina são resultados da interação entre o sistema imune inato e o metabolismo, a qual vem sendo cada vez mais debatida, pois evidências demonstram que células da imunidade inata, como os macrófagos, são solicitadas nos tecidos metabólicos em doenças caracterizadas por inflamação crônica de baixa intensidade [13]. Estas células respondem a estímulos de certas citocinas e ácidos graxos livres, produzindo assim, mais citocinas pró-inflamatórias, que irão agravar a resistência insulínica local [81]. As células de Küpffer são as principais produtoras de TNF-α no fígado [60], enquanto os macrófagos infiltrados no tecido adiposo promovem a maior produção desta mesma citocina [43]. Dessa forma, se torna importante avaliar os possíveis tecidos com macrófagos infiltrados, assim como a produção de citocinas pró-inflamatórias por estes. Além disso, estudos prévios [82] também citam a possibilidade de participação ativa de macrófagos que estão presentes no baço, um órgão do sistema imunológico, em interferir no metabolismo.

Recentemente, Swirski et al. [83] mostraram que o baço, órgão linfóide secundário, é um importante reservatório de monócitos não diferenciados e distintos dos macrófagos e células dendríticas presentes. Neste estudo, foi demonstrado que, ao se realizar um infarto agudo do miocárdio, potente estímulo inflamatório, os monócitos são recrutados do baço para a circulação sanguínea, e não da medula óssea, e chegam ao foco inflamatório para induzir o reparo tecidual coordenado, primeiramente com a presença de monócitos

inflamatórios (Ly-6C^{hi}), digerindo o tecido lesionado e em seguida, os monócitos de patrulhamento (Ly-6C^{low}), que participam do reparo da lesão [83].

Quando os camundongos eram esplenectomizados, o número de monócitos na circulação não aumentava após o infarto do miocárdio e a quantidade situada no coração, onde a inflamação foi induzida, estava bastante reduzida em relação à migração de monócitos provocada na presença do baço. Também foi observado que mesmo em camundongos CCR2$^{-/-}$, que em teoria induziria mobilização de monócitos apenas da medula óssea, houve aumento de monócitos circulantes e o número de células no baço foi comparativamente reduzido, embora não tenha havido acúmulo no miocárdio isquêmico, já que o CCR2 é necessário para a infiltração [83].

Nesse mesmo estudo [83], também foi demonstrado que o mecanismo que induz o recrutamento dos monócitos do baço para o sítio inflamatório é mediado pela angiotensina II, que exerce seus efeitos através dos receptores AT1 e AT2, presentes nos monócitos e macrófagos. Foi observado que no camundongo *Atgr1a*$^{-/-}$, deficiente para o receptor de angiotensina II, poucos monócitos migraram para o miocárdio após o infarto quando comparado com o animal controle que sofreu infarto. A administração exógena de angiotensina II, em camundongos controle, com o intuito de mimetizar a concentração circulante deste peptídeo durante o infarto do miocárdio, induziu migração de monócitos do baço, além da ativação dos receptores de angiotensina II [83].

Além de funcionar como um reservatório celular para possíveis eventos inflamatórios, o baço também pode contribuir para a expressão e secreção de proteínas que podem auxiliar na coordenação do estabelecimento de resistência à insulina. Um possível

candidato a promover estes efeitos é a *Glia Maturation Factor gamma* (GMF-γ). Esta proteína ainda não explorada no contexto de obesidade possui um alto nível expressão no baço, de acordo com o perfil tecidual de expressão gênica, e dados da literatura indicam que esta é uma proteína especificamente hematopoiética que medeia o comprometimento de linhagem de células-tronco hematopoiéticas humanas [84]. Outra função descrita é o envolvimento de GMF-γ na quimiotaxia de neutrófilos, sendo um importante regulador de migração e polaridade celular, já que neutrófilos que tiveram esta proteína inibida demonstraram possuir capacidade reduzida de polarização celular, assim como perda de direcionamento da migração em resposta a quimioatraentes [85].

OBJETIVOS

Objetivo geral

Avaliar a influência da flora intestinal e da esplenectomia sobre a resistência à insulina induzida por obesidade em roedores

Objetivos específicos

- Avaliar a modulação da flora intestinal com a utilização de um coquetel de antibióticos em camundongos submetidos à dieta hiperlipídica.
- Analisar a via de sinalização da insulina e inflamatória em fígado, músculo e tecido adiposo em camundongos em dieta rica em gordura e tratados com o coquetel de antibióticos.
- Investigar as alterações histológicas em fígado e tecido adiposo de camundongos tratados com antibióticos em dieta hiperlipídica e imuno-histoquímica para marcação de macrófagos ativados (proteína F4/80).
- Investigar a influência da esplenectomia na sensibilidade à insulina em camundongos submetidos à dieta hiperlipídica.
- Avaliar a via de sinalização da insulina e inflamatória em fígado, músculo e tecido adiposo de camundongos esplenectomizados e controle com obesidade induzida por dieta.
- Investigar as alterações histológicas em fígado e tecido adiposo de camundongos esplenectomizados submetidos à ração para roedores padrão e dieta hiperlipídica e imuno-histoquímica para marcação de macrófagos ativados (proteína F4/80).

CAPÍTULO 1

Diabetologia Springer

Gut microbiota modulation improves insulin signalling in high-fat fed mice

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Gut microbiota modulation improves insulin signalling in high-fat fed mice

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Aim/hypothesis A high-fat diet intake induces obesity and subclinical inflammation, which play important roles in insulin resistance. Recent studies have suggested that increased concentrations of circulating lipopolysaccharide (LPS), promoted by changes in intestinal permeability may have a pivotal role in the induction of insulin resistance. Thus, we investigated the effect of gut microbiota modulation on insulin resistance and macrophage infiltration of metabolically active tissues.

Methods Swiss mice were submitted to a high-fat diet with antibiotics or pair-feeding for eight weeks. Metagenome analyses were performed from mice faecal DNA samples. Blood was collected for determinations of glucose, insulin, LPS, cytokines, and acetate levels. Liver, muscle, and adipose tissue proteins were analysed by Western blotting. Additionally, liver and adipose tissue were analysed by histology and immunohistochemistry.

Results Antibiotic treatment greatly modified the gut microbiota, reducing levels of *Bacteroidetes* and *Firmicutes*, overall bacterial count and circulating LPS levels. This modulation reduced fasting glucose, insulin, TNF-α, and IL-6 levels; reduced TLR4, JNK, and IKKβ activation, and p-IRS-1 Ser307; and consequently improved insulin action in metabolically active tissues. Additionally, there was an increase in circulating acetate levels, thus inducing AMPK and ACC phosphorylation. We observed a striking reduction of Crown-like structures and $F4/80^{+}$ macrophage cells in the adipose tissue of antibiotic-treated mice.

Conclusions/interpretation These results suggest that modulation of gut microbiota in obesity can improve insulin signalling and glucose tolerance, by reducing circulating LPS

levels and inflammatory signalling; also, by increasing circulating acetate levels that activate AMPK, and finally lead to reduced macrophage infiltration.

A combination of environmental factors, including a high-fat, high-carbohydrate diet and a sedentary lifestyle, have led to dramatic increases in the incidence of obesity and its associated disorders worldwide [1]. The chronic low-grade inflammatory status present in obesity and type 2 diabetes is characterized by increased levels of proinflammatory cytokines, acute-phase reactants and other stress-induced molecules, and activation of a network of inflammatory signalling pathways [2].

Recent evidence has suggested that gut microbiota are important for the control of body weight and energy homeostasis. Several mechanisms have been proposed for this interaction, but they are not well defined. Increased energy harvesting from gut flora was suggested on the basis of studies where gut microbiota from lean mice were transplanted to germ-free animals, which then exhibited rapid weight gain, dramatic increases in total and epididymal fat content, and reduced lean body mass, even with reduced food intake. These animals went on to develop insulin resistance [3]. Focusing on gut microbiota profiles, Turnbaugh and colleagues [4] showed different bacteria were prevalent in lean versus obese mice, as well as humans, by metagenomic analysis, relying on increased energy harvesting from obese gut microbiota. Another proposed mechanism is suppression of intestinal fasting-induced adipose factor (Fiaf), which is an adipose tissue lipoprotein lipase (LPL) inhibitor that limits adipocyte uptake of fatty acids and triglyceride accumulation, and modulates peroxisomal proliferator-activated receptor coactivator 1α (PGC-1α); which in turn encodes key enzymes involved in mitochondrial fatty acid oxidation. AMPK is persistently activated in germ-free mice, and is reduced when microbiota are transplanted to these animals, consequently reducing mitochondrial fatty acid oxidation [3]. Most of these mechanisms may make contributions to the reasons by which gut microbiota can modulate

fat storage and energy homeostasis [4-7]. However, the connections between gut microbiota and insulin sensitivity and signalling are incompletely understood.

In theory, gut microbiota might regulate insulin sensitivity by multiple mechanisms, but clearly, lipopolysaccharides (LPS) and short chain fatty acids (SCFA) from gut microbiota, signalling through TLR4 and AMPK, respectively, in host tissues, are potentially important candidates for mediating this modulation. However, the correlation between changes in gut microbiota and the influence of these signalling pathways in insulin sensitivity has not yet been investigated.

It has been established that a high-fat diet (HFD) can modulate gut microbiota in mice, altering the percentages of particular bacteria species. In addition, antibiotics can downregulate gut microbiota dramatically, reducing number of colonies, and also modulate some bacterial species. Taking advantage of these modulations, the aim of the present study was to use these two approaches to modulating gut microbiota, HFD and/or antibiotics, to investigate correlations between changes in microbiota, and insulin sensitivity and signalling; and at the same time, the possible mechanism that accounts for these effects.

Methods

Materials Anti-phosphotyrosine, anti-IRβ, anti-pIR, anti-IRS-1, anti-pIRS-1 Ser307, anti-IKKβ, anti-JNK, anti-pJNK, anti-F4/80 and anti-MyD88 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Akt, anti-pAkt, anti-IκBα, anti-pAMPK, anti-AMPK and anti-TLR4 were purchased from Cell Signaling Technology (Bervely, MA, USA). Human recombinant insulin (Humulin R) was purchased from Eli Lilly (Indianapolis, IN, USA). Routine reagents were purchased from Sigma-Aldrich (Saint Louis, MO, USA), unless specified elsewhere.

Animals Six-week old male Swiss mice were obtained from the UNICAMP Central Animal Breeding Centre (Campinas, São Paulo, Brazil). Animals were housed in individual cages with free access to water and rodent chow under a 12h light/dark cycle. High-fat diet consisted of 55% of calories derived from fat, 29% from carbohydrates and 16% from protein. Animals were fasted for 8 hours before the experiments. All procedures were approved by the Ethics Committee from the State University of Campinas.

Antibiotic treatment Mice under high-fat diet were submitted to antibiotic treatment (Ampicillin, Neomycin and Metronidazole) at concentration of 1g/L each in place of drinking water for 8 weeks.

Pair-fed mice After observation that antibiotic therapy reduced mice food intake, animals under high-fat diet were submitted to pair-feeding to prevent weight differences between mice groups.

Metagenome profile Faecal samples were collected from metabolic cages with separated waste collectors, frozen in liquid nitrogen, and stored at -80°C until use. At this time, DNA

was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), and quantified. Libraries were synthesized from 500ng of total DNA following instructions with the Rapid Library Preparation Kit (Roche Applied Science, Mannheim, Germany). These libraries were analysed in a Bioanalyzer and High Sensitive DNA Kit (Agilent Technologies Inc., Santa Clara, CA, USA), and equimolar pools were made, titrated and submitted to large volume PCR following manufacturers' instructions (Roche Applied Science, Mannheim, Germany). Then, samples were sequenced in GS FLX Titanium, using the GS FLX Titanium PicoTiterPlate Kit combined with the GS FLX Titanium Sequencing Kit XLR70 (Roche Applied Science, Mannheim, Germany). The readouts obtained from the sequencing were analysed by bioinformatics using BLASTX, observed with METAREP software, and compared according to phylum prevalence among groups.

Culture-based microbial analysis of cecal contents Total aerobic and anaerobic bacteria were enumerated in selective media and incubation conditions according to Schumann *et al.* [8]. Briefly, cecal samples were diluted in Ringer medium, and total aerobic and anaerobic bacteria were investigated by plating onto nonselective media: TSS medium (Biomerieux, Lyon, France) for 24h to 48h at 37ºC in aerobic and anaerobic conditions. Bacterial numbers were expressed as colony forming units (CFU)/mg cecal content [9].

Short chain fatty acid determination Blood was withdrawn from the portal vein and centrifuged (2.800 RPM for 15 minutes). Serum obtained was deproteinized by the addition of methanol (1:5 dilution), and centrifuged at 50,000*g* for 10 minutes at 4 °C. Supernatant was separated chromatographically on an Acquity UPLC (Waters, Milford, MA, USA) using an Acquity UPLC BEH Amide Column (2.1x100 mm. 1.7μm). After chromatography, samples were injected in a Quattro Micro API mass spectrometer (Waters,

Milford, MA, USA) for measurement of acetate concentration. Acetate standards were submitted to the same procedures for calibration curve determination.

Insulin tolerance test Mice were fasted for 8 hours and submitted to a 30 minute insulin tolerance test. Briefly, 1.5 U/Kg insulin was injected intraperitoneally and glucose was measured every 5 minutes. The glucose disappearance rate (K_{itt}) was calculated from the formula $0{,}693/t_{1/2}$, where $t_{1/2}$ stands for time for glucose to reach 50% of the basal value. Glucose $t_{1/2}$ was calculated from the slope of the least square analysis of blood glucose concentration during the linear phase of decline.

Intraperiotoneal glucose tolerance test After 8 hours fasting, glucose tolerance test was performed. Glucose (1g/kg) was injected intraperitoneally and blood collected from the tail vein at different time points till 120 minutes for determination of blood glucose levels, using a glucometer (Optium Xceed, Abbott, Libertyville, IL, USA).

Assays Serum TNF-α and IL-6 were measured by commercially available ELISA kits (Thermo Fischer Scientific Inc., Rockford, IL, USA). Serum insulin was determined by ELISA (Millipore, St. Charles, MO, USA).

LPS determination Sterile serum samples, obtained from portal vein blood, were diluted to 20% with endotoxin-free water and then heated to 70°C for 10 minutes to inactivate serum proteins. Then, LPS was quantified using a commercially available Limulus Amebocyte Assay (Cambrex, Walkersville, MD, USA) according to the manufacturer´s protocol.

Tissue extraction, immunoprecipitation and protein analysis by immunoblotting Eight hours fasted mice were anesthetized and right after insurance of loss of pedal and corneal reflexes, the abdominal cavity was opened, the vena cava exposed and 0,1 mL of saline was injected with or without insulin (10^{-6}M). After insulin injection, liver, muscle and adipose

tissue were removed, minced coarsely and homogenized in specific extraction buffer. Tissue extracts were incubated overnight with MyD88 and IRS-1 antibody and immunoprecipitated by 6MB Protein A Sepharose (GE Healthcare, Uppsala, Sweden). The immunoprecipitates and whole-tissue extracts proteins were subjected by SDS-PAGE and immunoblotted with specific antibodies. Immunoreactive bands were detected by the enhanced chemiluminescense method (Supersignal West Pico Chemiluminescent Substrate, Thermo Fischer Scientific Inc., Rockford, IL, USA).

Liver and adipose tissue histology Liver and epididymal white adipose tissue depots were dissected and fixed by immersion in 4% parafolmaldehyde in 0.1M phosphate buffer, pH 7,4 for 24 hours, dehydrated, cleared and then embedded in paraffin. Sections (5 μm) were obtained and then stained by haematoxylin and eosin to assess morphology. Liver was analyzed for steatosis level estimating area covered by lipid droplets. Adipose tissue was evaluated by crown-like structure (CLS) density (average CLS within 10 high-power fields per animal) and mean adipocyte surface area (average surface area of 30 randomly sorted adipocytes per animal) were determined using Imagelab Analysis software, as described previously [10].

Liver and adipose tissue immunohistochemistry Five μm tissue sections were mounted on silanized glass slides, deparaffinized in xylene, rehydrated, antigen unmasked, quenched for endogenous peroxidase activity, blocked and incubated with F4/80 primary antibody, as described previously [11]. Antibody staining was performed using an IHC-peroxidase kit (ADVANCE^{TM}HRP, Dako CytoMation, Carpinteria, CA, USA) according to manufacturer´s instructions. Three different high-power fields from each three different

sections were evaluated. The total number of nuclei of F4/80 expressing cells were counted for each field and the area occupied by these cells estimated in each field.

Statistical analysis Results are expressed as mean ± SEM. The results of blots are presented as direct comparisons of bands in autoradiographs and quantified by optical densitometry (UN-SCAN-IT gel^{TM}, Silk Scientific Inc., Orem, UT, USA.). Statistical analysis were performed by two-way ANOVA with Bonferroni test for post hoc comparisons and Student´s t test where necessary. Level of significance adopted was, at least, $P < 0,05$.

Results

Effect of antibiotic treatment on gut microbiota Based on BLASTX analysis of metagenomic sequencing from faeces of all groups, we observed a profile change between rodents fed chow and animals fed a high-fat diet. *Bacteroidetes* prevalence increased in the gut of high-fat fed mice compared to control mice. In contrast, the presence of *Firmicutes* was significantly reduced in animals that received a high-fat diet. In addition, the latter group showed a marked increase in the prevalence of *Verrucomicrobia* bacteria, compared with control mice. Metagenomic studies revealed that after treatment with wide spectrum antibiotics, the gut microbiota profile was profoundly altered, showing almost no *Bacteroidetes* or *Verrucomicrobia*, and little prevalence of *Firmicutes*. These modifications were accompanied by a huge increase on the prevalence of *Proteobacteria* in the gut microbiota of mice under antibiotic treatment (Figure 1A-C). Besides this modulation of gut microbiota, bacterial quantification in faeces from antibiotic-treated mice showed striking overall reductions compared to untreated animals (Figure 1D).

Effect of antibiotic treatment on metabolic parameters of mice fed a high-fat Antibiotic-treated mice receiving a high-fat diet had reduced food intake compared with animals without antibiotics on drinking water, gaining significantly less weight during the eight weeks of the experiment (data not shown). In order to avoid this bias and not mask the treatment´s effect over insulin signalling, we submitted the non-treated animals to pair-feeding on a high-fat diet. The different groups of mice did not show significant weight differences among the groups (Figure 2A). Even under these experimental conditions, the pair-fed group presented with increased fasting blood glucose and serum insulin levels compared to control and antibiotic-treated animals (Figure 2B-C). In mice on HFD treated

with antibiotics, there was an improvement in insulin sensitivity, determined by the ITT, and in glucose tolerance (Figure 2D-E).

