ANGÉLICA C. A. CAMACHO PEREIRA

“EFEITO DE UM BLOQUEADOR DO RECEPTOR PDGF NA ADIPOGÊNESE DE CAMUNDONGOS TRATADOS COM DIETA HIPERLIPÍDICA”

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Mario José Abdalla Saad [Orientador]

Marco Antonio de Carvalho Filho

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"EFEITO DE UM BLOQUEADOR DO RECEPTOR PDGF NA ADIPOGÊNESE DE CAMUNDONGOS TRATADOS COM DIETA HIPERLIPÍDICA"

Angélica Costa Aranha Camacho Pereira

Dissertação de Mestrado apresentada à Pós-Graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas, para obtenção do título de Mestre em Ciências. Sob orientação do Prof. Dr. Mario José Abdalla Saad.

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Banca examinadora de Dissertação de Mestrado

Angélica Costa Aranha Camacho Pereira

Orientadora(a): Prof(a). Dr(a). Mario José Abdalla Saad

Membros:
Prof(a). Dr(a). Mario José Abdalla Saad -
Prof(a). Dr(a). Marco Antonio de Carvalho Filho-
Prof(a). Dr(a). Carla Roberta de Oliveira Carvalho -

Curso de pós-graduação em Fisiopatologia Médica da Faculdade de Ciências Médicas da Universidade Estadual de Campinas.

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SUMÁRIO

LISTA DE ABREVIATURAS

Akt/PKB	Proteína quinase B
BSA	albumina sérica bovina
CD29	Integrina beta-1
CD34	*Cluster of differentiation 34*
cDNA	DNA complementar
C/EBPα	Proteína α ligante ao amplificador CCAAT
CHOP	*C/EBP homologous protein*
DNA	Ácido desoxirribonucléico
DM2	Diabetes Mellitus tipo 2
DTT	ditiotritol
EDTA	Ácido etilenodiaminotetracético
GTT	*Glucose Tolerance Test*
IL-6	Interleucina-6
IRS-1	Substrato 1 do receptor de insulina
IR	Receptor da insulina
ITT	*Insulin Tolerance Test*

KLFs	Fatores semelhantes ao Krupel
mRNA	Ácido ribonucléico mensageiro
PDGFRβ	Receptor β de fatores de crescimento derivados de plaquetas
PPAR-γ	Receptor γ ativado por proliferador de peroxissomas
RNA	Ácido ribonucléico
RT- PCR	Reação em cadeia da polimerase- *Real Time*
SREBP-1c	Proteína 1 ligadora do elemento regulado por esteróis
TAG	Triacilglicerol
TNF-α	Fator de necrose tumoral alfa
SDS-PAGE	Eletroforese em gel de poliacrilamida com dodecil sulfato de sódio
Wnt	Proteínas Wingless e INT-1

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RESUMO

A obesidade é hoje considerada um problema de saúde pública. Essa condição é caracterizada pelo aumento do peso corporal, mais especificamente do tecido adiposo branco.

A adipogênese (diferenciação do pré-adipócito em adipócito) é um fenômeno complexo e não muito bem caracterizado. Recentes estudos mostraram que os pré-adipócitos estão localizadas nas paredes dos vasos que irrigam o tecido adiposo. Estas células estão presentes exclusivamente neste tecido e expressam alguns marcadores, dentre eles o PDGFRβ.

O PDGFRβ é um receptor tirosina quinase cujo papel na migração, proliferação e diferenciação de diversos tipos celulares tem sido extensivamente estudado.

O AG1296 (6,7- dimetoxi-2-fenil-quinoxalina) é um potente inibidor do receptor PDGF, pertencente à classe da quinoxalinas.

Deste modo, levando-se em consideração o papel do receptor PDGF no crescimento e proliferação celulares e o fato de que as células PDGFRβ positivas provenientes do tecido adiposo possuem alto potencial adipogênico, neste estudo investigamos o efeito do AG1296 na adipogênese de camundongos submetidos à dieta hiperlipídica e à dieta padrão. Nós também investigamos se essa inibição afetaria a sensibilidade à insulina desses grupos estudados.

Para tanto, camundongos *Swiss* machos com seis semanas de vida foram divididos em quatro grupos: o grupo Controle que recebeu dieta padrão, o grupo C+AG1296 que recebeu dieta padrão e tratamento com AG1296, o grupo DH que recebeu dieta hiperlipídica somente e o grupo DH+AG1296 que recebeu dieta hiperlipídica e tratamento com AG1296. Peso corpóreo e ingestão alimentar foram

medidos diariamente durante o tratamento (7 ou 15 dias). Através de Western blot, foram quantificadas as principais proteínas pró-adipogênicas (SREBP-1c, C/EBPα e PPARγ) e a fosforilação das principais proteínas da via da insulina (IR, IRS1 e AKT).

Nossos resultados indicaram que nos animais controle, após 15 dias de tratamento com AG1296, houve uma redução nas três frações de tecido adiposo, associada a uma redução em algumas das proteínas adipogênicas, além de uma melhora na sinalização insulínica em fígado e músculo e uma redução na glicemia de jejum.