Effect of antibiotic treatment on LPS, proinflammatory cytokines and circulating acetate levels Mice fed a high-fat diet under pair-fed regimen showed increased levels of LPS in their portal circulation ($p < 0{,}00001$) compared to the control group, which was prevented by the antibiotic treatment (Figure 3A). The serum TNF-α and IL-6 levels showed increases in untreated mice given the high-fat diet compared with the control group, and antibiotic treatment blocked these increases in cytokine levels (Figure 3B-C). In addition, concentrations of serum acetate were significantly reduced in mice fed the high-fat diet compared to mice receiving standard chow; but after antibiotic treatment, levels remained close to those in control animals (Figure 3D).

Effect of antibiotic treatment on the TLR4 signalling pathway in HFD mice The increased circulating concentration of LPS absorbed from the gut upregulated the TLR4 signalling pathway in liver, muscle, and adipose tissue, in mice submitted to a pair-feeding regimen. This event resulted in increased JNK activation and IκBα degradation, consequently enhancing IRS-1 Ser307 phosphorylation, which in turn induces insulin resistance. Modulation of gut microbiota by antibiotic treatment reduced TLR4 activation by reducing levels of circulating LPS, consequently decreasing JNK activation, IκBα degradation and IRS-1 Ser307 phosphorylation (Figure 4A-L).

Effect of antibiotic treatment on the insulin signalling pathway and AMPK Pair-fed mice showed inflammation-induced impairment of the insulin signalling pathway after stimulation in liver, muscle, and adipose tissue, represented by reductions in insulin-induced IRβ, IRS-1 and Akt phosphorylation. After antibiotic treatment, insulin-induced phosphorylation of these proteins increased, following reductions in LPS concentration and in observed inflammation, promoting amelioration of insulin sensitivity (Figure 5A-I). In mice fed a HFD, there was a decrease in AMPK and ACC phosphorylation, which were improved after antibiotic treatment (Figure 6A-F).

Effect of antibiotic treatment on histology and macrophage infiltration in liver Microscopic assessment of liver samples revealed that the steatosis induced by the HFD in the antibiotic-treated group was slightly reduced in comparison to livers from its pair-fed control group, showing reduced triglyceride content in the hepatic parenchyma. The number of Küpffer cells increased 2-fold in pair-fed mice, as detected by $F4/80^{+}$ staining immunohistochemistry. This macrophage infiltration was inhibited by antibiotic treatment, remaining at a basal count of Küpffer cells (Figure 7A-C).

Effect of antibiotic treatment on histology and macrophage infiltration in adipose tissue Evaluation of adipose tissue cells revealed no differences in adipocyte size between pair-fed and antibiotic-treated groups, probably due to the absence of body weight differences (data not shown). On the other hand, a striking increase in crown-like structures (CLS), defined by an intense infiltration of innate immune cells around inflamed and compromised adipocytes, was found in adipose tissue of pair-fed mice when compared to the treatment

group. Striking increases in the number of infiltrated macrophages, determined by F4/80^{+} staining, were also observed in adipose tissue of pair-fed mice, but such cells were virtually eliminated from the vicinity of adipocytes by the antibiotic treatment (Figure 8A-D).

Discussion

Here we show that HFD changed gut microbiota in mice, inducing an increase in the percentage of *Bacteroidetes*, which was associated with an increase in circulating LPS levels, a reduction in circulating acetate, and a decrease in whole body insulin sensitivity. When these animals were treated with antibiotic as a strategy of intestinal flora modulation, they showed marked reductions in gut bacteria content and circulating LPS, and increases in SCFA levels, thus attenuating TLR4 activation; the latter resulting in improved insulin signalling and inflammatory profiles, and activation of AMPK in hepatic, muscular, and adipose tissue. In addition, striking reductions in macrophage infiltration into liver and fat pad were noted, culminating in prevention of insulin resistance.

Our metagenomic analysis of gut microbiota in HFD fed mice showed a slight increase in *Bacteroidetes* prevalence (78.5% versus 61.8%) and a significant decrease in *Firmicutes* (6.1% versus 31.6%), compared to the control group. This result was similar to previous studies [12-14] that analysed gut microbiota composition in obese and overweight subjects and mice with nonalcoholic steatohepatitis (NASH), but was not in agreement with other previously published data [4, 6]; this is evidence that the gut microbiota profile in obesity is still controversial, relying on methodological details to explain differences among published data. The *Verrucomicrobia* phylum, composed of two Gram negative sister phyla, *Chlamydiae* and *Lentisphaerae,* which has not been much explored in the literature, is present in low percentages in control mice, but showed considerable prevalence in animals submitted to a HFD (0.03% versus 13.65%). Mice submitted to antibiotic treatment showed a huge difference in gut microbiota profiles compared to control and pair-fed groups. Almost all *Bacteroidetes* and *Firmicutes* were annihilated from the intestinal

lumen, the same fate as *Verrucomicrobia* in antibiotic-treated mice, but these changes were accompanied by a striking increase in the *Proteobacteria* phylum, almost the sole phylum present (97%). Even with the high prevalence of Gram negative bacteria (*Proteobacteria*), the reduction in LPS circulating levels is related to the striking reduction in overall bacteria content in intestinal lumen, consequently reducing its uptake, in addition to the improving intestinal barrier function.

Our data, showing an increase in portal LPS circulating levels in mice fed a HFD, suggest that the source of this molecule is the gut microbiota. Available data indicates that translocation of TLR4 agonists, such as LPS, from the gut is an important feature in metabolic diseases such as obesity and NASH, promoting inflammation and insulin resistance in metabolically active tissues [14, 15].

A great deal of data has shown the importance of LPS in inflammation and induction of insulin resistance, using LPS injection and sepsis models [16-22]. The primary mechanism induced by LPS is initiated when it binds TLR4, its receptor, which in turn associates with MyD88 and triggers its signalling pathway; this in turn activates JNK and IKKβ through IRAK, leading to serine phosphorylation of IRS-1, NF-κB activation, and transcription of proinflammatory cytokines and insulin resistance [23, 24].

Cani and colleagues [25] showed that antibiotic treatment improved inflammation, oxidative stress, and macrophage infiltration markers based on adipose tissue mRNA of rodents fed either regular chow or a HFD, with or without antibiotics. They also showed marked augmentation of ZO-1 (Zonula Occludens-1) mRNA, important intestinal barrier protein, which correlated with diminished intestinal permeability, resulting in reduced

circulating LPS levels in antibiotic-treated mice. All those experiments were performed in HFD fed animals with differences in body weight, not excluding the body weight reduction after antibiotic treatment, an important "insulin-sensitizing" feature, from the study, as performed in the present study. Our experiments were conducted using pair-feeding in order to exclude body weight differences, a consequence of reduced food intake induced by the antibiotic treatment, and show, evidently, the insulin sensitivity increase after gut microbiota modulation of antibiotic-treated mice on HFD.

Our results, similar to previous studies performed on genetically obese *ob/ob* mice [9, 26], showed an improvement in whole metabolism of mice under a HFD treated with antibiotics, based on increases in insulin sensitivity and glucose tolerance, and reductions in fasting glucose and insulin. We also showed that the amelioration of metabolism is mainly related to a reduction in circulating LPS levels, which made TNF-α and IL-6 serum levels remain in the same levels of the animals fed a standard chow diet. All these effects induced by antibiotic treatment must be mediated by modulation of gut microbiota and improvement in intestinal barrier function.

We also report, for the first time, the influence of reduced LPS concentration after gut microbiota modulation by antibiotic treatment upon downregulation of the TLR4-MyD88 association in liver, muscle, and adipose tissue; leading to reduced activation of JNK, IKKβ, and serine phosphorylation of IRS-1. Inversely, but directly related, increased activation of the insulin signalling pathway, as determined by tyrosine phosphorylation of IRβ, IRS-1 and serine phosphorylation of Akt, was seen in all three tissues [27]. These observations show an increased control over inflammation and metabolism mediated by augmented insulin activity in target tissues.

Another important issue regarding the relationship between obesity and gut microbiota is short chain fatty acid (SCFA) production based upon bacterial fermentation of low- and non-digestable carbohydrates in the diet. Reduction of circulating acetate levels in HFD fed mice may be related to a reduced dietary carbohydrate:fat ratio, affecting SCFA production [28]. Modifications in the gut microbiota profile promoted by antibiotic therapy induced an increase in the circulating acetate levels. Acetate has the capacity to activate AMPK in liver and muscle [29], increasing fatty acid oxidation [30] and energy expenditure; it also induces GLUT4 translocation to the cell membrane and glucose uptake [31]. There is evidence that organic acids such as butyrate and acetate can contribute to intestinal immune and barrier functions by increasing numbers of immune cells in the gut epithelial layers cells [32], and stimulating mucin production in the gut [33]. Thus, they could also contribute to the reduced intestinal permeability and lower circulating LPS levels seen in antibiotic-treated mice compared to pair-fed mice.

Macrophage activity is related to inflammation and induction of insulin resistance in metabolically active tissues in obesity and type 2 diabetes, enhancing TNF-α and IL-6 production and activity, mainly in liver and adipose tissue; these are sites preferentially infiltrated by innate immune cells in order to maintain homeostasis and prevent metabolic dysfunctions provoked by high levels of circulating free-fatty acids, glucose, and LPS observed in obese individuals [34, 35].

The reductions in macrophage infiltration in liver and adipose tissue, as determined by F4/80 staining, in antibiotic-treated mice are due to reductions in circulating levels of LPS; whereas this molecule can cause inflammation, inducing macrophages to secrete TNF-α and IL-6, promoting insulin resistance; it also induces expression and activity of

chemoattractant proteins like MCP-1, and other chemokines, whose role is to promote innate cellular immune responses and translocation against stimuli, like HFD induced adipose tissue hypertrophy and liver steatosis [36, 37]. It is important to halt the production of inflammatory molecules, as promoters of insulin resistance, so that macrophage infiltration is prevented, or already-infiltrating macrophages do not receive stimuli to remain infiltrated, as occurs with some drugs or with weight loss [38-41].

Taken together, our data show that a HFD promotes alterations in the gut microbiota profile, which favour an inflammatory and insulin resistant status, with intestinal barrier disruption and high circulating LPS levels, in addition to intense macrophage activity. All these obesity related features were reversed by changes in the gut microbiota profile induced by an antibiotic therapy in mice submitted to a HFD. These observations lead us to suggest that utilization of strategies that target modification of the gut microbiota profile could bring immense benefits in preventing and attenuating obesity related symptoms and metabolic derangement.

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Author contribution B.M.C. researched data, contributed to discussion, wrote, edited and reviewed the article; D.G. researched data; D.M.L.T researched data; A.A.S. researched data; P.L.F. researched data; J.C.D. researched data; L.T.K. researched data; J.V. researched data; J.B.C.C. contributed to discussion; M.J.A.S. contributed to discussion, edited and reviewed the article.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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Figure Legends

Figure 1: Metagenomic analysis of gut microbiota prevalences in control (A), pair-fed (B) and antibiotic-treated mice (C). Bacterial counts were taken from control, pair-fed and antibiotic-treated mice faeces (D). Phylum charts were extracted using METAREP software based on BLASTX analysis of DNA pyrosequencing from mouse stool. Bars represent mean ± SEM from six mice per group. * $P < 0,05$.

Figure 2: Effect of antibiotic treatment in mice under a HFD regimen. Body weight (A), fasting blood glucose (B), portal serum insulin (C), insulin sensitivity based on Kitt (D), and glucose tolerance testing (E) of control, pair-fed, and antibiotic-treated mice. Bars represent mean ± SEM from six mice. * $P < 0,05$.

Figure 3: Effect of antibiotics in portal vein serum levels of lipopolysaccharide - LPS (A), TNF-α (B), IL-6 (C), and acetate (D) from control, pair-fed, and antibiotic-treated mice. Bars represent mean ± SEM from six mice. *$P < 0,001$.

Figure 4: Effect of antibiotics on TLR4 activation and its downstream effectors JNK and IKKβ, leading to serine307 phosphorylation of IRS-1 in liver (A-D), muscle (E-H), and adipose tissue (I-L). Bars represent densitometry quantification of blots as mean ± SEM from three different experiments. *$P < 0,01$.

Figure 5: Effect of antibiotics on the insulin signalling pathway in liver (A-C), muscle (D-F), and adipose tissue (G-I) of mice under pair-feeding regimen and antibiotic treatment. Bars represent densitometry quantification of blots as mean ± SEM from three different experiments. *$P < 0,01$.

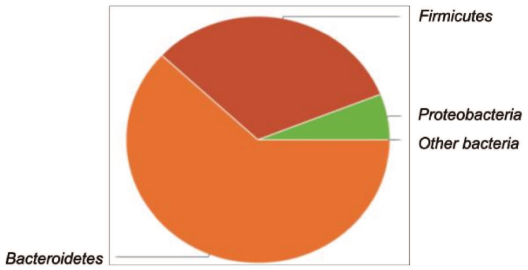
Figure 6: Effect of antibiotics on AMPK and ACC activation in liver (A-B), muscle (C-D), and adipose tissue (E-F). Bars represent densitometry quantification of blots as mean ± SEM from three different experiments. *P < 0,01.

Figure 7: Effect of antibiotics on liver steatosis (above) and macrophage infiltration (below) (A-B), and quantification of $F4/80^{+}$ cells (C) from pair-fed or antibiotic-treated mice. Bars represent mean ± SEM from three different experiments. HE staining (above) and F4/80 immunostaining (below). Magnification of 400x and scale bars are highlighted in the figure. *P < 0,01.

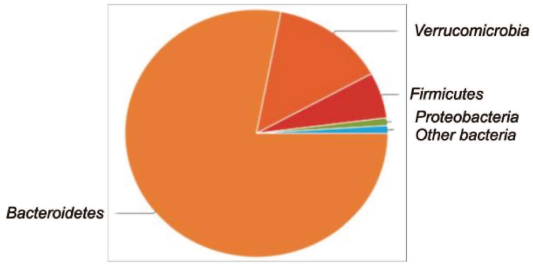
Figure 8: Effect of antibiotics on prevalence of Crown-like structures (CLS) and macrophage infiltration in epididymal adipose tissue (A-C) and quantification of the frequency of $F4/80^{+}$ cells in tissues (D) from pair-fed and antibiotic-treated mice. Bars represent mean ± SEM from three different experiments. HE staining (above) and F4/80 immunostaining (below). Magnification of 400x and scale bars are highlighted in the figure. *P < 0,01.

Figure 1

A.



B.



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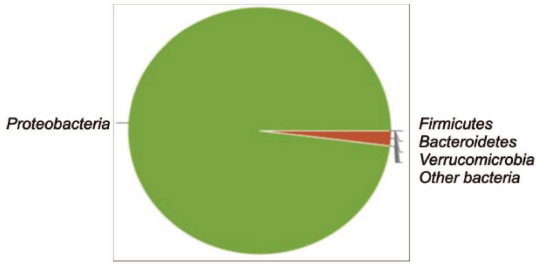


D.

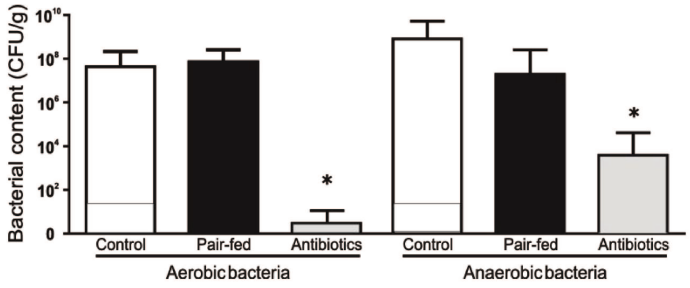


Figure 2

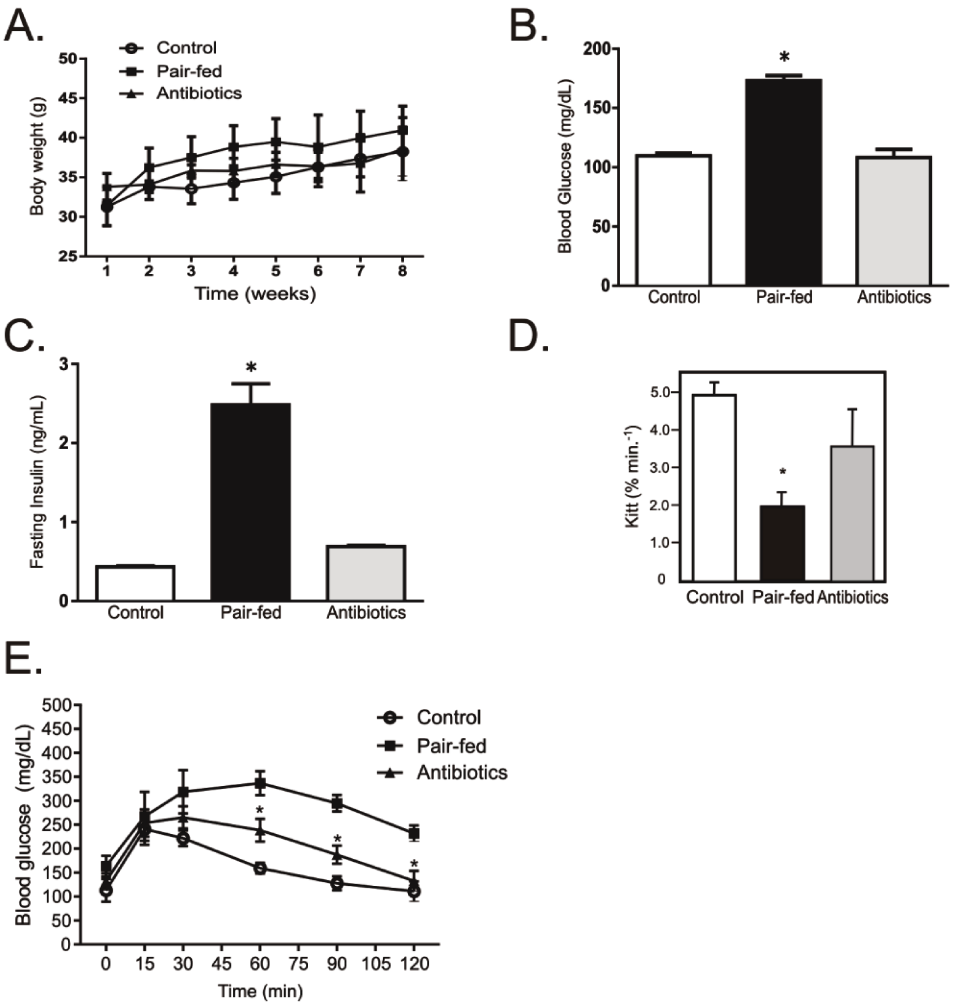

A.
Control
Pair-fed
Antibiotics
Body weight (g)
Time (weeks)
B.
Blood Glucose (mg/dL)
Control
Pair-fed
Antibiotics
C.
Fasting Insulin (ng/mL)
Control
Pair-fed
Antibiotics
D.
Kitt (% min.-1)
Control
Pair-fed
Antibiotics
E.
Control
Pair-fed
Antibiotics
Blood glucose (mg/dL)
Time (min)