Além disso, nos animais submetidos à dieta hiperlipídica, após 7 dias de tratamento com AG1296, foi possível observar uma redução nas proteínas adipogênicas e uma redução na fração epididimal do tecido adiposo. Houve também uma melhora na sinalização insulínica e na tolerância à glicose.

Com isso, podemos sugerir que a inibição do PDGFRβ pode ter um papel importante na adipogênese e na sinalização insulínica e pode ser um alvo potencial para prevenção da obesidade e resistência à insulina.

Palavras-chave: Adipogenia, Receptor Tipo beta para Fator de Crescimento Derivado de Plaquetas, Resistência à Insulina

ABSTRACT

Obesity can be defined as a disease in which body fat is excessively accumulated. Adipogenesis is a complex and not completely known phenomenon.

Recent studies showed that adipocyte progenitor cells are exclusively found in adipose tissue and express some markers like PDGFRβ (Platelet-derived growth factor β).

AG1296 (6,7-dimethoxy-2-phenyl-quinoxaline) is a potent and selective inhibitor of PDGF receptor kinase.

In this context, the main objective of this work was to investigate if the inhibition of PDGF receptor through AG1296 would be able to affect white adipose tissue generation in high-fat-diet-fed and standard-chow-fed mice. We also investigated if this inhibition would have an effect on the insulin sensitivity in these studied groups.

For this purpose, six-week-old male *Swiss* mice were divided into four groups and assigned to receive the following diet and/or treatment: the control group (C) received standard rodent diet, the second group (C + AG1296) received standard rodent diet plus AG1296 (50 mg/Kg/day by gavage), the third group (HFD) received high fat diet (55% calories from fat, 29% calories from carbohydrate and 16% from protein) and the fourth group (HFD+AG1296) received high fat diet plus AG1296. Body weight and food intake were measured during the treatment (7 and 15 days). After that, tissues (epididymal, retroperitoneal and mesenteric adipose tissue, liver and muscle) were extracted and processed.

Through *Western blot* analysis, we were able to quantify the main proteins related to adipogenesis (SREBP-1c, C/EBPα e PPARγ) and the phosphorylation of the main proteins from insulin pathway (IR, IRS1 and Akt).

Our results indicated that on control mice, after 15 days of treatment with AG1296, there was a reduction on adipose fat pad, associated with reduction in some adipogenic proteins, an increase in insulin signaling in liver and muscle and a reduction in fasting plasma glucose.

Futhermore, on mice fed a high fat diet, after 7 days of treatment with AG1296, it was possible to observe a reduction on adipogenesis proteins and a reduction in epididymal fat pad. Also, there was an improvement in insulin signaling pathway and in glucose tolerance.

In conclusion, our results suggest that PDGFRβ inhibition might have an important role in adipogenesis and in insulin signaling and could be a potential target for preventing obesity and insulin resistance.

Key words: Adipogenesis; Receptor, Platelet- derived growth factor receptor beta; Insulin resistance

INTRODUÇÃO

1-INTRODUÇÃO

Obesidade

Atualmente, a obesidade tem atingido índices alarmantes no mundo todo, o que nos leva a crer que estamos em meio a uma epidemia com conseqüências devastadoras para a saúde pública. Projeções da Organização Mundial de Saúde (OMS) apontam que no ano de 2025, mais de 50% da população será composta de indivíduos obesos nos Estados Unidos e, no Brasil, esta será de mais de 25% dos indivíduos adultos (Kopelman, 2000).

Essa condição é definida cientificamente como um aumento do peso corporal, especificamente do tecido adiposo, de tal magnitude, que causa sérios danos ao organismo (Spiegelman and Flier, 2001).

A obesidade pode ser o fator determinante no desenvolvimento de algumas doenças muito comuns na atualidade, como: diabetes tipo 2, dislipidemias, doenças cardiovasculares, Doença de Alzheimer, e até alguns tipos de câncer (Sunyer and Xavier, 2002; Grundy, 2004).

Na obesidade, o aumento do tecido adiposo induz a secreção de vários tipos de hormônios (como, por exemplo, leptina e adiponectina), citocinas (IL-6, TNFα, dentre outras) e outras proteínas (como adipsina) que podem modular a homeostase energética (Hotamisligil et al, 1993; Hotamisligil, 2003; Warne, 2003; Ahima RS and Flier JS, 2000; Kershaw and Flier, 2004; Kern et al., 2003; Wisse, 2004). Algumas dessas substâncias possuem efeitos sistêmicos e podem causar resistência à insulina em várias células e tecidos responsivos a esse hormônio (Bastard et al, 2006). O TNFα, por exemplo, age estimulando a fosforilação do IRS-1 em serina, reduzindo sua fosforilação em tirosina em resposta à insulina e a habilidade deste de

se associar ao receptor de insulina (IR), atenuando a cascata de sinalização e ação da insulina (Hotamisligil, 2003; Wellen and Hotamisligil, 2005).