Figure 3

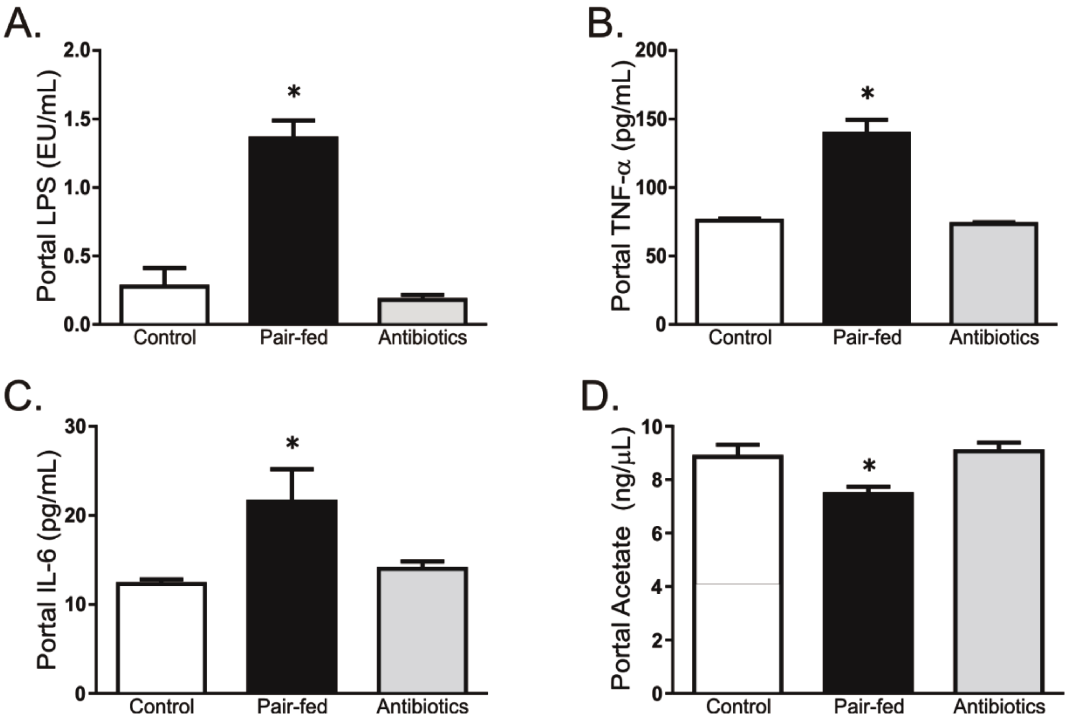

A.
Portal LPS (EU/mL)
2.0
1.5
1.0
0.5
0.0
*
Control
Pair-fed
Antibiotics
B.
Portal TNF-α (pg/mL)
200
150
100
50
0
*
Control
Pair-fed
Antibiotics
C.
Portal IL-6 (pg/mL)
30
20
10
0
*
Control
Pair-fed
Antibiotics
D.
Portal Acetate (ng/μL)
10
8
6
4
2
0
*
Control
Pair-fed
Antibiotics

Figure 4

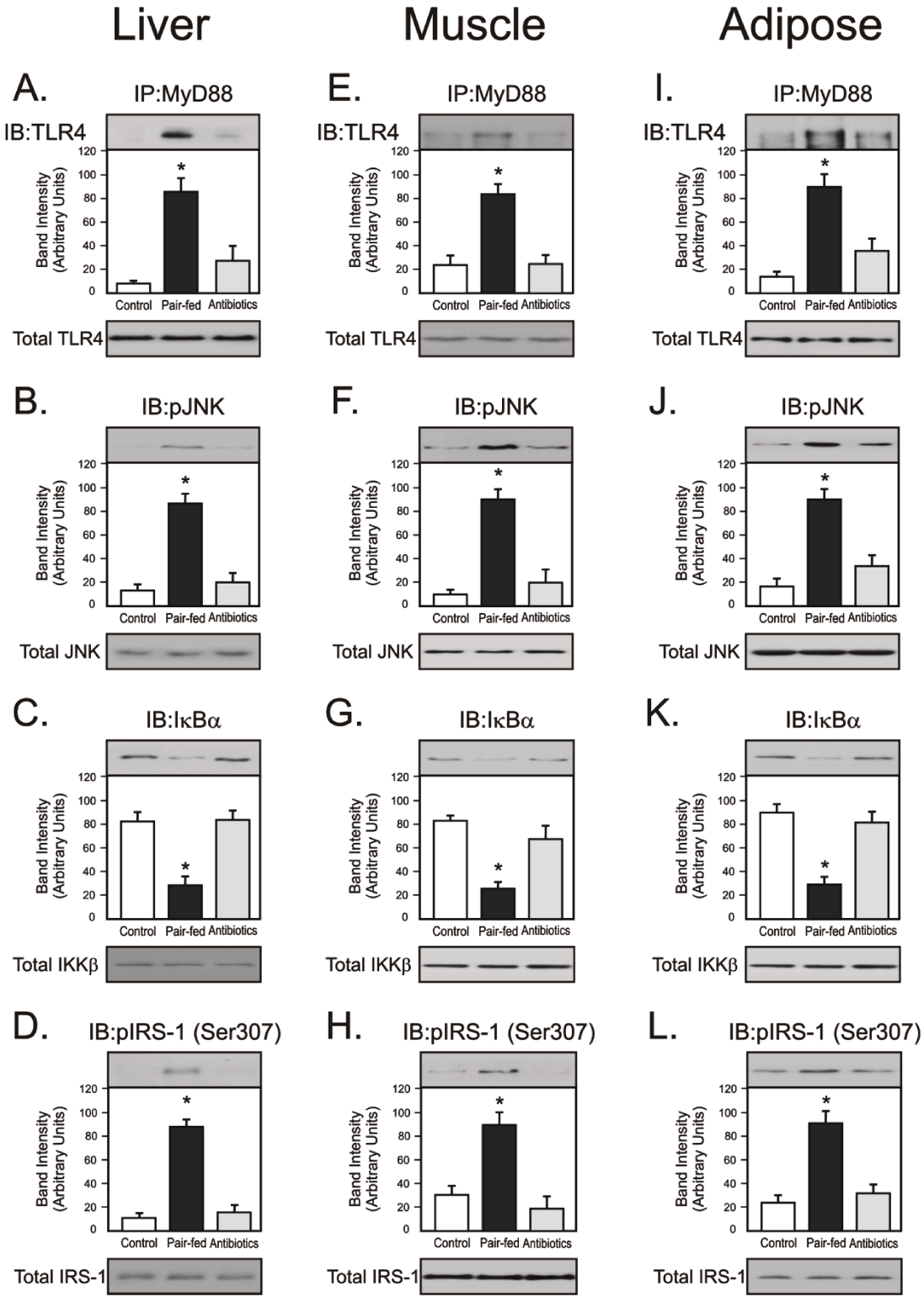

Liver
Muscle
Adipose
A.
IP:MyD88
IB:TLR4
Band Intensity (Arbitrary Units)
Control
Pair-fed
Antibiotics
Total TLR4
E.
IP:MyD88
IB:TLR4
Total TLR4
I.
IP:MyD88
IB:TLR4
Total TLR4
B.
IB:pJNK
Total JNK
F.
IB:pJNK
Total JNK
J.
IB:pJNK
Total JNK
C.
IB:IκBα
Total IKKβ
G.
IB:IκBα
Total IKKβ
K.
IB:IκBα
Total IKKβ
D.
IB:pIRS-1 (Ser307)
Total IRS-1
H.
IB:pIRS-1 (Ser307)
Total IRS-1
L.
IB:pIRS-1 (Ser307)
Total IRS-1

Figure 5

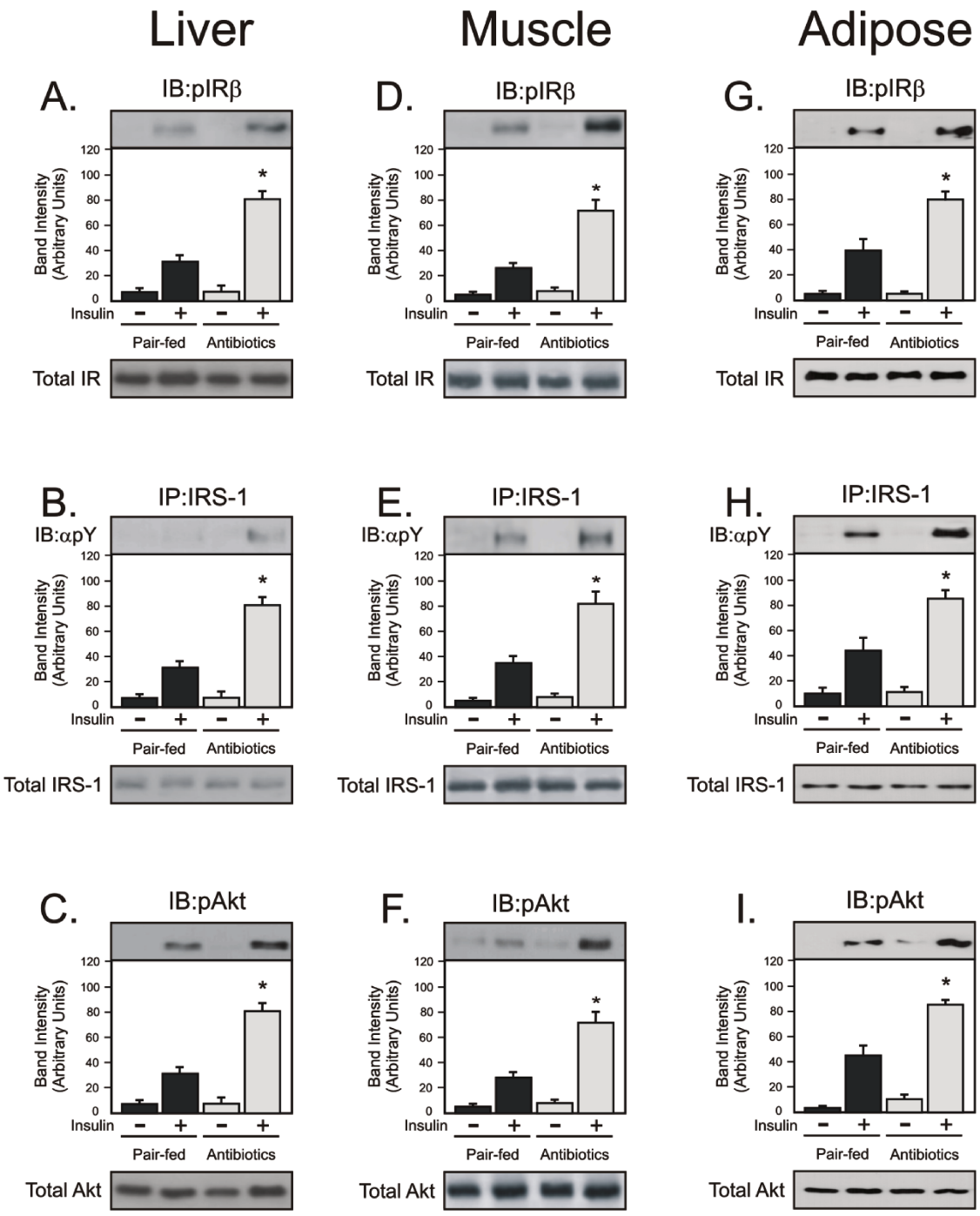

Liver
Muscle
Adipose
A.
IB:pIRβ
D.
IB:pIRβ
G.
IB:pIRβ
Band Intensity (Arbitrary Units)
Insulin
Pair-fed
Antibiotics
Total IR
B.
IP:IRS-1
IB:αpY
E.
IP:IRS-1
IB:αpY
H.
IP:IRS-1
IB:αpY
Total IRS-1
C.
IB:pAkt
F.
IB:pAkt
I.
IB:pAkt
Total Akt

Figure 6

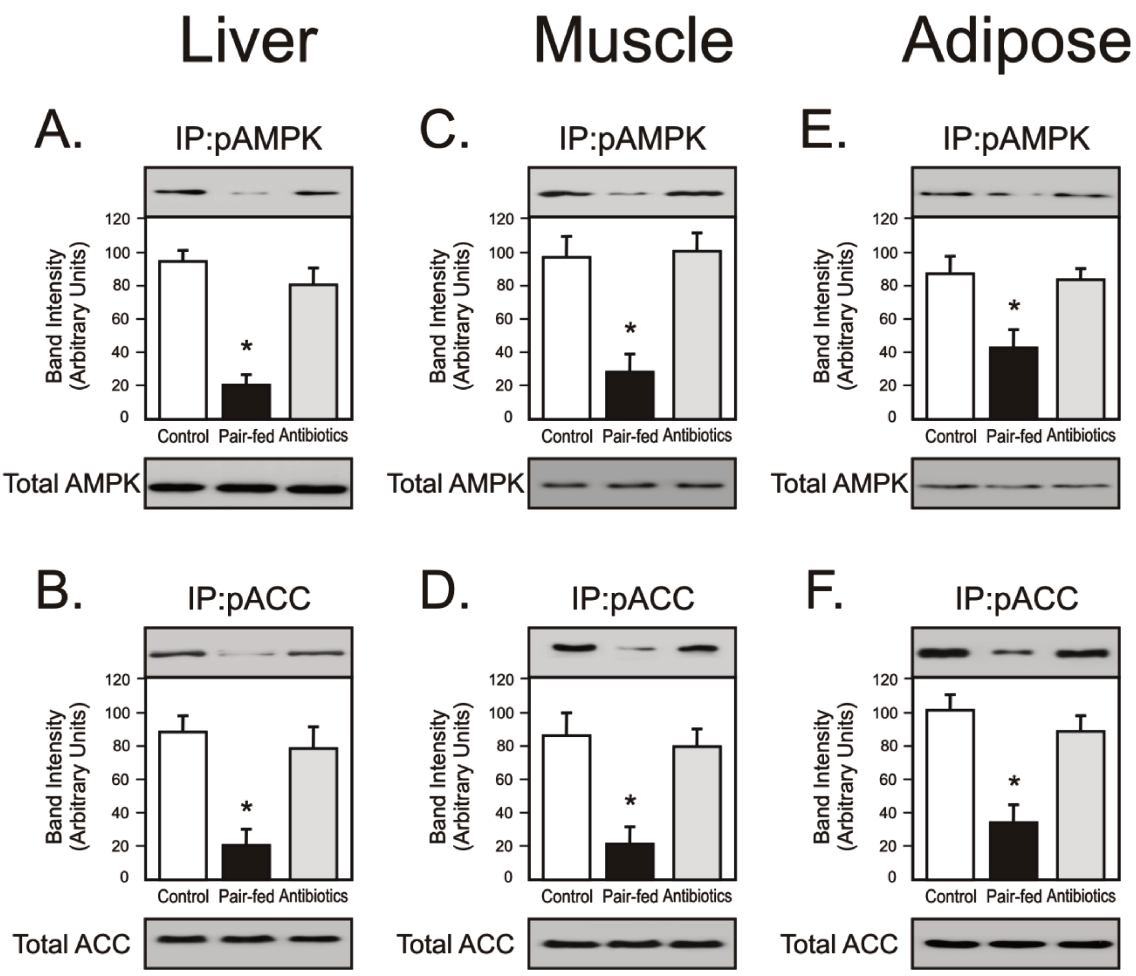
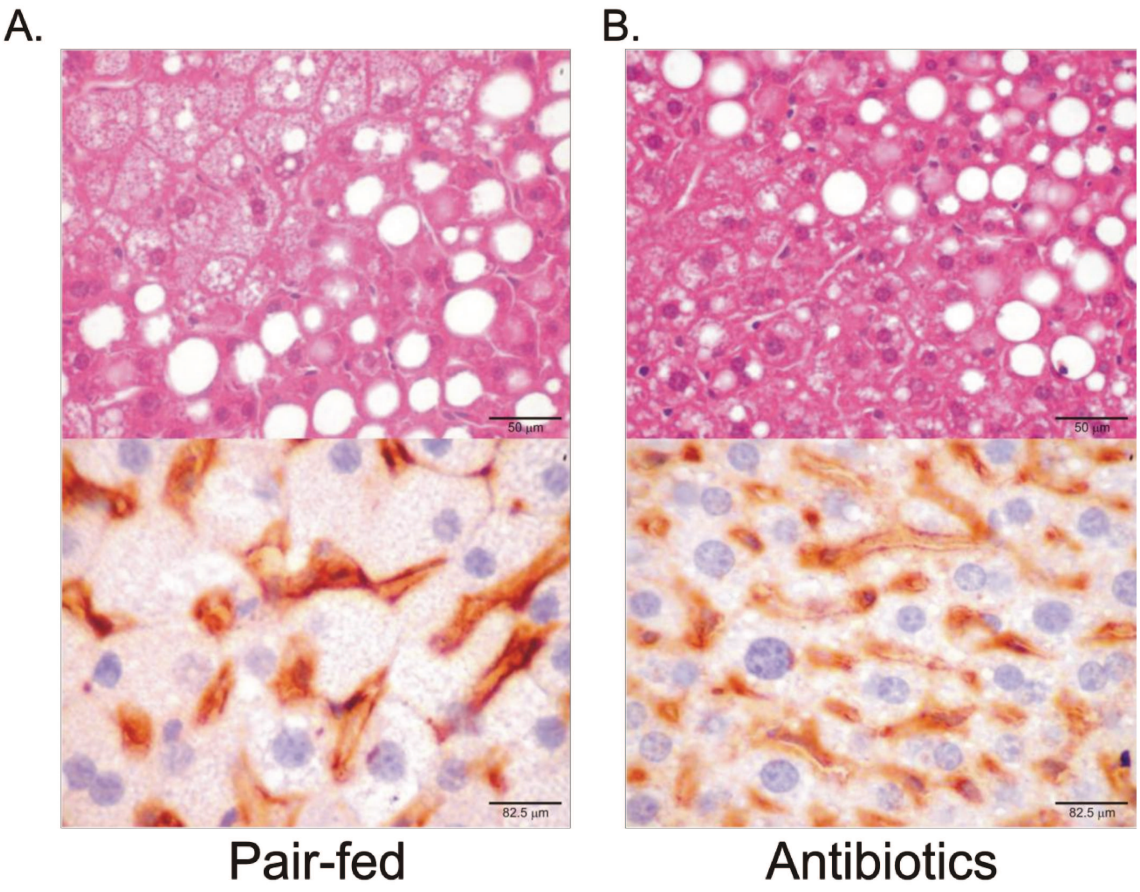