Hoje se sabe que a obesidade é caracterizada por um estado inflamatório crônico, sub-clínico, do tecido adiposo branco, resultado da ativação do sistema imune inato, que pode subseqüentemente levar à resistência à insulina, intolerância à glicose e diabetes tipo 2 (Xu et al, 2003). Essa condição é caracterizada por uma intensa infiltração de macrófagos no tecido adiposo branco. Esses macrófagos são a principal fonte de citocinas pró-inflamatórias TNFα e IL-6 que contribuem para o desenvolvimento de resistência à insulina em órgãos como músculo e fígado. Além disso, na obesidade, ocorre um aumento dos níveis de leptina, que pode regular e ativar a produção de TNFα pelos macrófagos. Ocorre também uma redução da produção de adiponectina pelos adipócitos. Essa adipocina tem o efeito de reduzir a secreção de TNFα pelos macrófagos, possuindo um efeito antiinflamatório (Figura 1) (Hotamisligil, Arner et al. 1995, Bastard et al, 2006).

Tecido Adiposo

O número de células do tecido adiposo apresenta um aumento significativo durante a infância (Prins and O'Rahilly, 1997) porém, esse número se estabiliza na idade adulta, embora essas células sofram uma constante renovação (Spaldin et al, 2008). Indivíduos com obesidade na fase adulta apresentam aumento no tamanho dos adipócitos, enquanto a obesidade apresentada na juventude é resultado de ambos, hipertrofia (um aumento no tamanho dos adipócitos) e hiperplasia (um aumento no número de células) (Hirsch and Batchelor, 1976; Lane and Tang, 2005).

Esse conjunto de adipócitos, que formam o tecido adiposo, é o principal reservatório energético do organismo. Os adipócitos são as únicas células

especializadas no armazenamento de lipídios na forma de triacilglicerol (TAG) em seu citoplasma, sem que isto seja nocivo para sua integridade funcional. Essas células possuem todas as enzimas e proteínas reguladoras necessárias para sintetizar ácidos graxos (lipogênese) e estocar TAG em períodos em que a oferta de energia é abundante, e para mobilizá-los pela lipólise quando há déficit calórico (Frayn et al, 2003).

Além dos adipócitos, o tecido adiposo contém uma matriz de tecido conjuntivo (fibras colágenas e reticulares), tecido nervoso, células do estroma vascular, nódulos linfáticos, células imunológicas (leucócitos, macrófagos), fibroblastos e pré-adipócitos (células adiposas indiferenciadas) (Kershaw and Flier, 2004).

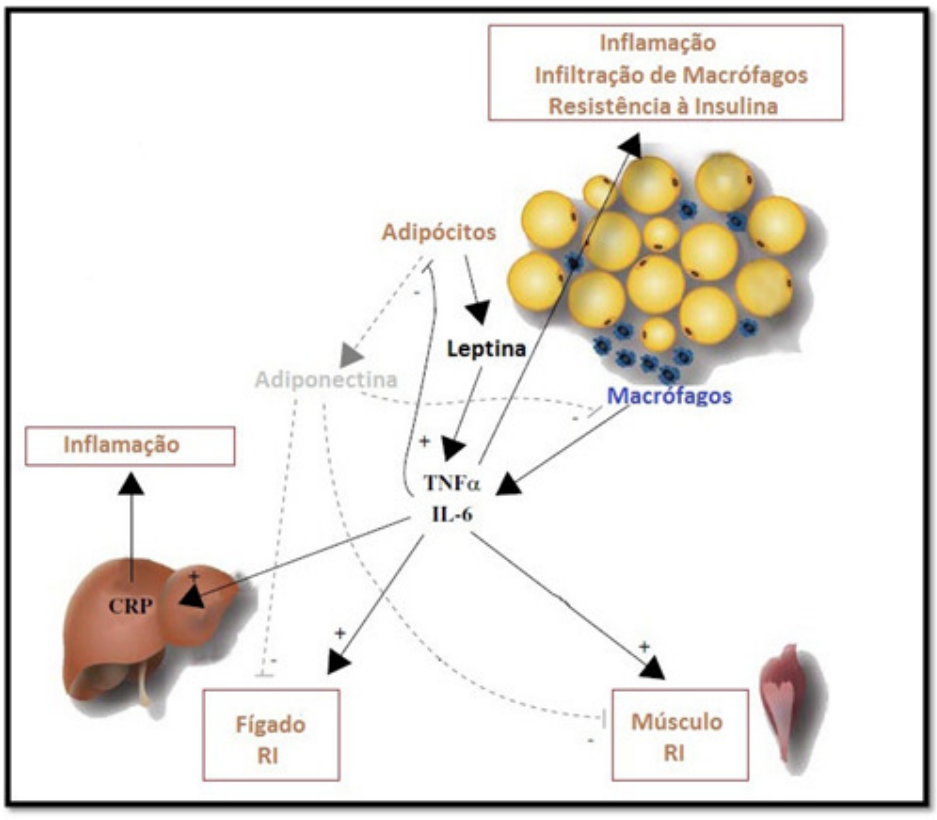

Figura 1: Relação entre Obesidade, Inflamação e Resistência à Insulina. Adaptado de Adaptado de Bastard JP et al, 2006.

Adipogênese

A adipogênese inicia-se antes do nascimento. O tecido adiposo, assim como o muscular e o ósseo, tem origem mesodérmica, a partir de células-tronco mesenquimais (Konieczny and Emerson, 1984; Jiang Y et al, 2002; Otto and Lane; 2005).

Células troco mesenquimais são células indiferenciadas que podem dar origem a diversos tipos de células como adipócitos, osteoblastos, mioblastos e tecido conectivo dependendo do estímulo a que são submetidas (Otto and Lane, 2005). Acredita-se que essas células originam um precursor (adipoblasto), que se desenvolve em pré-adipócitos já predestinados a produzirem adipócitos do tecido branco ou marrom. A transição de pré-adipócito para adipócito envolve quatro estágios: crescimento, expansão clonal, diferenciação prematura e diferenciação terminal (Gesta et al, 2007).

A diferenciação do pré-adipócito em adipócito é um processo altamente controlado. Fatores de transcrição adipogênicos, incluindo o PPARγ (receptor gama ativado por proliferadores de peroxissomas), a SREBP-1c (proteína 1c ligadora do elemento regulado por esteróis) e as C/EBP (proteinas ligantes ao amplificador CCAAT) desempenham papel-chave na complexa cascata transcricional da adipogênese. Sinais hormonais e nutricionais afetam a diferenciação do adipócito positiva ou negativamente, e componentes envolvidos na interação célula-célula ou na matriz celular também são importantes na regulação do processo (Gregoire et al, 1998). Muitos outros fatores pró-adipogênicos e anti-adipogênicos também estão envolvidos nesta complexa cascata de eventos (dentre eles as wnt e β–cateninas,KLFs e outras) assim como está sendo elucidada a maneira como eles

se inter-relacionam e afetam a formação do tecido adiposo (Lefterova and Lazar, 2009).

A família C/EBP consiste em 5 diferente membros: C/EBPα, C/EBPβ, C/EBPδ, C/EBPγ e CHOP. Durante a adipogênese, ocorre a transcrição seqüencial destes fatores, sendo que a transcrição de C/EBPβ e C/EBPδ promovem a expressão de C/EBPα e PPARγ (Farmer, 2006).

Estudos comprovaram que C/EBPα é necessária para formação do tecido adiposo branco (Linhart et al, 2001). Apesar de ambos, C/EBPα e PPARγ controlarem a diferenciação dos adipócitos, PPARγ parece ser dominante neste processo. O receptor PPARγ tem, portanto, um papel fundamental na adipogênese e na manutanção do estado diferenciado do adipócito maduro. Estudos envolvendo indução de knockout dos receptores PPARγ nos adipócitos maduros *in vivo* levaram a morte de ambos os tecidos adiposos branco e marrom (Gesta et al, 2007).

O PPARγ pertence a uma superfamília de receptores nucleares. É altamente expresso no tecido adiposo e estimula a transcrição de muitos genes específicos do adipócito, assim como os passos iniciais críticos da adipogênese (Tontonoz et al, 1995).

Existem duas isoformas de PPARγ (PPARγ-1 e -2) geradas por promotores distintos e mecanismos alternativos de *splicing*. O PPARγ-1 está presente nos adipócitos e em menor proporção em várias outras células (macrófagos, pneumócitos, epitélio do cólon, etc.). O PPARγ-2 é exclusivo do tecido adiposo (Rosen and MacDougald, 2006). Além de sua função na adipogênese, esse receptor parece ter grande importância na sensibilidade à insulina em todos os tecidos (Sharma and Staels, 2007, Camp et al, 2002).

Os C/EBP pertencem à família b-zip (domínio básico de ligação do DNA), que contém um *zipper* de leucina necessário para a dimerização. O C/EBPβ também induz adipogênese, possivelmente por estimular a expressão do PPARγ, cujo gene contém sítios para C/EBP na região promotora. C/EBPα e PPARγ se ligam à região promotora e ativam genes específicos do tecido adiposo branco, levando a ativação da cascata da adipogênese (Fonseca-Alaniz et al, 2007; Lane et al, 1999). Essa inter-relação entre C/EBPα e PPARγ parece ser de fundamental importância para o controle transcricional da adipogênese e também para a sensibilidade à insulina nos adipócitos, já que estudos comprovaram que fibroblastos provenientes de camundongos knockout para C/EBPα produzem adipócitos defeituosos, que acumulam menos lipídios e apresentam ausência de transporte de glicose estimulada por insulina e tem expressão gênica e fosforilação de IR e IRS-1 reduzidos (Wu et al, 1999).

O fator de transcrição SREBP-1c é altamente expresso no tecido adiposo e tem papel fundamental na formação dos adipócitos. Essa proteína é capaz de induzir o PPARγ e também um ligante endógeno deste fator de transcrição, além da expressão de vários genes críticos na biossíntese de lipídios. Estudos revelaram que ele é regulado pelos fatores de transcrição C/EBPβ e C/EBPδ inicialmente e posteriormente pelo C/EBPα durante a adipogênese (Payne et al, 2009).