Liver
Muscle
Adipose
A.
IP:pAMPK
Band Intensity (Arbitrary Units)
Control
Pair-fed
Antibiotics
Total AMPK
C.
IP:pAMPK
E.
IP:pAMPK
B.
IP:pACC
Total ACC
D.
IP:pACC
F.
IP:pACC

Figure 7


A.
B.
50 µm
82.5 µm
50 µm
82.5 µm
Pair-fed
Antibiotics

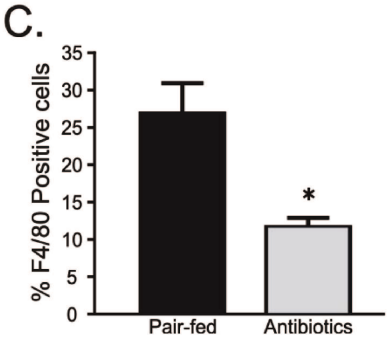
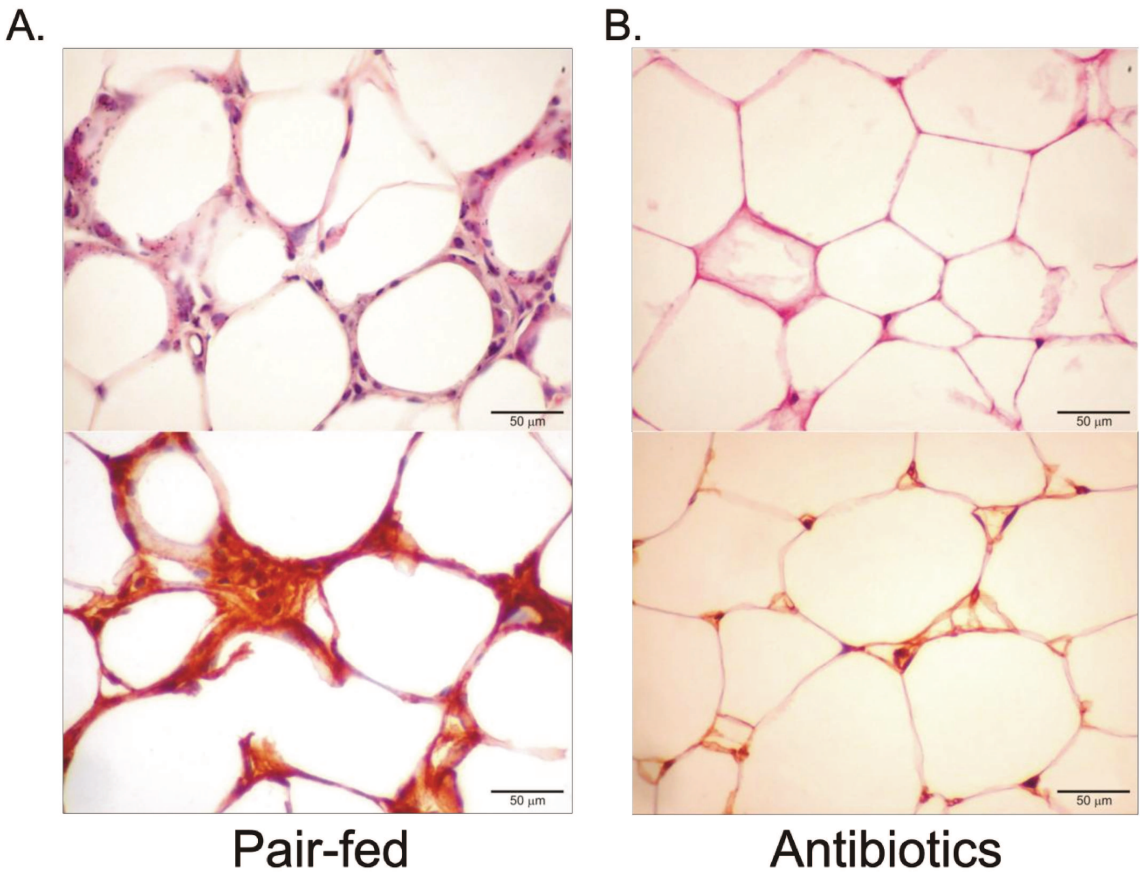
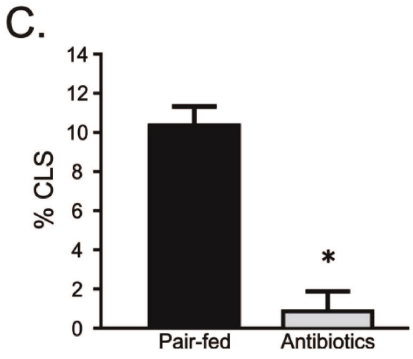
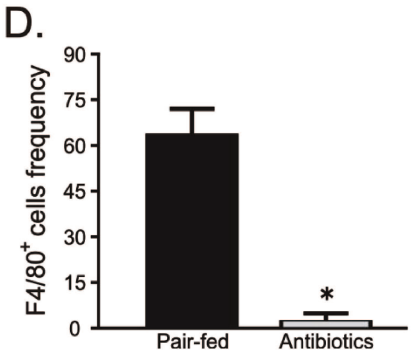

C.
% F4/80 Positive cells
35
30
25
20
15
10
5
0
*
Pair-fed
Antibiotics

Figure 8


A.
B.
50 µm
50 µm
50 µm
50 µm
Pair-fed
Antibiotics


C.
% CLS
14
12
10
8
6
4
2
0
*
Pair-fed
Antibiotics


D.
F4/80+ cells frequency
90
75
60
45
30
15
0
*
Pair-fed
Antibiotics

CAPÍTULO 2

Splenectomy improves insulin sensitivity in high-fat fed mice

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Abstract

Obesity is characterized by a chronic subclinical inflammatory state which induces insulin resistance in metabolic tissues, leading to defects in insulin signaling pathway, and consequently reduced insulin sensitivity and glucose homeostasis. Increased circulating metabolic components related to obesity, such as fatty acids, activates innate immune system elements, triggering inflammatory signaling pathways and cytokine expression, responsible for defenses against pathogens, showing an intimate relation of innate immune system activation and metabolic diseases. In order to investigate possible novel inflammation sources, we evaluated insulin sensitivity on several models of insulin resistance after splenectomy by hyperinsulinemic-euglycemic clamp. Then, liver, muscle and adipose tissue proteins from male *Swiss* mice submitted to surgery and fed a high-fat diet for 8 weeks were evaluated by immunoblotting for insulin and inflammatory signaling pathway. Additionally, liver and adipose tissue were evaluated by histology and F4/80 staining by immunohistochemistry, for macrophage infiltration observation. In splenectomized animals, the insulin sensitivity was significantly improved when compared to animals submitted to *sham* surgery on high-fat diet. In diet-induced obese *Swiss* mice, proinflammatory proteins were activated (JNK and IKKβ), leading to IRS-1 serine 307 phosphorylation and insulin resistance, which were reduced by spleen removal in all metabolic tissues evaluated. Following this improvement, insulin-induced signaling pathway (IR, IRS-1 and Akt) was significantly more activated when compared to *sham* animals under high-fat diet, restoring metabolic homeostasis. On liver evaluation by hematoxylin and eosin staining, reduced levels of steatosis and significant less infiltrated macrophages detected by F4/80 staining were observed. In adipose tissue, a striking

reduction of *crown-like structures* (CLS) and F4/80^{+} infiltrated cells was observed in splenectomized compared to *sham* mice, indicating improvement on tissue inflammation, corroborating with insulin signaling amelioration. In summary, spleen has a potential role on metabolism, as its surgical removal propitiate protection against obesity-induced inflammation and insulin resistance, enhanced by reduction on macrophage migration to metabolic tissues.

Introduction

Over the last decades, obesity and its associated abnormalities and metabolic diseases, such as insulin resistance and type 2 diabetes, have reached epidemic proportions worldwide (1, 2). The close relationship between nutrient excess and derangements in the cellular and molecular mediators of immunity and inflammation is responsible for the obesity-induced chronic low-grade inflammatory response (3).

Obesity-induced activation of immune system components by increased circulating intestinal products and fatty acids, mainly by TLR4 activation (4-6), induces the activation of inflammatory signaling pathways in metabolically active tissues, such as c-Jun NH_2-terminal kinase (JNK) and inhibitor of IκB kinase β (IKKβ), that culminates in IRS-1 Ser307 phosphorylation in mice, thus promoting impairment of the insulin signaling by attenuated activation of PI3K and Akt phosphorylation, recognized as insulin resistance (7-10). Additionally, ablation of IKKβ in myeloid cells are also important for the insulin resistance resolution, leading to a global improvement in insulin sensitivity, and suggested as the main responsible for the crosstalk between insulin-responsive tissues involving proinflammatory cytokines (9).

Expansion of adipose tissue mass that occurs in obesity, induces accumulation of immune cells, mainly macrophages, which are functionally and numerically dominant (11, 12) in the adipose tissue, which contributes to both local and systemic inflammation, with increased production of proinflammatory molecules, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and is highly correlated with insulin resistance. Available data suggest that the increased number of macrophages infiltrated in the adipose tissue of obese

mice is due to increased bone marrow-derived monocytes that differentiate into mature macrophages.

Recent evidence showed that, although the bone marrow produces and contains numerous (pro)monocytes (13), the spleen was an important non-differentiated monocyte reservoir, distinct of the present macrophages and dendritic cells, which are demanded in rapid-onset inflammation. The monocyte migration mechanism from the spleen to inflammatory sites was ruled by the angiotensin II, where angiotensin receptor 1 (AT1) deficient mice showed reduced monocyte accumulation after an inflammatory stimuli (14). Additionally, previous studies suggested that the TNF-α production by the splenic macrophages is increased in obese individuals and it could have a relevant role in inflammation-induced insulin resistance (15).

Relying on these data, there is an influence of the spleen on the metabolism and glucose homeostasis, but it is not well understood. Thus, the present study aimed on evaluate the effect of splenectomy on high-fat diet induced obesity (DIO) in mice. We show that splenectomized DIO mice presented reduced inflammation and infiltrated macrophages in the liver and adipose tissue, which promoted increased insulin activity in the liver, muscle and adipose tissue, and insulin sensitivity, suggesting that the spleen has great importance for insulin resistance induced by obesity.

Materials and Methods

Materials Anti-phosphotyrosine, anti-IRβ, anti-pIR, anti-IRS-1, anti-pIRS-1(Ser307), anti-IKKβ, anti-JNK, anti-pJNK, anti-F4/80 and anti-AT1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Akt, anti-pAkt and anti-IκBα were purchased from Cell Signaling Technology (Bervely, MA, USA). Human recombinant insulin (Humulin R) was purchased from Eli Lilly (Indianapolis, IN, USA). Routine reagents were purchased from Sigma-Aldrich (Saint Louis, MO, USA), unless specified elsewhere.

Animals Eight-week old male Swiss, *ob/ob* mice and Wistar rats were obtained from the UNICAMP Central Animal Breeding Centre (Campinas, São Paulo, Brazil). Animals were housed in individual cages with free access to water and rodent chow under a 12h light/dark cycle. Obesity was induced by feeding mice and rats with a high-fat diet (HFD) consisted of 55% of calories derived from fat, 29% from carbohydrates and 16% from protein for 12 weeks. Halothane anesthetized mice were submitted to surgical removal of the spleen and its controls, to Sham surgery (C). Eight weeks after surgery, the animals were evaluated. All procedures were approved by the Ethics Committee from the State University of Campinas.

Hyperinsulinaemic-euglycaemic clamp studies. After an overnight fast, mice were anesthetized and catheters inserted into the jugular vein and femoral artery. Prime continuous insulin and bolus were infused through the jugular vein. When blood glucose reached 90-100 mg/dL, we started a variable glucose infusion to maintain euglycemia at a steady-state plateau (90-120 min) and evaluated the glucose infusion rate, which determined the insulin sensitivity status.

Intraperiotoneal glucose tolerance test After an overnight fasting, glucose tolerance test was performed. Glucose (1g/kg) was injected intraperitoneally and blood collected from the tail vein at different time points till 120 minutes for determination of blood glucose levels, using a glucometer (Optium Xceed, Abbott, Libertyville, IL, USA).

Assays Serum insulin (Millipore, St. Charles, MO, USA), portal vein serum TNF-α and IL-6 (Thermo Fischer Scientific Inc., Rockford, IL, USA) were measured by commercially available ELISA kits following manufacturer´s instructions.

Cell migration induction Sham and splenectomized mice (8 per group) were treated with CL316,243 or saline intraperitoneally twice, with a 4-hour interval between injections, and sacrificed 14 hours after the second injection as described (16). Spleen and epididymal adipose tissue were weighed, processed for tissue histology and protein extraction.

Tissue extraction and protein analysis by immunoblotting Overnight fasted mice were anesthetized and right after insurance of loss of pedal and corneal reflexes, the abdominal cavity was opened, the vena cava exposed and 0,1ml of saline was injected with or without insulin (10^{-6}M). After insulin injection, liver, muscle and adipose tissue were removed, minced coarsely and homogenized in extraction buffer as previously described (17). Whole-tissue extracts proteins were subjected by SDS-PAGE and immunoblotted with specific antibodies. Immunoreactive bands were detected by the enhanced chemiluminescense method (Supersignal West Pico Chemiluminescent Substrate, Thermo Fischer Scientific Inc., Rockford, IL, USA).

Liver and adipose tissue histology Liver and epididymal white adipose tissue depots were dissected and fixed by immersion in 4% parafolmaldehyde in 0.1M phosphate buffer, pH 7,4 for 24 hours, dehydrated, cleared and then embedded in paraffin. Sections (5 μm) were

obtained and then stained by hematoxylin and eosin to assess morphology. Liver was analyzed for estimating area (%) covered by F4/80$^+$ cells. Adipose tissue was evaluated by crown-like structures (CLS) density (average CLS within 10 high-power fields per animal) and frequency for F4/80$^+$ cells (average cell count in 10 high-power fields per animal).

Liver and adipose tissue immunohistochemistry Five μm tissue sections were mounted on silanized glass slides, deparaffinized in xylene, rehydrated, antigen unmasked, quenched for endogenous peroxidase activity, blocked and incubated with F4/80 primary antibody. Antibody staining was performed using an IHC-peroxidase kit (ADVANCE^{TM}HRP, Dako CytoMation, Carpinteria, CA, USA) according to manufacturer´s instructions. Three different high-power fields from each three different sections were evaluated. The total number of nuclei of F4/80 expressing cells were counted for each field and the area occupied by these cells estimated in each field.

Peptide labeling and mass spectrometry analysis Lean and obese mice spleens were excised, frozen in liquid nitrogen, powdered and proteins isolated in specific extraction buffer (8M urea, 75mM NaCl, 50mM Tris, pH 8.2, 1mM NaF, 1mM β-glycerophosphate, 1mM sodium orthovanadate, 10mM sodium pyrophosphate, 1mM PMSF, 1 tablet of protease inhibitor cocktail per 10 ml of lysis buffer- Complete Mini EDTA-free). Protein content was measured (Bradford Protein Assay kit, Thermo Fischer Scientific Inc., Rockford, IL, USA) and 300 μg were reduced and alkylated, and then digested overnight with sequencing grade trypsin (Promega, Madison, WI, USA) at 37 °C. Peptides were desalted using SepPak solid phase extraction cartridges (Waters, Milford, MA, USA) and dried in Speed-Vac. Then, iTRAQ (Applied Biosystems, Foster City, CA, USA) labeling was performed by peptides resuspension in dissolution buffer and label incubation for 1

hour at room temperature: reagent 114 was added to lean and 115 to obese spleen peptides. Reverse reaction was also carried out (115 to lean and 114 to obese spleen peptides). After desalting, labeled peptides were resuspended, injected in an offline HPLC and separated in 45 fractions, which were injected in an Acquity UPLC and ionized by an electrospray source connected to a QTOF Premier mass spectrometer (Waters, Milford, MA, USA). Each fraction was analyzed for 45 minutes and peptide fingerprint search performed with Mascot (Matrix Science Inc., Boston, MA, USA) using IPI Mouse 3.83 database.

Statistical analysis Results are expressed as mean ± SEM. The results of blots are presented as direct comparisons of bands in autoradiographs and quantified by optical densitometry (UN-SCAN-IT gel^{TM}, Silk Scientific Inc., Orem, UT, USA.). Statistical analysis were performed by two-way ANOVA with Bonferroni test for post hoc comparisons and Student´s t test where necessary. Level of significance adopted was, at least, $P < 0,05$.

Results

Splenectomy improves insulin sensitivity in obesity models

Several obesity models were submitted to hyperinsulinemic-euglycemic clamp. We observed that in *ob/ob* and high-fat fed Swiss mice, and in HFD-fed Wistar rat for 12 weeks, the glucose infusion rate was significantly increased ($p<0,01$) in splenectomized (SPL+DIO) animals compared to *ob/ob* and diet-induced obese (DIO) animals that kept the spleen and suffered Sham surgery (Figure 1A-C). Unexpectedly, in splenectomized Swiss mice (SPL) that received standard rodent chow, the glucose infusion rate was also significantly increased compared to its control (C) (Figure 1A).