Em resumo, o controle transcricional da adipogênese envolve a ativação de várias famílias de fatores de transcrição. Estas proteínas são expressas em uma cadeia na qual C/EBPβ e C/EBPδ são detectados primeiro, seguidos pelo PPARγ, que por sua vez ativa a proteína C/EBPα e um gama de outras proteínas envolvidas na complexa cadeia da adipogênese. C/EBPα exerce um feedback positivo na proteína PPARγ, para manter o estado de diferenciação. SREBP-1c é regulada

pela insulina no tecido adiposo e pode ativar PPARγ pela indução da sua expressão assim como pela expressão de um ligante endógeno deste receptor. SREBP-1c também é capaz de ativar muitos genes da lipogênese. Todos esses fatores contribuem para a expressão de genes que vão caracterizar o fenótipo do adipócito maduro (Figura 2) (Spiegelman and Flier, 2001).

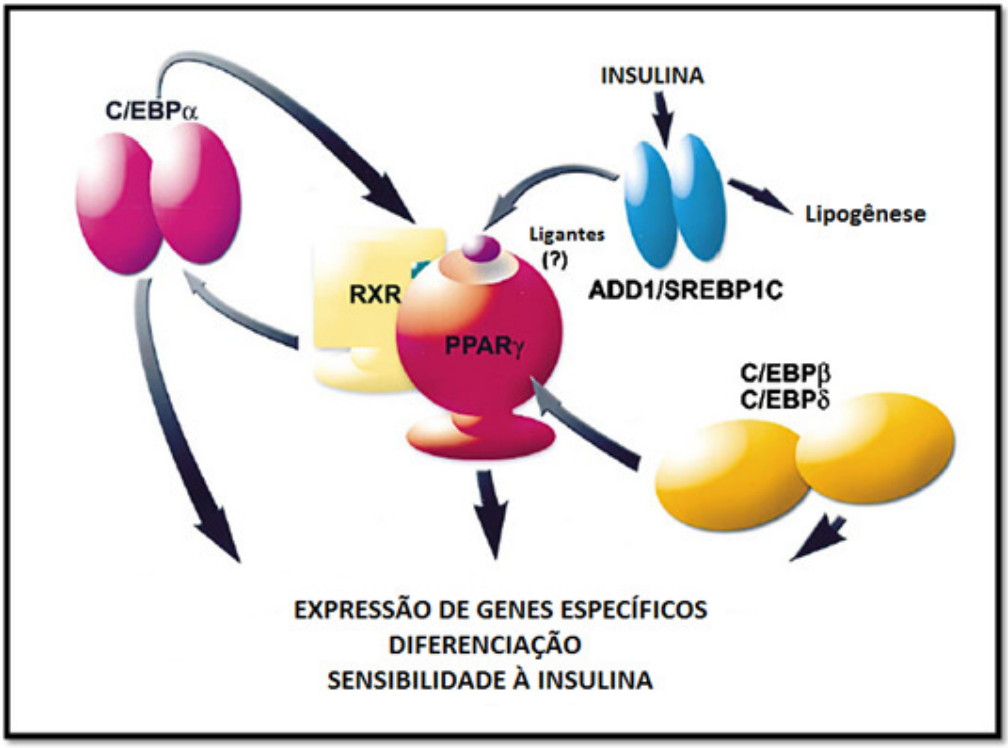

Figura 2: Controle Transcricional da Adipogênese. Adaptado de Spiegelman BM and Flier JS, 2001

Até há pouco tempo, não se sabia muito a respeito da natureza e da localização das células precursoras dos adipócitos. Neste contexto, dois estudos começaram a responder a esses questionamentos (Rodeheffer et al, 2008; Tang et al, 2008) . Utilizando as técnicas de citometria de fluxo em combinação com transplante de células *in vivo*, identificaram-se as células precursoras dos adipócitos no estroma vascular do tecido adiposo branco. Foram utilizados marcadores já

conhecidamente expressos em células precursoras de outros tecidos. Primeiramente, utilizaram-se os marcadores CD29 e CD34. Mostrou-se que somente as células $CD29^+$ e $CD34^+$ tinham potencial adipogênico. Posteriormente, utilizando-se as mesmas técnicas e outros marcadores (Sca 1 e $CD24^+$) determinou-se que as células $CD29^+$: $CD34^+$: Sca 1: $CD24^+$ (0,08% do total de células do estroma vascular) eram adipogênicas e após serem transplantadas, in vivo, em camundongos lipodistróficos, formaram um tecido adiposo normal e funcional (Rodeheffer et al, 2008).