Splenectomized mice has improved metabolic parameters with no body weight change

Even with increased insulin sensitivity, measured by hyperinsulinemic-euglycemic clamp, SPL mice showed no differences in glucose homeostasis parameters compared to group C (Figure 2A-E; $p>0,05$). DIO mice showed increased body weight, blood glucose, serum insulin and glucose intolerance compared to mice under chow (Figure 2A-E; $p<0,01$). The SPL+DIO group showed no body weight difference when compared to DIO. On the other hand, blood glucose, serum insulin and glucose intolerance were significantly decreased ($p<0,01$), determining an improvement in glucose metabolism proportioned by spleen removal (Figure 2A-E). Serum TNF-α levels were significantly increased in DIO mice compared to mice under standard chow, which was inhibited when splenectomized mice were fed a HFD (Figure 2F; $p<0,01$). IL-6 serum levels were elevated in both groups

that received HFD when compared to standard diet fed mice ($p<0,01$), without significant differences between the HFD groups (Figure 2G).

Insulin signaling is improved in splenectomized mice

Then, we evaluated the insulin signaling proteins in liver, muscle and adipose tissue of sham and splenectomized mice. We observed that in DIO mice, the insulin-stimulated signaling pathway was impaired, determined by reduced activation of IR, IRS-1 and Akt in all tissues compared to control group, determining insulin resistance status. When splenectomized mice were submitted to HFD for the same period, the tyrosine phosphorylation of IR, IRS-1 and serine phosphorylation of Akt were significantly increased, almost restoring control phosphorylation levels in liver, muscle and adipose tissue indicating obesity-induced insulin resistance protection. Interestingly, in SPL mice, as happened on the hyperinsulinemic-euglycemic clamp, showed increased phosphorylation of IR and Akt, at least in liver, giving rise to an additional effect of splenectomy over glucose homeostasis (Figure 3A-I).

Splenectomy impairs inflammation and insulin resistance induced by obesity

After observe that the insulin sensitivity and signaling were improved in splenectomized mice, we investigated proinflammatory proteins in liver, muscle and adipose tissue and IRS-1 serine307 phosphorylation, a known insulin resistance marker. In all tissues, in activated proinflammatory proteins, there was no difference between groups that were fed with chow. Conversely, in DIO mice, JNK and IKKβ activation were increased, same as the IκBα degradation. Following proinflammatory proteins reduction,

the IRS-1 serine307 phosphorylation in liver, muscle and adipose tissue of splenectomized mice under HFD was reduced compared to DIO group (Figure 4A-C). All those inflammatory alterations provoked by obesity were inhibited by spleen absence during the obesity induction, that, in consequence, promoted increase in insulin sensitivity and signaling.

Obesity-induced liver and adipose tissue macrophage infiltration is reduced in splenectomized DIO mice

After observe that the inflammation was blunted and insulin resistance improved in splenectomized mice, we then evaluate the liver and adipose tissue for macrophage infiltration. In liver, we evaluated area occupied by macrophages ($F4/80^+$ cells), and detected no differences between standard chow fed groups. In DIO mice, there was a significant increase ($p<0,01$) on the area populated by macrophages when compared to sham mice, and this increase is completely prevented by splenectomy, which maintained the area covered by macrophages the same as C and SPL mice (Figure 5A-B). In adipose tissue, we evaluated the area compromised by crown-like structures (CLS) and the frequency of macrophages ($F4/80^+$ cells), which are related to tissue inflammation. C and SPL groups showed no differences in both evaluations, where no CLS and little number of macrophages were observed. DIO mice showed significantly increased ($p<0,01$) area compromised by CLS and a striking increase in $F4/80^+$ cells infiltrated in adipose tissue when compared to C group. In mice without spleen under HFD (SPL+DIO), the area compromised by CLS was reduced enormously, accompanied by an impressive reduction in $F4/80^+$ cells frequency in adipose tissue (Figure 5C-E).

Adipose tissue macrophage infiltration induced by lipolysis is impaired in splenectomized mice

We treated the mice with CL316,243, an lipolysis inductor, in order to induce immune cell infiltration in the adipose tissue. Treated mice showed marked macrophage infiltration and increased MCP-1 expression in the adipose tissue compared to control group (vehicle only). In splenectomized mice, the macrophage infiltration and MCP-1 expression in the adipose tissue were almost completely prevented, suggesting that the spleen is responsible for the cellular immune response mediated by obesity components, such as lipolysis and insulin resistance (Figure 6A-B).

Angiotensin II is involved in the adipose tissue macrophage infiltration

Obese adipose tissue showed increased expression of angiotensin type 1 receptor (AT1), however, its expression was strikingly reduced in splenectomized mice, probably by reduced number of macrophages infiltrated in the fat pad (Figure 7A).

GMF-γ is an adipose tissue macrophage infiltration mediator produced in spleen

After 2D-LC/MS/MS proteomic evaluation of mice spleens, we found a target protein, which expression was 2-fold increased in obese mice compared to lean animals. This protein was identified by Mascot as Glia Maturation Factor gamma (GMF-γ). The increased expression of GMF-γ in obese mice was confirmed by spleen immunoblot when compared to lean animal (Figure 7B).

Discussion

Here we show, for the first time, evidence that the spleen, an organ previously limited to immunological and hematological functions, can definitely influence on metabolism and glucose homeostasis through induction of inflammation that reach metabolically active tissues, such as liver, muscle and adipose tissue, and immune cell migration in liver and adipose tissue, promoting insulin resistance and macrophage infiltration in obese mice. Those features were attenuated by surgical removal of the spleen in DIO mice, which presented an increased insulin sensitivity and insulin activity, and prevention of inflammation and macrophage infiltration.

Our results showed that the insulin sensitivity, measured by the hyperinsulinemic-euglycemic clamp, was increased in several insulin resistance models (diet-induced and genetically obese) that were splenectomized when compared to obese animals that maintained the spleen intact. Interestingly, in Swiss mice, even the splenectomized lean mice showed significantly increased glucose uptake when compared to its control. Even in lean healthy animals there are resident populations of leukocytes, which indicates that the immune system is ready to respond to any inflammatory signals (18, 19), and the spleen contains resident monocytes that outnumbers the blood monocytes (14), thus, we speculate that those cells maintain some inflammation level to respond immediately to nutrient or pathogen stimuli and, after splenectomy, the resident monocytes, macrophages and dendritic cells are removed and promoted increase in glucose consumption of splenectomized lean mice. In Wistar rats, evaluated glucose infusion rate showed a slight increase in glucose consumption in splenectomized lean rats, but it was not statistically significant.

There was no body weight nor food intake differences between sham and splenectomized mice. The groups fed their distinct diets maintained the same body weight throughout the experiments. In DIO mice, even with the same body weight, the blood glucose, serum insulin and glucose tolerance were improved in splenectomized, compared to DIO mice, which indicates the insulin resistance attenuation. Previous studies also show that female $TLR4^{-/-}$ mice on HFD were heavier than wild-type and showed increased insulin sensitivity (4).

Following the improvement in insulin resistance in splenectomized obese mice, we found that the portal circulation TNF-α levels were reduced compared to DIO mice, but not IL-6 levels and no difference between lean animals. Probably, it is one of the mechanisms that increased insulin sensitivity in splenectomized obese mice, since TNF-α was the first inflammatory component shown to downregulate insulin sensitivity (20, 21). It is also important to highlight that previous studies suggested that splenic macrophages were important for systemic TNF-α production in obese mice, which production is reduced by spleen removal (15). In accordance with this, mice deficient for TNF-α receptor (TNFR) also had increased insulin sensitivity and glucose tolerance (22-24).

Insulin signaling evaluated by immunoblotting showed a significant increased insulin activity in SPL+DIO mice compared to DIO animals in the liver, muscle and adipose tissue observed by the higher tyrosine phosphorylation levels of IRβ, IRS-1 and serine phosphorylation of Akt, which induces increased glucose uptake by GLUT4 in the muscle and adipose tissue, and an improved hepatic glucose production control (25, 26). Unexpected, like hyperinsulinemic-euglycemic clamp results, there was increased insulin activity in the liver of splenectomized lean mice, evaluated by the IR and Akt

phosphorylation. On the other hand, IRS-1 tyrosine phosphorylation was not increased compared with the control group, probably by the higher importance of IRS-2 in liver insulin signaling (27, 28).

Activation of immune system components, such as TLR4, triggers an inflammatory signaling cascade that induces phosphorylation and activation of JNK and IKKβ, which culminates in IRS-1 Ser307 phosphorylation and insulin resistance (4, 5). Downregulation of TLR4, by several strategies (4, 5, 29, 30), induces improvement of insulin signaling by reducing JNK and IKKβ phosphorylation allowing PI3K activation and Akt phosphorylation. In our study, we observed reduction in JNK and IKKβ activity and prevention of IκBα degradation in the liver, muscle and adipose tissue of SPL+DIO mice compared to DIO animals, which reduced IRS-1 Ser307 phosphorylation, attenuating proinflammatory cytokines transcription, mediated by the NF-κB transcription factor, and insulin resistance.

The liver contains resident macrophages, known as Küpffer cells, which are the main producer of cytokines in hepatic tissue (31), and they are increased in obese mice, as we can see in our results. In splenectomized mice, the number of $F4/80^{+}$ cells, that characterize activated macrophages, was significantly reduced, indicative of reduced inflammation and increased liver insulin sensitivity. There were also reduction in steatosis level as evidenced by the number and size of the lipid droplets into the hepatic parenchyma, also promoting improvement of the insulin sensitivity.

In lean adipose tissue, alternatively activated macrophages have a crucial role in maintaining insulin sensitivity of adipocytes by IL-10 secretion (12, 32, 33). Inversely, in obese adipose tissue, macrophages are classically activated, which secrete proinflammatory

cytokines, such as TNF-α and induce insulin resistance via iNOS, JNK and IKKβ (34, 35). In the adipose tissue of obese mice, $F4/80^+$ cells are classically activated and are almost half of the cells in the adipose tissue, clearly altering the macrophage to adipocytes ratio promoting inflammation and insulin resistance (34). Our results showed a huge increase in the number of macrophages in the adipose tissue of DIO mice, and this cell migration to inflamed adipose tissue was almost fully prevented by the splenectomy in SPL+DIO mice, therefore reducing inflammation and insulin resistance, and improving the adipocyte metabolic status.

Some inflammation and macrophage sources have been investigated. One of the hypothesis is that the preadipocytes present in the adipose tissue stromal vascular fraction, could be converted in macrophages under certain conditions (36) and that TNF-α could induce increase on the expression of some inflammation genes by preadipocytes, hypothesizing that the macrophage infiltration could be a combination of preadipocytes conversion to macrophages and migrated circulating monocytes (37). Previous data show that the main source for TNF-α producing macrophages that infiltrate the obese adipose tissue is derived from the bone marrow, through transplant experiments using wild-type and TNF-α deficient mice (38). There is also the hypothesis that splenic macrophages are great inflammation inducers and may play an important role in the insulin resistance (15).

In order to test an acute stimuli for adipose tissue macrophage infiltration, we treated the animals with CL316,243, a β3-adrenergic agonist, which increase lipolysis of stored triglycerides in adipocytes, as previously described (16). In obesity, the basal lipolysis is increased, which increases extracellular lipid concentrations that drives macrophages accumulation in the adipose tissue (39). With increased adiposity, the

infiltrated macrophages form multinucleated syncytia that contain large lipid droplets, suggesting that the macrophages phagocytose or take up the excess lipid (40, 41). Lean mice were treated with CL316,243 and there was a significant influx of macrophages in the adipose tissue. In splenectomized mice, this influx was completely impaired, indicating that the spleen could be the main donor for adipose tissue macrophage infiltration in acute inflammatory stimuli, such as the rapid lipolysis induced by the β3-adrenergic agonist.

Monocytes express angiotensin receptor, by which angiontesin II induces cellular cytoskeletal rearrangement and migration in vitro (42) and augments monocyte-mediated inflammation (43). We found that AT1 expression was strikingly increased in the adipose tissue of obese mice compared to lean animals, and splenectomized obese mice showed a great reduction compared to sham obese animals, demonstrating the impaired adipose tissue macrophage infiltration by splenectomy. Recent data showed that the angiotensin II mediates the expel of splenic monocytes, whereas *Atgr1a*$^{-/-}$ mice subjected to inflammatory stimuli did not made it efficiently, promoting accumulation of only few monocytes in inflammation site (14). We hypothesized that in the absence of the spleen, inflammation is reduced and fewer monocytes migrate to the adipose tissue, reflecting reduced AT1 expression and insulin resistance improvement.

In addition to TNF-α secretion and immune cell reservoir, we speculate that the spleen produces other products that aid the inflammation and insulin resistance mediated by this tissue. GMF-γ became a candidate for those features after proteomic overexpression identification in obese spleen, corroborated by the protein tissue levels, as well as its function already described, where it is needed for the actin debranching modulation, crucial for neutrophil polarization and migration towards inflammatory sites (44). We speculate

that similar processes are needed for resident splenic monocytes enter the circulation and infiltrate adipose tissue, turning into macrophage and propagating inflammation and insulin resistance.

Our results suggest that the spleen is an important site for inflammation-induced insulin resistance, ruling the TNF-α induced inflammation, as the SPL+DIO mice had reduced levels of this cytokine, and, mainly, as a great source of the macrophages that infiltrated the liver and adipose tissue of obese mice. Additionally, we speculated that other spleen products, like GMF-γ, which is overexpressed in obese mice, could have a role in cell migration and macrophage infiltration in the liver and adipose tissue. In summary, splenectomized obese mice showed reduced inflammation and macrophage infiltration and, consequently, improved insulin sensitivity and signaling, giving rise to the hypothesis that the spleen has an important role in the glucose metabolism regulation in rodents.

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Conflict of interest The authors declare no duality of interest associated with this manuscript.

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Figure Legends

Figure 1 - **Splenectomy induces increase in the insulin sensitivity in mice fed a HFD.** Glucose Infusion Rate measured by the hyperinsulinemic-euglycemic clamp in (A) Swiss mice, (B) Wistar rats and (C) genetically obese *ob/ob* mice. Bars represent mean ± SEM from 6 animals per group. $P < 0,05$. * versus C; # versus DIO; § versus SPL.

Figure 2 - Splenectomy induces improvement of the metabolic parameters in obese mice without body weight change. (A) Body weight, (B) blood glucose, (C) serum insulin, (D) intraperitoneal glucose tolerance test (GTT), (E) area under the curve of the GTT, portal vein serum measurement of (F) TNF-α and (G) IL-6. Bars represent mean ± SEM from 8 animals per group $P < 0,05$. * versus C; # versus DIO; § versus SPL.

Figure 3 - **Splenectomy induces increased insulin signaling activity under insulin stimulation in obese mice.** Insulin signaling pathway was evaluated by the tyrosine phosphorylation of IR, IRS-1 and serine phosphorylation of Akt in the (A, D and G) liver, (B, E and H) muscle and (C, F and I) adipose tissue. Bands are representatives of 3 independent experiments and bars represent blot densitometry quantification ± SEM. $P < 0,05$. * versus C; # versus DIO.

Figure 4 - Splenectomy promotes reduction in the inflammatory signaling in the liver, muscle and adipose tissue of obese mice. Proinflammatory signaling measured by JNK and IKKβ activation and IRS-1 Ser 307 phosphorylation in the (A) liver, (B) muscle and (C) adipose tissue. Bands are representatives of 3 independent experiments.

Figure 5 - Splenectomy prevents macrophage infiltration in the liver and adipose tissue of obese mice. (A) Liver micrographs stained with HE (above) and immunostained for F4/80 (below). Magnification: 400x. (B) Area occupied by $F4/80^+$ cells in the liver of

mice. Bars represent mean ± SEM from 4 animals. P < 0,05. * versus C; # versus DIO. (C) Adipose tissue micrographs stained with HE (above) and immunstained for F4/80 (below). Magnification: 660x. (D) Estimated adipose tissue area occupied by Crown-like Structures (CLS). (E) $F4/80^+$ cells frequency in the adipose tissue. Bars represent mean ± SEM from 4 animals. P < 0,05. * versus C; # versus DIO; § versus SPL.

Figure 6 - Splenectomy prevents macrophage infiltration in the adipose tissue stimulated by CL316,243-induced lipolysis. (A) Adipose tissue micrographs stained with HE. Magnification: 400x. (B) Estimated adipose tissue area occupied by Crown-like Structures (CLS). Bars represent mean ± SEM from 4 animals. P < 0,05. * versus C.

Figure 7 - AT1 and GMF-γ are higher expressed in obese mice adipose tissue and spleen, respectively, and splenectomy reduced AT1. (A) AT1 expression in the adipose tissue of obese and splenectomized obese mice. (B) Splenic GMF-γ expression evaluated by immunoblotting. Bands are representatives of 3 independent experiments.