Além disso, sabe-se, no momento, que as células progenitoras dos adipócitos estão localizadas nas paredes dos vasos que irrigam o tecido adiposo. Utilizando camundongos manipulados geneticamente, foi demonstrado que as células progenitoras dos adipócitos são pericitos (células que envolvem os capilares sangüíneos). Estas células estão presentes exclusivamente no tecido adiposo e expressam alguns marcadores, dentre eles o PDGFRβ. As células $PDGFR\beta^+$ provenientes do tecido adiposo branco tiveram maior potencial adipogênico do que as células negativas para este receptor (Tang et al, 2008). Essa adipogênese foi estimulada por tiazolidinedionas, que são drogas antidiabéticas que ativam o PPARγ, muito utilizadas no tratamento do diabetes tipo 2 (Mundaliar and Henry, 2001). Em contraste, células $PDGFR\beta^+$ isoladas de outros órgãos não promoveram adipogênese na mesma proporção que as provenientes do tecido adiposo e não foram responsivas as tiazolidinedionas (Tang et al, 2008).

PDGFR e seus inibidores

PDGF (fatores de crescimento derivados de plaquetas) e seus receptores são bastante conhecidos por seu papel no crescimento celular e fisiopatologia de vários tipos de câncer.

Esses receptores foram inicialmente identificados em fibroblastos células da glia e células do músculo esquelético. Atualmente, sabe-se que muitos outros tipos de células expressam esse receptor (Raines and Ross, 1993).

Existem dois tipos de receptores PDGF (α e β). O PDGFRβ possui 1106 resíduos de aminoácidos e um único domínio transmembrana localizado no meio do polipeptídio. Seu domínio extracelular pode ser dividido em cinco subdomínios e a porção intracelular possui três regiões: o domínio justamembrana, o domínio tirosina quinase e um domínio C-terminal (Claesson-Welsh, 1996).

Quando da ligação da molécula de PDGF, esse receptor sofre dimerização (Heldin, 1995). Essa dimerização do receptor tirosina quinase é seguida pela sua autofosforilação que ocorre principalmente através da fosforilação de uma molécula do receptor pela outra no dímero formado (UIlrich and Schlessinger, 1990).

A maior parte dos sítios de fosforilação dos receptores PDGF está localizada fora do domínio tirosina quinase. No receptor PDGFβ, um sítio de fosforilação, o Y857, foi identificado no domínio tirosina quinase e este parece regular sua atividade (Cooper and Kazlauskas, 1993).

Drogas que interfiram diretamente na fosforilação deste receptor (inibidores da PDGFR quinase) têm sido muito testadas ultimamente na terapia de vários tipos de câncer e têm mostrado bons resultados (Pietras et al. 2003)

O AG 1296 (6,7- dimetoxi-2-fenil-quinoxalina) é um potente inibidor da PDGFR quinase pertencente à classe da quinoxalinas (Figura 3) (Kovalenko et al,

1994). Essa substância não interfere com a ligação do PDGF ao seu receptor, nem com a dimerização do mesmo, mas abole sua atividade tirosina quinase. Essa droga se liga ao domínio quinase do receptor PDGF quando este está ativado, em um sítio diferente daquele ao qual se liga a molécula de ATP, inibindo a reação catalítica (Kovalenko et al, 1997). As quinoxalinas são altamente seletivas para os receptores PDGF, já que estas se ligam a áreas que são exclusivas destes receptores (Levitzki, 2004).

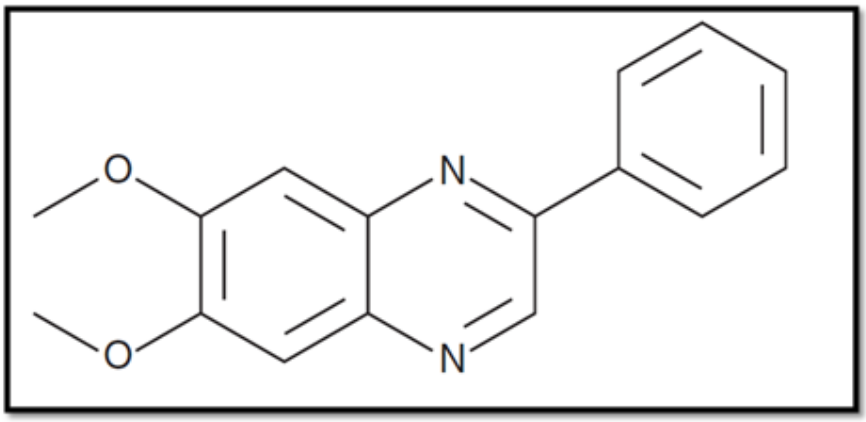

Figura 3: Estrutura Molecular do AG1296

Considerando-se que o receptor PDGFRβ foi identificado como um dos marcadores das células precursoras dos adipócitos, as drogas que agem, especificamente, inibindo esse receptor podem ter uma função importante na inibição da adipogênese e nas doenças que decorrem da formação do tecido adiposo, como é o caso da obesidade e das doenças associadas a ela.

OBJETIVOS

2-OBJETIVOS

Objetivo Geral:

- Investigar o efeito de um inibidor do receptor PDGF na adipogênese, através de estudos morfológicos e da expressão de proteínas em tecido adiposo epididimal, mesentérico e retroperitoneal de camundongos submetidos à dieta hiperlipídica.

Objetivos específicos:

Investigar o efeito de um inibidor do receptor PDGF:

- Na adipogênese in vitro através de cultura de 3T3-L1 pré-adipócitos.
- Na expressão das proteínas ligadas à adipogênese (SREBP-1c, C/EBPα e PPARγ) no tecido adiposo de camundongos tratados com dieta hiperlipídica e dieta padrão.
- Na sinalização insulínica (proteínas p-IR, p-IRS-1, p-AKT) em fígado, músculo e tecido adiposo de camundongos tratados com dieta hiperlipídica e dieta padrão.
- Nos níveis séricos de TNFα, IL-6, adiponectina e leptina de camundongos tratados com dieta hiperlipídica e dieta padrão.
- Na histologia do tecido adiposo de camundongos tratados com dieta hiperlipídica e dieta padrão.

CAPÍTULO

PDGFRβ KINASE INHIBITOR REDUCES ADIPOGENESIS AND IMPROVES INSULIN SENSITIVITY IN MICE

Angelica Camacho Pereira, Francine Mittestainer, Kelly Lima Calisto, Bruno M. Carvalho, Alexandre G Oliveira, Dioze Guadagnini, Joseane Morari, Renata A. Bagarolli, Licio Velloso, José Vassallo, José B C Carvalheira, Mario J A Saad

Department of Internal Medicine, State University o f Campinas, 13081-970, Campinas, SP, Brazil.

Please address correspondence to: Mario J. A. Saad, M.D., Departamento de Clínica Médica, FCM-UNICAMP, Cidade Universitária Zeferino Vaz, Campinas, SP, Brazil, 13081-970,

ABSTRACT

Obesity can be defined as a disease in which body fat is excessively accumulated. Adipogenesis is a complex and not completely known phenomenon.

Recent studies showed that adipocyte progenitor cells are exclusively found in adipose tissue and express some markers like PDGFRβ (Platelet-derived growth factor β).

AG1296 (6,7-dimethoxy-2-phenyl-quinoxaline) is a potent and selective inhibitor of PDGF receptor kinase.

In this context, the main objective of this work was to investigate if the inhibition of PDGF receptor through AG1296 would be able to affect white adipose tissue generation in high-fat-diet-fed and standard-chow-fed mice. We also investigated if this inhibition would have an effect on the insulin sensitivity in these studied groups.

For this purpose, six-week-old male *Swiss* mice were divided into four groups and assigned to receive the following diet and/or treatment: the control group (C) received standard rodent diet, the second group (C + AG1296) received standard rodent diet plus AG1296 (50 mg/Kg/day by gavage), the third group (HFD) received high fat diet (55% calories from fat, 29% calories from carbohydrate and 16% from protein) and the fourth group (HFD+AG1296) received high fat diet plus AG1296. Body weight and food intake were measured during the treatment (7 and 15 days). After that, tissues (epididymal, retroperitoneal and mesenteric adipose tissue, liver and muscle) were extracted and processed.

Through *Western blot* analysis, we were able to quantify the main proteins related to adipogenesis (SREBP-1c, C/EBPα e PPARγ) and the phosphorylation of the main proteins from insulin pathway (IR, IRS1 and Akt).

Our results indicated that on control mice, after 15 days of treatment with AG1296, there was a reduction on adipose fat pad, associated with reduction in some adipogenic proteins, an increase in insulin signaling in liver and muscle and a reduction in fasting plasma glucose.

Futhermore, on mice fed a high fat diet, after 7 days of treatment with AG1296, it was possible to observe a reduction on adipogenesis proteins and a reduction in epididymal fat pad. Also, there was an improvement in insulin signaling pathway and in glucose tolerance.

In conclusion, our results suggest that PDGFRβ inhibition might have an important role in adipogenesis and in insulin signaling and could be a potential target for preventing obesity and insulin resistance.

Key words: Adipogenesis, AG1296, PDGFRβ, Insulin resistance

INTRODUCTION

Obesity is considered nowadays an epidemic of dramatic proportions. The WHO estimated that in 2008, 1.5 billion adults aged 20 years and older were overweight and over 200 million men and 300 million women — approximately 10% of adults — were obese. In 2010, about 43 million children under five years of age were overweight (1).

This condition is characterized by an increase in body weight, specifically of white adipose tissue, in such a way that can undergo serious health problems (2). Among the obesity-related pathologies, the more important are type 2 diabetes, cardiovascular diseases, dyslipidemia and some kinds of cancer (3).

Adipose tissue is thought to be derived from the embryonic mesoderm which gives rise to mesenchymal stem cells that are the precursors of the adipoblasts. These cells in their turn give rise to the preadipocytes and lately to the mature adipocytes (4).

Adipogenesis (the process in which preadipocytes turn to mature adipocytes) is a complex cascade of events that involves the activation of several families of transcription factors (5). These proteins are expressed in a sequence where C/EBPβ and C/EBPγ are the first to be detected, followed by PPARγ which is the responsible to activate C/EBPα. This protein exerts a positive feedback in PPARγ, to maintain its activated state. Another protein that participates in this network is SREBP-1c that activates PPARγ directly or through the induction of an endogenous ligand. All these events are going to result in the mature adipocyte phenotype (6).