Figure 1

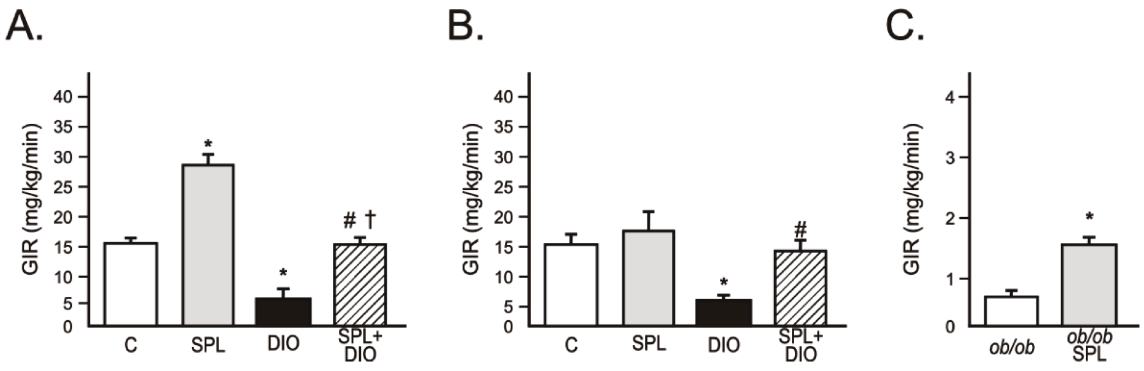

A.
B.
C.
GIR (mg/kg/min)
40
35
30
25
20
15
10
5
0
*
†
C
SPL
DIO
SPL+ DIO
#
4
3
2
1
ob/ob
ob/ob SPL

Figure 2

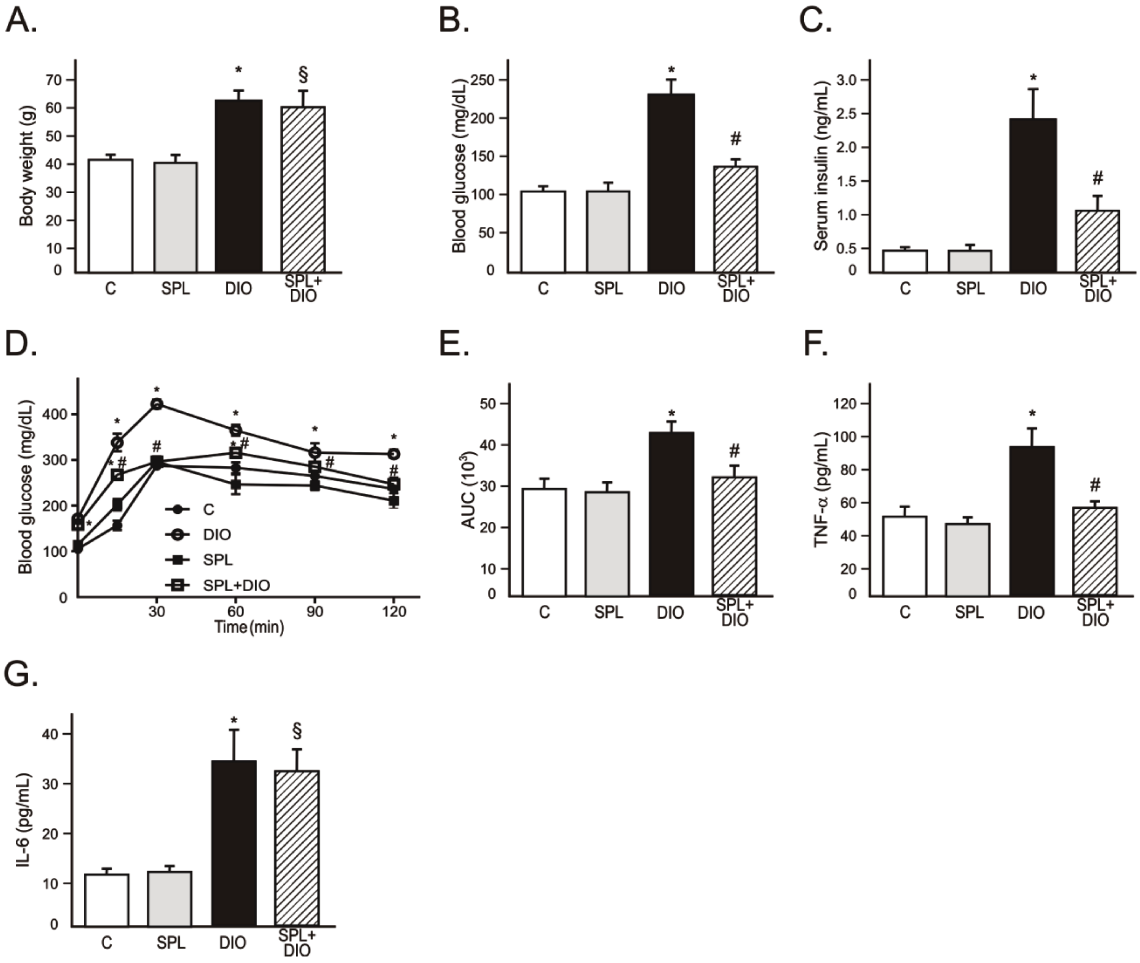

A.
Body weight (g)
C
SPL
DIO
SPL+ DIO
B.
Blood glucose (mg/dL)
C.
Serum insulin (ng/mL)
D.
Blood glucose (mg/dL)
Time (min)
C
DIO
SPL
SPL+DIO
E.
AUC (10^3)
F.
TNF-α (pg/mL)
G.
IL-6 (pg/mL)

Figure 3

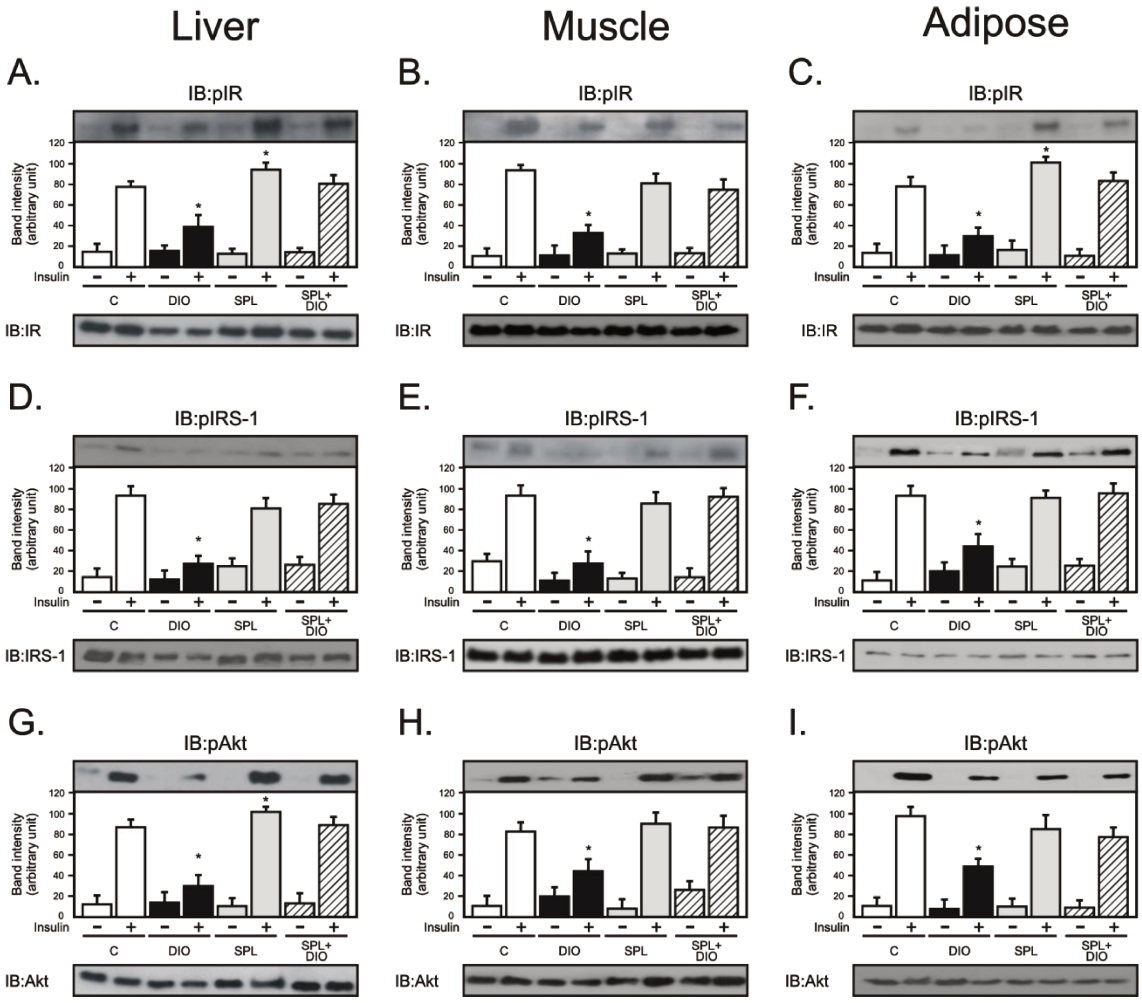

Liver
Muscle
Adipose
A.
B.
C.
IB:pIR
D.
E.
F.
IB:pIRS-1
G.
H.
I.
IB:pAkt
Band intensity (arbitrary unit)
Insulin
C
DIO
SPL
SPL+ DIO
IB:IR
IB:IRS-1
IB:Akt

Figure 4

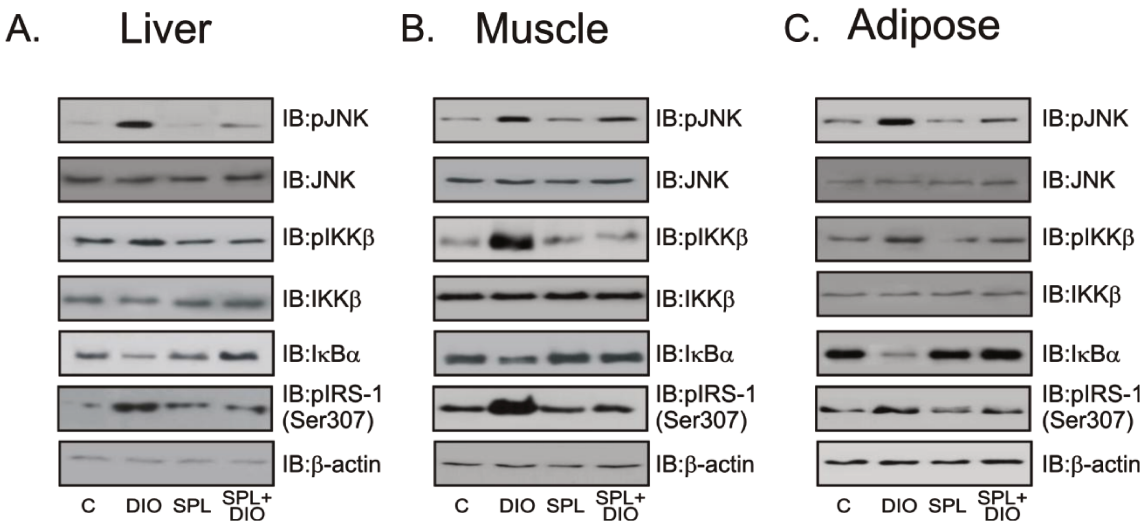

A. Liver
B. Muscle
C. Adipose
IB:pJNK
IB:JNK
IB:pIKKβ
IB:IKKβ
IB:IκBα
IB:pIRS-1 (Ser307)
IB:β-actin
C
DIO
SPL
SPL+ DIO

Figure 5

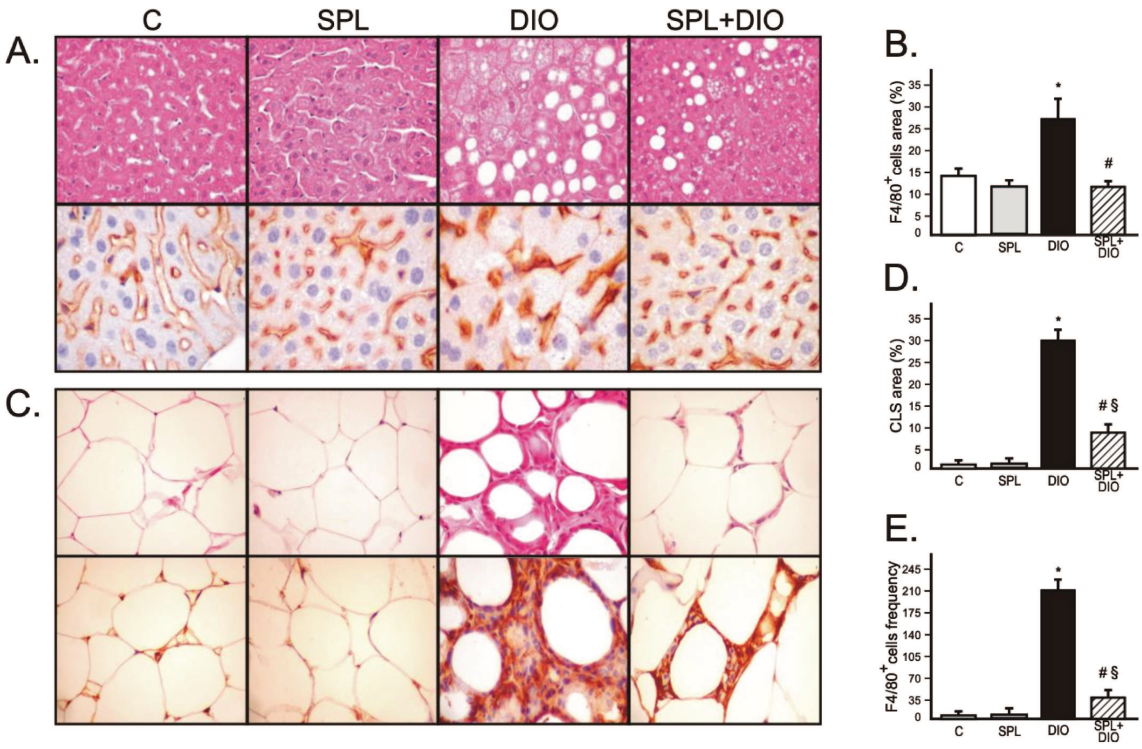

C
SPL
DIO
SPL+DIO
A.
C.
B.
F4/80+cells area (%)
D.
CLS area (%)
E.
F4/80+cells frequency
C
SPL
DIO
SPL+ DIO
*
#
§

Figure 6

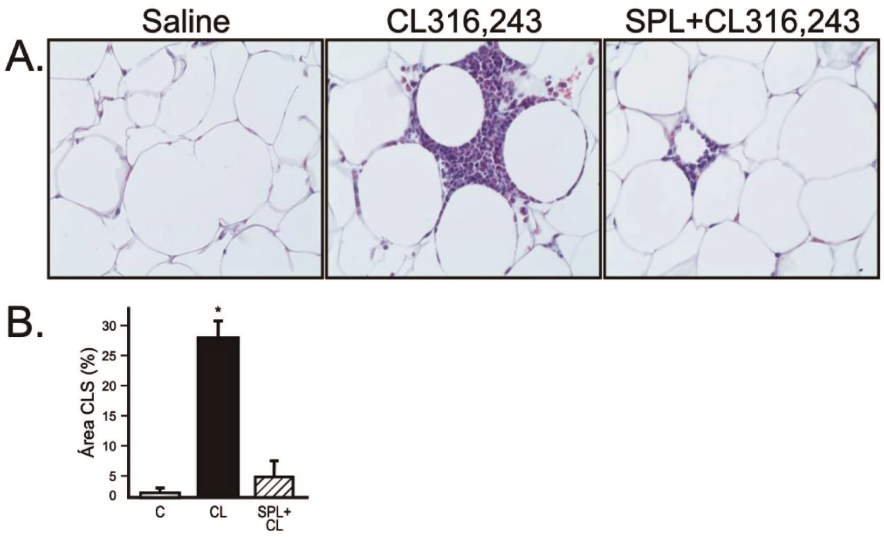
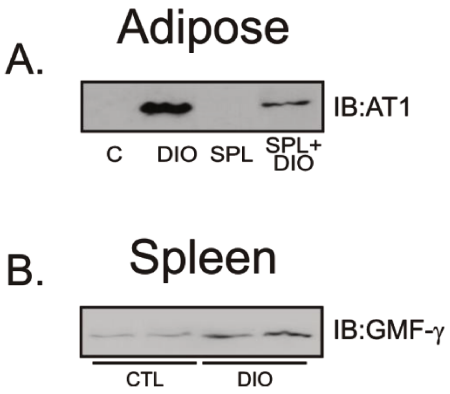

A.
Saline
CL316,243
SPL+CL316,243
B.
Área CLS (%)
30
25
20
15
10
5
0
*
C
CL
SPL+ CL

Figure 7



DISCUSSÃO

No presente estudo, avaliamos dois fenômenos completamente diferentes que poderiam influenciar na ocorrência de resistência à insulina induzida por obesidade: a flora intestinal e a atividade do baço, e descrever alguns dos mecanismos que estes realizariam na obesidade.

Em relação à flora intestinal, demonstramos que a dieta rica em gordura alterou a microbiota em camundongos, com indução no aumento da proporção de bactérias do filo *Bacteroidetes*, que está associado com aumento nos níveis circulantes de lipopolissacarídeos (LPS), redução nos níveis séricos de acetato e da sensibilidade à insulina. No momento em que estes animais foram tratados com antibióticos como estratégia de modulação da flora intestinal, eles apresentaram grandes reduções na quantidade de bactérias e nos níveis circulantes de LPS, com aumento nos níveis de ácidos graxos de cadeia curta, assim atenuando a ativação de TLR4; este último fenômeno propiciou melhora na sensibilidade à insulina e no perfil inflamatório, além da ativação de AMPK nos tecidos hepático, muscular e adiposo. Além disso, surpreendentes reduções no nível de infiltração de macrófagos no fígado e no tecido adiposo foram observados, culminando na prevenção de resistência à insulina.

A análise metagenômica da flora intestinal em camundongos em dieta hiperlipídica mostrou um leve, mas significante aumento na prevalência de *Bacteroidetes* (78,5% contra 61,8%) e uma grande redução de *Firmicutes* (6,1% contra 31,6%), comparado aos animais controle. Esta variação foi similar a outros estudos na literatura [69, 70] que analisaram a composição da microbiota em pacientes com sobrepeso e obesos e em camundongos com

esteato-hepatite não alcoólica (NASH), mas foram discrepantes em relação a outros dados previamente publicados [62, 68]; isto evidencia que o perfil bacteriano em estudos de obesidade permanece controverso, baseando-se em detalhes metodológicos para explicar as diferenças nos dados já publicados. O filo *Verrucomicrobia*, composto por dois filos-irmãos de bactérias Gram negativas, *Chlamydiae* e *Lentisphaerae*, os quais não foram devidamente investigados pela literatura, está presente em baixas porcentagens em camundongos alimentados com ração, mas apresentaram considerável prevalência nos camundongos alimentados com dieta rica em gordura (0,03% contra 13,65%).

Por outro lado, os animais tratados com antibióticos apresentaram enormes diferenças no perfil metagenômico comparados aos animais controle e mantidos em regime de *pair-feeding*. Praticamente todas as bactérias pertencentes aos filos *Bacteroidetes* e *Firmicutes* foram aniquiladas do intestino, seguindo o mesmo destino do filo *Verrucomicrobia* nos camundongos tratados com antibióticos, mas estas mudanças foram acompanhadas por um grande aumento na prevalência do filo *Proteobacteria*, praticamente o único grupo bacteriano presente (97%). Mesmo com a alta prevalência de bactérias Gram negativas (*Proteobacteria*), houve redução nos níveis circulantes de LPS, relacionada à grande redução no número de bactérias presentes no intestino e consequentemente menor absorção desta molécula e melhora da função da barreira intestinal.

Nossos dados que demonstraram aumento nos níveis de LPS na circulação portal em camundongos alimentados com dieta hiperlipídica sugerem que a fonte dessa molécula é a flora intestinal. Dados disponíveis indicam que a translocação de agonistas do TLR4, como o LPS, a partir do intestino é um importante componente em doenças metabólicas

como a obesidade e NASH, promovendo inflamação e resistência à insulina em tecidos-alvo deste hormônio [69, 73].

Uma grande quantidade de dados demonstrou a importância do LPS na inflamação e indução de resistência à insulina utilizando injeções de LPS e modelos de sepse [29-31, 86-89]. O mecanismo primário induzido por LPS é iniciado quando este se liga ao TLR4, seu receptor, o qual se associa com o MyD88 e desencadeia sua via de sinalização; isto, por sua vez, ativa JNK e IKKβ através da IRAK, levando à fosforilação em serina do IRS-1, ativação de NF-κB e transcrição de citocinas pró-inflamatórias e resistência à insulina [90, 91].

Cani e colaboradores [92] demonstraram que um tratamento com antibióticos melhorou a expressão de marcadores de inflamação, de estresse oxidativo e de infiltração de macrófagos no tecido adiposo de camundongos alimentados com dieta rica em gordura. Além disso, eles mostraram acentuado aumento no RNAm de ZO-1 (*Zonula Occludens-1*), uma importante proteína da barreira intestinal, a qual é correlacionada com redução na permeabilidade intestinal, o que resulta em menores níveis circulantes de LPS nos animais tratados com antibióticos. Todos os experimentos foram realizados em camundongos alimentados com dieta hiperlipídica, sem excluir a redução de peso causada pelo tratamento com antibiótico, a qual é um importante fator sensibilizante à ação da insulina, do estudo, como realizado no presente trabalho. Nossos experimentos foram conduzidos utilizando regime de *Pair-feeding* com o intuito de eliminar as diferenças de peso, uma consequência da redução na ingestão induzido pelo tratamento com antibióticos, e demonstrar, de forma clara, o aumento na sensibilidade à insulina causada pela modulação da flora intestinal de animais alimentados com dieta hiperlipídica tratados com antibióticos.

Nossos resultados, similares a estudos realizados em camundongos geneticamente obesos *ob/ob* [93, 94], demonstraram melhora no metabolismo de camundongos em dieta rica em gordura tratados com antibióticos, baseados em aumento da sensibilidade à insulina e tolerância à glicose, além de reduções na glicemia e insulinemia de jejum. Também observamos que a melhora no metabolismo é principalmente relacionada com a redução nos níveis circulantes de LPS, os quais fizeram os níveis séricos de TNF-α e IL-6 permanecerem semelhantes aos animais alimentados com ração. Todos estes efeitos induzidos pelo tratamento com antibióticos devem ser mediados pela modulação da flora intestinal e melhora na função da barreira intestinal.

Também relatamos, pela primeira vez, a influência da redução na concentração de LPS, após modulação da microbiota pelo tratamento com antibióticos, na regulação negativa da associação de TLR4-MyD88 em fígado, músculo e tecido adiposo; levando à reduzida ativação de JNK e IKKβ, e fosforilação em serina do IRS-1. De maneira contrária, mas diretamente ligada, foi observado aumento na ativação da via de sinalização da insulina, determinada pela fosforilação em tirosina de IRβ, IRS-1 e fosforilação em serina de Akt, nos três tecidos [22]. Estas observações mostram um aumento no controle da inflamação e do metabolismo mediada pela aumentada atividade da insulina em tecidos-alvo do hormônio.

Outra importante questão a respeito da relação entre obesidade e flora intestinal é a produção de ácidos graxos de cadeia curta (SCFA) proveniente da fermentação bacteriana de carboidratos não ou pouco digeríveis presentes na dieta. A queda nos níveis circulantes de acetato de camundongos alimentados com dieta hiperlipídica pode ser relacionada à redução na razão carboidrato:gordura da dieta, assim, afetando a produção SCFA [95]. As

modificações no perfil da microbiota promovidas pela terapia com antibióticos induziram aumento nos níveis circulantes de acetato. Este ácido graxo possui a capacidade de ativar AMPK no fígado e músculo [80], promovendo aumento da oxidação de ácidos graxos [96] e gasto energético; também ocorre indução da translocação de GLUT4 para a membrana plasmática e captação de glicose [97]. Há evidências de que ácidos orgânicos, como butirato e acetato, podem contribuir com as funções de imunidade e barreira intestinal pelo aumento no número de células do sistema imune na camada de células epiteliais [98], e estimulando a produção de mucina no intestino [99]. Assim, os SCFA também poderiam contribuir para a redução na permeabilidade intestinal e nos níveis circulantes de LPS observados nos camundongos tratados com antibióticos quando comparados aos animais em regime de *pair-fed*.

A atividade de macrófagos está relacionada com inflamação e resistência à insulina em tecidos metabolicamente ativos na obesidade e diabetes do tipo 2, proporcionando aumento na produção de atividade de TNF-α e IL-6, principalmente no fígado e tecido adiposo, locais preferencialmente infiltrados por células do sistema imunológico com o intuito de manter a homeostase e prevenir disfunções metabólicas provocadas pelos altos níveis circulantes de ácidos graxos, glicose e LPS observados em indivíduos obesos [100, 101].

As reduções na infiltração de macrófagos no fígado e tecido adiposo, determinadas pela marcação com F4/80, em camundongos tratados com antibióticos, ocorreram devido a reduções nos níveis circulantes de LPS; ao passo que essa molécula pode causar inflamação, induzindo macrófagos a secretarem TNF-α e IL-6, promovendo resistência à insulina; este cenário também induz a expressão e atividade de proteínas quimioatraentes,

como a MCP-1 e outras quimiocinas, cujo papel é promover respostas celulares da imunidade inata e translocação em direção ao estímulo, como a hipertrofia do tecido adiposo e esteatose hepática induzida por dieta rica em gordura [102, 103]. É importante bloquear a produção de moléculas inflamatórias, como os promotores de resistência à insulina, para a prevenção da infiltração de macrófagos ou o impedimento do recebimento de estímulos por parte dos macrófagos já infiltrados, como ocorre no tratamento com algumas drogas ou com a redução do peso [104-107].

Em síntese, nossos dados demonstraram que a dieta rica em gordura promoveu alterações no perfil da flora intestinal, a qual favoreceu um estado inflamatório e de resistência à insulina, provocada por redução na função protetora da barreira intestinal e níveis circulantes elevados de LPS, além de intensa atividade de macrófagos. Todos estes processos relacionados à obesidade foram revertidos por mudanças na flora intestinal, induzida por terapia com antibióticos, em camundongos alimentados com dieta hiperlipídica. Estas observações nos levam a sugerir que a utilização de estratégias que focam em alterar o perfil da flora intestinal podem trazer imensos benefícios na prevenção e atenuação de transtornos metabólicos.

Para a análise do outro fenômeno proposto por esta tese, que seria a participação do baço em processos metabólicos, nossos experimentos trazem, de forma inédita, evidências de que este órgão, até o momento limitado a funções imunológicas e hematológicas, deve apresentar influência sobre o metabolismo e homeostase glicêmica através da indução de inflamação que alcança tecidos metabolicamente ativos, como o fígado, o músculo e o tecido adiposo, e a migração de células do sistema imune para o fígado e tecido adiposo, que culmina na promoção de resistência à insulina e infiltração de macrófagos em

camundongos obesos. Estes processos são atenuados após a remoção cirúrgica do baço em animais submetidos à obesidade induzida por dieta, apresentando aumento na sensibilidade à insulina e atividade da insulina, além de prevenção da inflamação e infiltração de macrófagos.

Nossos resultados demonstraram que a sensibilidade à insulina, avaliada pelo *clamp* hiperinsulinêmico-euglicêmico, estava aumentada em alguns modelos de resistência à insulina (obesidade induzida por dieta e genética) que foram esplenectmizados quando comparados aos animais obesos em que os baços foram mantidos intactos. Surpreendentemente, em camundongos *Swiss*, até mesmo o grupo de animais magros esplenectomizados apresentaram consumo de glicose aumentado quando comparados aos animais alimentados com ração que apenas sofreram a cirurgia *sham*. Mesmo em animais sadios e magros, há populações residentes de leucócitos, indicando que o sistema imune está de prontidão para responder a qualquer estímulo inflamatório [108, 109], assim como o baço que possui monócitos residentes que superam o número de células desse tipo na circulação [83]; dessa maneira, especulamos que estas células mantêm algum nível de inflamação para induzir resposta imediata a estímulos nutricionais ou patogênicos e que, após a esplenectomia, estes monócitos, macrófagos e células dendríticas residentes foram removidos, o que pode ter acarretado em aumento do consumo de glicose em camundongos magros esplenectomizados. Em outras linhagens de camundongos, como o C57BL/6, C3H/HePas e o camundongo deficiente da iNOS, também apresentaram aumento na sensibilidade à insulina avaliada pelo *clamp* hiperinsulinêmico-euglicêmico (dados não divulgados). Em ratos *Wistar*, a razão de infusão de glicose avaliada apresentou um

aumento modesto no consumo de glicose em ratos magros esplenectomizados, mas não foi estatisticamente significante.

Não houve variação nem no ganho de peso, nem na ingestão de alimentos entre os camundongos *sham* e esplenectomizados. Os grupos alimentados com suas dietas distintas mantiveram o mesmo peso por todo o experimento. Nos camundongos obesos, mesmo com o peso similar, houve melhora na glicemia e insulinemia de jejum, e na tolerância à glicose nos animais esplenectomizados comparados aos animais com baço, o que indica atenuação da resistência à insulina nesses animais. Em estudos prévios, foi demonstrados que camundongas deficientes de TLR4 sob dieta hiperlipídica tinham maior peso que seus controles, contudo, apresentavam maior sensibilidade à insulina [30].

Em seguida à constatação da melhora da resistência à insulina em camundongos obesos esplenectomizados, nós observamos que os níveis de TNF-α, na circulação portal, estavam reduzidos quando comparados aos animais *sham*, entretanto, os níveis de IL-6 permaneceram os mesmo, assim como não houve diferença nos níveis de citocinas entre os grupos de animais magros. Provavelmente, este é um dos mecanismos que levam ao aumento da sensibilidade à insulina nos camundongos obesos esplenectomizados, já que o TNF-α foi o primeiro componente da resposta inflamatória com comprovada ação indutora de resistência à insulina [24, 26]. Em conformidade com o exposto, camundongos deficientes do receptor de TNF (TNFR) também obtiveram aumento na sensibilidade à insulina e na tolerância à glicose [28, 110, 111]. Também é importante destacar que estudos anteriores sugeriram que os macrófagos presentes no baço seriam importantes para a produção sistêmica de TNF-α em animais obesos, a qual é reduzida pela remoção do baço [82].

A sinalização da insulina, avaliada por técnica de *Western Blotting*, apresentou significativo aumento de sua atividade no grupo de camundongos obesos esplenectomizados comparado aos animais obesos nos tecidos hepático, muscular e adiposo, avaliado pela maior fosforilação em tirosina de IRβ, IRS-1 e fosforilação em serina da Akt, o que induz aumento da captação de glicose por GLUT4 em músculo e tecido adiposo, e aumentado controle da produção hepática de glicose [19, 22]. De maneira inesperada, assim como os resultados do clamp hiperinsulinêmico-euglicêmico, houve aumento na atividade da insulina no fígado dos camundongos magros esplenectomizados quando observadas as fosforilações de IRβ e Akt. Por outro lado, a fosforilação em tirosina do IRS-1 não estava aumentada quando comparada ao animal magro, possivelmente pela maior importância do IRS-2 na sinalização da insulina no fígado [112, 113].

A ativação de componentes do sistema imune, como o TLR4, inicia uma cascata de sinalização inflamatória que acaba por induzir a fosforilação e ativação da JNK e IKKβ, que culmina na fosforilação em Ser307 do IRS-1 e resistência à insulina [29, 30]. Contudo, a regulação negativa do TLR4, por várias estratégias [29-31, 88], induz aumento da sinalização da insulina através da inibição da fosforilação da JNK e IKKβ, de forma que permite a ativação da PI3K e fosforilação da Akt. Em nosso estudo, nós observamos redução na atividade de JNK e IKKβ, além da prevenção da degradação de IκBα em fígado, músculo e tecido adiposo dos camundongos obesos esplenectomizados comparados aos animais com baço, os quais demonstraram reduzida fosforilação do IRS-1 em Ser307, o que atenuou a transcrição de citocinas pró-inflamatórias pelo NF-κB e a resistência à insulina.

O fígado contém macrófagos residentes, conhecidos como células de Küpffer, que são os principais produtores de citocinas no tecido hepático [60], as quais estão em maior número em camundongos obesos, como podemos observar em nossos resultados. Em camundongos esplenectomizados, o número de células $F4/80^+$, que caracteriza a presença de macrófagos ativados, está significativamente reduzido, indicativo de inflamação reduzida e aumentada sensibilidade hepática à insulina. Também houve redução nos níveis de esteatose evidenciado pelo número e tamanho das gotículas de gordura presentes no parênquima hepático, também promovendo melhora da sensibilidade à insulina.

No tecido adiposo magro, macrófagos alternativamente ativados tem um papel crucial na manutenção da sensibilidade à insulina dos adipócitos através da secreção de IL-10 [46, 55, 114]. Inversamente, no tecido adiposo obeso, os macrófagos estão classicamente ativados, condição que induz secreção de citocinas pró-inflamatórias, como o TNF-α e induzem resistência à insulina via iNOS, JNK e IKKβ [43, 47]. Em camundongos obesos, as células $F4/80^+$ estão classicamente ativadas e representam praticamente metade das células presentes no tecido adiposo, claramente alterando a razão de macrófagos para adipócitos, com a promoção de inflamação e resistência à insulina [43]. Nossos resultados apresentaram enorme aumento no número de macrófagos no tecido adiposo de camundongos obesos, e esta migração celular para o tecido adiposo inflamado foi quase que completamente prevenida pela esplenectomia em camundongos obesos, portanto, reduzindo inflamação e resistência à insulina, e promovendo melhora no padrão metabólico dos adipócitos.

Algumas fontes de inflamação e macrófagos já foram investigadas. Uma das hipóteses levantadas pela literatura aponta que os pré-adipócitos presentes na fração de

estroma vascular do tecido adiposo possa ser convertida em macrófagos sob algumas condições [115] e que TNF-α poderia induzir o aumento na expressão de alguns genes inflamatórios nestes pré-adipócitos, formando a ideia de que a infiltração de macrófagos seria uma combinação da transformação de pré-adipócitos em macrófagos e da migração de monócitos circulantes [44]. Estudos anteriores indicam que a principal fonte de macrófagos produtores de TNF-α que infiltram o tecido adiposo é a medula óssea, através de transplantes de medula utilizando camundongos normais e deficientes para a produção de TNF-α [116]. Adicionalmente, há a hipótese de que os macrófagos provenientes do baço são grandes indutores de inflamação e podem possuir um papel importante no processo de resistência à insulina [82]. Nossos resultados sugerem que o baço seja uma importante fonte das células que infiltram o tecido adiposo, talvez até com mais destaque que a própria medula óssea, já que em camundongos esplenectomizados obesos a infiltração de macrófagos encontrada seria contribuição da medula óssea, a qual apresentou-se bastante reduzida quando comparada com a infiltração de macrófagos sob influência conjunta da medula óssea com o baço, indicando potencial fonte de inflamação e células inflamatórias para indução de resistência à insulina.

Possivelmente, durante o período necessário para indução de obesidade e infiltração de macrófagos no tecido adiposo, a medula óssea é capaz de repovoar o baço, substituindo as células que foram solicitadas a migrarem para o tecido adiposo. Dessa maneira, com o intuito de provocar um estímulo agudo para indução de infiltração de macrófagos, nós tratamos os camundongos com o CL316,243, um agonista β3-adrenérgico capaz de induzir intensa lipólise dos triglicerídeos armazenados nos adipócitos como descrito previamente [117]. Na obesidade, os níveis basais de lipólise estão aumentados, o que aumenta as

concentrações de lipídeos extracelulares que conduzem ao acúmulo de macrófagos no tecido adiposo [118]. Com o aumento da massa adiposa, os macrófagos infiltrados formam um sincício multinucleado que contem grandes gotículas de gordura, sugerindo que os macrófagos estariam fagocitando ou captando o excesso de lipídeos [56, 119]. Assim, animais magros foram tratados com CL316,243 e foi observado significativa infiltração de macrófagos no tecido adiposo. Diferentemente, em camundongos esplenectomizados, este influxo de células foi completamente enfraquecido, indicando que o baço seria a principal fonte dos macrófagos infiltrados no tecido adiposo em estímulos inflamatórios agudos como a rápida lipólise induzida pelo agonista β3-adrenérgico.

De acordo com o exposto, nossos resultados sugerem que o baço seria a principal fonte da indução de resistência à insulina provocada por inflamação, coordenando a inflamação induzida por TNF-α, já que o camundongo obeso esplenectomizado apresentou reduzidos valores desta citocina, e, principalmente, como uma grande fonte de macrófagos que infiltraram o fígado e o tecido adiposo de camundongos obesos. Em resumo, os camundongos obesos esplenectomizados apresentaram reduzida inflamação e infiltração de macrófagos e, consequentemente, aumentada sensibilidade e sinalização da insulina, dando origem à hipótese de que o baço possui um importante papel na regulação do metabolismo da glicose em roedores.

CONCLUSÃO

A flora intestinal de camundongos submetidos à dieta hiperlipídica apresenta alterações na prevalência de filos bacterianos, com aumento nos níveis circulantes de LPS e citocinas pró-inflamatórias, e indução de resistência à insulina. A modulação da microbiota utilizando antibióticos modificou drasticamente o perfil da microbiota, que somando-se à redução na permeabilidade intestinal, dos níveis circulantes de LPS e citocinas e o aumento nos níveis circulantes de acetato, acarretaram inibição da atividade do TLR4, JNK e IKKβ com consequente aumento da sensibilidade à insulina e tolerância à glicose.

Camundongos esplenectomizados não desenvolveram resistência à insulina induzida por obesidade quando submetidos a dieta rica em gordura, apresentando níveis circulantes de TNF-α, assim como a ativação de proteínas inflamatórias, como JNK e IKKβ, mantendo a sensibilidade à insulina em níveis similares aos camundongos em ração. Além disso, o número de macrófagos infiltrados no fígado e tecido adiposo está bastante reduzido, assim como os camundongos que receberam estímulo agudo para infiltração de macrófagos no tecido adiposo quando comparados aos animais com baço. Dessa forma, nossos resultados sugerem que o baço possui uma importante atividade na resistência à insulina induzida por inflamação e na regulação do metabolismo em camundongos.

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ANEXOS

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Comissão de Ética na Experimentação Animal
CEEA/Unicamp

CERTIFICADO

Certificamos que o Protocolo nº **1358-1**, sobre **"Resistência à insulina e doença hepática gordurosa não alcoólica: influência da esplenectomia da flora intestinal e do receptor Toll-like 4 (TLR4)"**, sob a responsabilidade de **Prof. Dr. Mário José Abdalla Saad /Bruno de Melo Carvalho** , está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal – CEEA/Unicamp em **26 de setembro de 2007**.

CERTIFICATE

We certify that the protocol nº **1358-1**, entitled **"Insulin resistance and nonalcoholic fatty liver disease : influence of splenectomy gut flora and Toll-like receptor 4 (TLR4)"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - Unicamp) on **September 26, 2007**.

Campinas, 26 de setembro de 2007.

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APÊNDICE

PKR is a key modulator of insulin resistance in humans

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Context. Molecular integration of nutrient- and pathogen-sensing pathways is of great interest in understanding the mechanisms of insulin resistance in obesity. The double-stranded RNA-dependent protein kinase (PKR) was recently implicated in regulating this integration in obese mice. However, the modulation of this protein in human tissues in situations of insulin resistance has not been investigated.

Objective. The present study was performed to first determine the tissue expression and phosphorylation levels of PKR in the liver, muscle and adipose tissue in obese humans, and also the modulation of this protein in the adipose tissue of obese patients after bariatric surgery, which is an effective treatment for morbid obesity.

Patients. Eleven obese subjects (mean BMI 45.4 ± 3.6 kg/m^2) who were scheduled to undergo Roux-en-Y Gastric Bypass surgery (RYGBP) participated in this study.

Intervention. All patients were submitted to an euglycemic-hyperinsulinemic clamp, open RYGBP procedure, and tissue biopsies from liver, muscle and adipose tissue, and another fat biopsy 6 months after RYGBP.

Results. PKR activity was increased in the liver, muscle and adipose tissue of obese patients. After bariatric surgery, increased PKR activity as well as insulin resistance and metabolic profile were reversed.

Conclusions. Our data show that PKR is activated in the liver, muscle and adipose tissue of obese humans and, after bariatric surgery, there is a clear reduction in PKR activation in adipose tissue in obesity. Thus, PKR is an important mediator of obesity and insulin resistance and a potential target for the therapy of these medical conditions.

Obesity is characterized by insulin resistance and chronic activation of inflammatory pathways (1). Evidence coming from different sources has shown that these activated inflammatory pathways are causally linked to insulin resistance in obesity, through cross-talk between inflammatory and metabolic pathways (2, 3). In this regard, intracellular kinases such as c-Jun N-terminal kinase (JNK) and IκB kinase β (IKKβ), linked to inflammatory signaling pathways, have important roles in insulin resistance (4, 5). These kinases can be activated through the activation of TLR4 and endoplasmic reticulum (ER) stress, conditions present in obese and diabetic humans, who show increased TLR4 expression in muscle and also ER stress in liver and adipose tissue (6, 7).

Recently, the double-stranded RNA-dependent protein kinase (PKR) was described as playing an important role in the connection between the inflammatory and metabolic pathways (8). This kinase is a critical component of the innate immune response against virus infection, and its cellular actions are mediated by modulating cell signaling and translational regulation. To be enzymatically active, PKR needs to be activated by binding to dsRNA or other RNAs or activators (9). It is relevant to mention that PKR is able to activate the two serine kinases that are important mediators of insulin resistance: JNK and IKKβ (10, 11). JNK is also activated in situations of ER stress which plays an important role in the development of insulin resistance and diabetes. In this regard, PKR also has an important role in ER stress, and the blockade of PKR activity protects against ER stress (12), suggesting that PKR may, at least in part, integrate the mechanisms of insulin resistance. Nakamura et al. (8) showed that PKR can respond to nutrient signals as well as ER stress and coordinate the activity of other critical inflammatory kinases, such as JNK, to regulate insulin activity and metabolism, suggesting that PKR is a critical component of the inflammatory complex that responds to nutrients and organelle dysfunction (8).

However, the modulation of this protein in human tissues in situations of insulin resistance has not yet been investigated. The present study was performed to first determine the tissue expression and phosphorylation levels of PKR in the liver, muscle and adipose tissue in obese

humans, and also the modulation of this protein in adipose tissue in obesity after bariatric surgery, which is an effective treatment for morbid obesity.

Patients and Methods

Patients. Eleven obese subjects (mean BMI 45.4 ± 3.6 kg/m^2; 2 male and 9 female) who were scheduled to undergo Roux-en-Y Gastric Bypass surgery (RYGBP) participated in this study. We also included nine apparently healthy lean subjects as a control group (mean BMI 24.2 ± 2.8 kg/m^2, 3 male and 6 female). The study was approved by the Institutional Ethics Review Board at the State University of Campinas. All subjects completed a comprehensive medical evaluation. No subjects were diabetic, had other metabolic diseases, or were taking medications that interfere with insulin activity or glucose and fatty acid metabolism.

RYGBP. All RYGBP procedures were performed by the same surgeon (J. C. P.) using standard surgical techniques. All patients had an open RYGBP procedure, which involved constructing a small (~20 ml) proximal gastric pouch by stapling across stomach. A 150-cm Roux-Y limb was constructed by transecting the jejunum 30 cm distal to the ligament of Treitz and creating a jejunojejunostomy 150 cm distal to the transection.

Tissue extraction and procedures. Liver tissue samples, muscle from the rectus abdominal and subcutaneous and visceral adipose tissue samples were obtained during RYGBP surgery, and in the obese group subcutaneous abdominal adipose tissue was also obtained by percutaneous needle biopsy 6 months after RYGBP surgery and were immediately frozen in liquid nitrogen. Evaluations were performed at baseline and 6 months after surgery for insulin sensitivity, biochemistry, anthropometric measures, and body composition by electric bioimpedance (BiodynamicsCorp., Seattle, WA, USA).

Euglycemic-hyperinsulinemic clamp studies. To quantify insulin sensitivity, a 180-min euglycemic-hyperinsulinemic clamp (13) was performed. Insulin sensitivity was calculated as the glucose infusion rate (GIR) in the last 60 min (steady state), corrected for the glucose distribution space and adjusted to fat-free mass, resulting in the M value. Other indices of insulin sensitivity were

calculated from the M value: the glucose metabolic clearance rate (MCRg = M/steady state glycemia) and M adjusted for steady-state insulin (M/I).

Assays. Serum samples were analyzed for basal and steady-state insulin levels (Bayer Corp., Tarrytown, NY, USA) as well as leptin, ultrasensitive C-reactive protein (us-CRP) and adiponectin by ELISA (R&D Systems Inc., Minneapolis, MN, USA).

Protein analysis by immunoblotting. The liver, muscle and adipose tissue fractions were homogenized in specific extraction buffer. Whole tissue protein extracts were resolved by SDS-PAGE and immunoblotted with specific antibodies. Immunoreactive bands were detected by the enhanced chemiluminescense method (Supersignal West Pico Chemiluminescent Substrate, Thermo Scientific Inc., Rockford, IL, USA). Blots results are presented as direct comparisons of bands in autoradiographs and quantified by optical densitometry (UN-SCAN-IT gel^{TM}, Silk Scientific Inc., Orem, UT, USA).

Statistical analysis. Results were analyzed by Wilcoxon signed-rank tests to compare baseline to post surgery data. Data are presented as mean ± SD (SPSS 12; SPSS Inc., Chicago, IL, USA). Statistical significance, was set at $P < 0.05$.

Results

Anthropometric and metabolic characteristics of obese patients before and after bariatric surgery.

The anthropometric and metabolic data of controls and obese humans are presented in Table 1. The metabolic data of the obese patients clearly show that they presented insulin resistance, characterized by a marked reduction in M during the glucose-clamp (Table 1). The obese patients also presented an increase in leptin and us-CRP and reduced adiponectin levels. Six months after bariatric surgery, the patients showed a marked reduction in BMI and a complete reversion of metabolic parameters and hormone levels. The glucose intolerance observed in three patients before surgery was also reversed (data not shown).

Effect of obesity on PKR, JNK and IRS-1 serine phosphorylation.

In order to investigate whether the modulation of PKR and JNK observed in the tissues of obese mice could also be observed in humans, we evaluated the modulation of these proteins in the liver, muscle and subcutaneous and visceral adipose tissue of obese humans, and also in the tissues of lean subjects. We also investigated the modulation of these proteins in the adipose tissue of obese humans before and 6 months after bariatric surgery.

In insulin resistance, there is a clear increase in PKR phosphorylation, which is accompanied by an increase in JNK, c-jun and IRS-1 Ser^{312} phosphorylation in the muscle and liver. There was also a clear increase in PKR, JNK and IRS-1 serine phosphorylation in the visceral and superficial adipose tissue in obese patients and, by comparison, no clear differences were observed in the modulation of these proteins between these two adipose tissues in obesity (Figure 1A-C).

Effect of bariatric surgery on PKR, JNK and IRS-1 serine phosphorylation in subcutaneous adipose tissue.

Before surgery, there was an increase in the PKR protein level and a more marked increase in the PKR phosphorylation level associated with an increase in JNK and IRS-1 Ser^{312} phosphorylation levels. These alterations in tissue protein and phosphorylation levels were completely reversed at 6 months after bariatric surgery (Figure 1D).

Discussion

Our data show that the PKR, originally identified as a pathogen sensor and a proposed regulator of the innate immune response against viral infections in higher eukaryotes, is activated in the liver, muscle and adipose tissue of obese humans. Furthermore, after bariatric surgery, there is a clear reduction in PKR activation in the adipose tissue of obese individuals.

One important point in the relationship between insulin resistance and inflammatory pathways is related to the integration of signals from different molecules with similar biological functions. In this regard, the study by Nakamura et al. (8) and our study suggest that PKR may have an important role in coordinating the relationships between inflammatory pathways and insulin activity. Our data show that, in the liver, muscle, subcutaneous and visceral adipose tissue of obese humans, there was an increase in PKR phosphorylation, in parallel with an increase in JNK and c-Jun phosphorylation and in IRS-1 serine phosphorylation. Interestingly, in obese patients submitted to bariatric surgery, 6 months after surgery, we showed a clear reduction in PKR, JNK and IRS-1 serine phosphorylation in the adipose tissue, suggesting that the downmodulation of these two kinases is accompanied by an improvement in insulin sensitivity. Recently it was demonstrated that in addition to JNK, PKR is also able to bind and induce IRS-1 serine phosphorylation (8). Serine phosphorylation of IRS-1 has been proposed as a molecular mechanism of attenuated insulin signaling, and Ser^{312} phosphorylation has become a molecular indicator of insulin resistance. Thus, we may suggest that alterations in insulin sensitivity and glucose metabolism in obese humans may be, at least in part, due to PKR activity. PKR also has an important role in ER stress, which has an impact on the development of insulin resistance and diabetes; the blockade of PKR activity protects against ER stress (8, 12). In this regard, at least in liver and adipose tissue, tissues in which ER stress plays an important role in insulin resistance in obesity, PKR and ER stress may integrate the molecular mechanisms of insulin resistance.

Skeletal muscle insulin resistance is a well-known metabolic complication of obesity affecting glucose and fatty acid metabolism. In the past few years, several groups have demonstrated an increase in JNK activity in the skeletal muscle in obesity (14, 15). As previously mentioned in the liver and adipose tissue, the activation of JNK in obesity is related, at least in part, to an increase in ER stress (16). However, it is well established that obesity does not induce ER stress in muscle in humans (7, 17, 18). In this regard, the activation of JNK may be related to different pathways, but certainly PKR may have an important role in JNK activity in the muscle of obese patients.

In addition to dsRNA, PKR is also activated by TLR1, TLR3, TLR4, TNFR, fatty acids and ceramides (19, 20). The activation of PKR by different stimuli suggests that PKR may have an important role in other situations of insulin resistance and may have a determining role in the metabolic abnormalities that emerge during the course of many infections (8). In this regard, the activation of PKR seems to be a common mechanism of insulin resistance, since PKR can sense nutrients, infections and probably other metabolic products produced during metabolic or ER stress.

In summary, our data show that PKR may represent a central mechanism for the integration of the pathogen response and innate immunity with insulin action and metabolic pathways that are critical in obesity in humans. The potential for PKR as a target for new therapeutic approaches against insulin resistance deserves further exploration.

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The authors reported no potential conflicts of interest relevant to this article.

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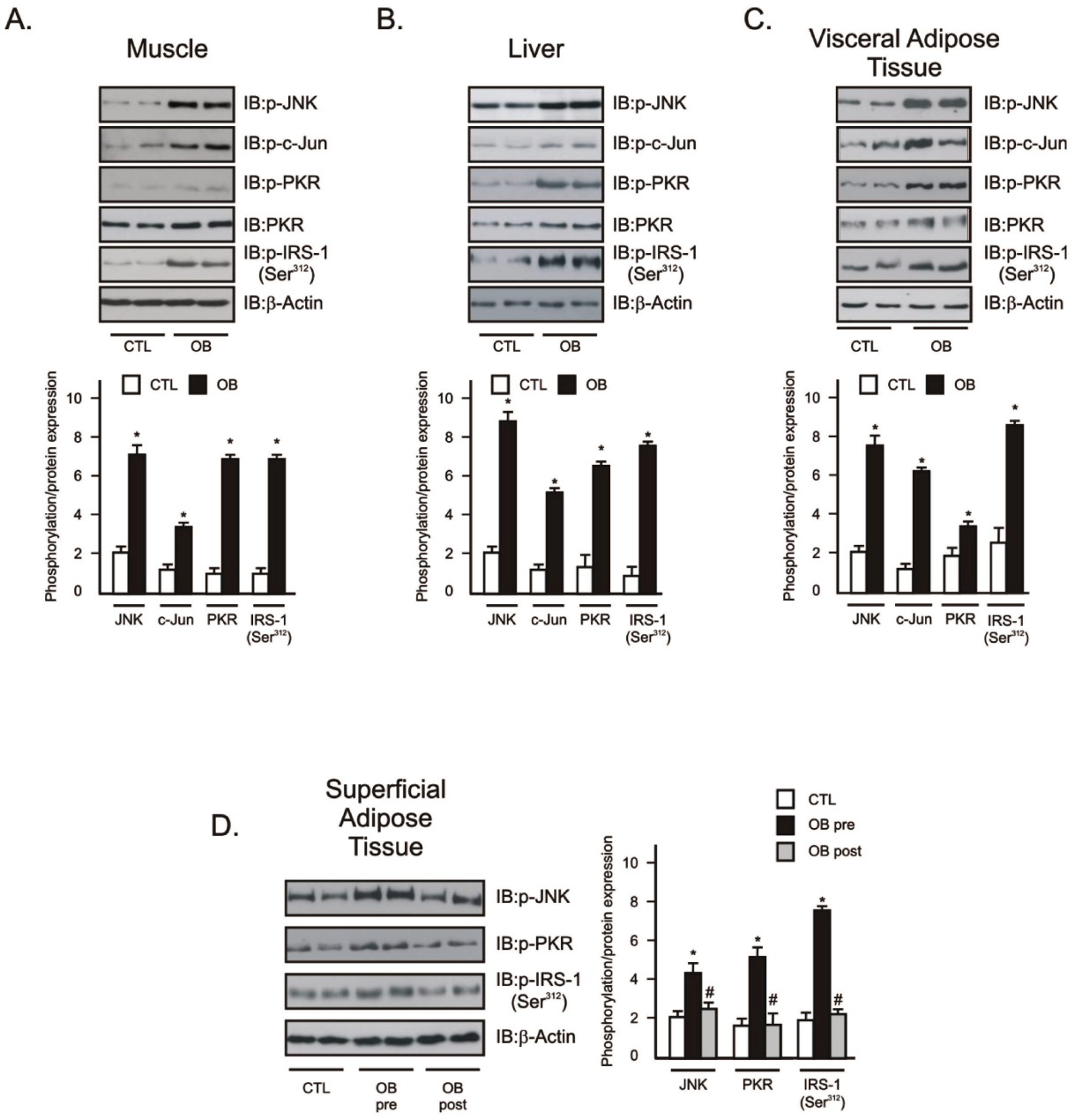
Figure 1 - Obesity consequences over JNK, PKR, c-Jun and IRS-1 Serine^{312} phosphorylation in the muscle, liver and visceral adipose tissue and its reversal in superficial adipose tissue in humans submitted to bariatric surgery. (A-C) Inflammatory profile in the muscle, liver and visceral adipose tissue of control and obese humans. Graphs represent the phosphorylation/protein expression of JNK, c-jun, PKR and IRS-1^{Ser312} in the muscle, liver and visceral adipose tissue of control and obese humans. Error bars indicate SEM. $P < 0.01$. * vs. CTL. (D) Phosphorylation of JNK, PKR and IRS-1 serine^{312} in the superficial adipose tissue of humans before and 6 months after bariatric surgery. Phosphorylation/protein expression of JNK, PKR and IRS-1^{Ser312} in the superficial adipose tissue of humans before and 6 months after bariatric surgery. Error bars indicate SEM. $P < 0.01$. * vs. CTL; # vs. OB pre.

Table 1

	Age (years)	BMI (kg/m^2)	Glucose (mg/dL)	M-clamp (mg/kg.FFM/min)	Leptin (ng/ml)	Adiponectin (ng/dl)	us-CRP (mg/dl)
LEAN	37.7 ± 8.1	24.2 ± 2.8	74 ± 9	40 ±15	16 ± 5	6.7 ± 2.3	0.23 ± 0.09
OBESE PRE	35.4 ± 6.5	45.4 ± 3.6*	98 ± 10*	27 ± 7*	70 ± 36*	4.9 ± 1.3*	1.37 ± 0.16*
OBESE POST	36.1 ± 6.6	31.0 ± 4.1#	77 ± 7#	42 ±12#	21 ± 16#	6.2 ± 1.4#	0.33 ± 0.07#

Results are expressed as mean ± SD. P < 0,05, * versus Lean, # versus Obese pre.

Figure 1


A.
Muscle
IB:p-JNK
IB:p-c-Jun
IB:p-PKR
IB:PKR
IB:p-IRS-1 (Ser312)
IB:β-Actin
CTL
OB
Phosphorylation/protein expression
JNK
c-Jun
PKR
IRS-1 (Ser312)
B.
Liver
C.
Visceral Adipose Tissue
D.
Superficial Adipose Tissue
OB pre
OB post