PDGFRβ is a tyrosine kinase receptor and binding of PDGF, its ligand, leads to activation of the kinase and autophosphorylation. This receptor and its ligand are known to induce migration, proliferation and differentiation of different cell types (7). It has been reported that preadipocytes express PDGFRβ which seems to be important for adipogenesis since PDGFRβ negative cells have small adipogenic potential (8).

AG1296 (6,7-dimethoxy-2-phenyl-quinoxaline) is a potent and specific inhibitor of platelet-derived growth factor receptor (9). This quinoxalin neither interferes with the ligand (PDGF) binding with the receptor nor has any effect on receptor dimerization but binds in areas outside the ATP binding domain in the PDGF receptor inhibiting the catalytic reaction (10, 11).

Since PDGF receptor β has important role in adipogenesis, the aim of this work was to investigate in vivo the effect of AG1296, a PDGFR β tyrosine kinase inhibitor, in adipogenesis and some aspects that are altered in an installed obesity state.

RESEARCH DESIGN AND METHODS

Materials

Male Swiss mice were obtained from University of Campinas, São Paulo. Mice were bred under specific pathogen-free conditions at the Central Breeding Center of University of Campinas (Brazil). All antibodies were from Santa Cruz Technology (Santa Cruz, CA), except anti-Akt, anti-phospo-Akt and anti-α-tubulin which were obtained from Cell Signaling Technology (Beverly, MA). Human recombinant insulin was from Eli Lilly and Co. (Indianapolis, IN). Tyrphostin AG1296 was obtained from Enzo Lifescience (Farmingdale, NY). Routine reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless specified elsewhere.

3T3-L1 cell culture and differentiation

3T3-L1 mouse embryonic fibroblasts were grown to 100% confluence. Adipogenesis was induced with a mixture of insulin (5 µg/ml), dexamethasone (0.25 µM), methylisobutylxanthine (0.5 mM) in Dulbecco's modified Eagle's (DMEM) supplemented with 10% fetal bovine serum (FBS) at days 2, 4 and 6. AG1296 was dissolved in dimethylsulfoxide and added to cell culture medium at concentrations of 0, 0.5, 1, 2 and 4 µM at the same days. From day 8 the induction medium was removed and replaced by DMEM containing 10% FBS, changed every other day. At all time, cells were maintained at 37°C in 5% of CO_2 atmosphere. All experiments, unless otherwise indicated, were performed at least in triplicate.

Oil Red O Staining

For Oil-Red O staining, cells were washed gently with PBS and fixed with 10% formaldehyde for 30 minutes. After being washed twice with water, cells were stained with Oil-Red O solution (60% isopropanol and 40% water) for at least 1 hour at room temperature.

Real Time RT-PCR

Total RNA was obtained from cells at day 4 of differentiation using RNeasy Micro Kit (Quiagen). Samples were submitted to DNase treatment to eliminate contamination. After that, 1 µg of each sample was submitted to reverse transcription reaction according to ThermoScript™ RT-PCR System (Invitrogen Life Technologies) protocol. Twenty ng of each cDNA sample were used in the reaction with 300nM of especific *primers* and 12,5µl de SYBR® Green Master Mix PCR (Aplied Biosystems). The following primers were used for amplification: *Mouse* PPARγ *forward*: 5'-AGGCCGAGAAGGAGAAGCTGTTG-3', *Mouse* PPARγ *reverse*: 5'-TGGCCACCTCTTTGCTCTGCTC-3', *Mouse* C/EBPα *forward:* 5'-CCTTGAGACCGAGAGACTTTCC-3', *Mouse* C/EBPα *reverse*: 5'-AGGAGCGAGTTTTGAGTATCCA-3'. Real-time detection of amplification was performed in an ABI 5700 Sequence Detector System (Applied Biosystems). GAPDH expression was used as endogenous control. A negative "No Template Control" was also included. The dissociation protocol was performed at the end of each run to check for non-specific amplification. Two replicas were run on the same plate for each sample. Results were expressed as relative expression values.

Animal characterization

All experiments were approved by the Ethics Committee of State University of Campinas. Six-week-old male *Swiss* mice were maintained under specific pathogen-free (SPF) conditions in a regimen of 12-h dark/light cycles and room temperature of 21°C. The animals were randomly divided into four groups with similar body weights and assigned to receive the following diet or treatment: a standard rodent chow (C), a standard rodent chow plus AG1296 (C+ AG1296), high fat diet (HFD), consisting of 55% calories from fat, 29% from carbohydrate and 16% from protein and high fat diet plus AG1296 (HFD + AG1296). Food and water were *ad libitum. B*ody weight and food intake were measured daily. For the group that received high fat diet, it started at same time of the treatment with AG1296 and they were maintained in this regimen for 7 and 15 days.

AG1296 administration protocol

AG1296 was suspended in a mixture of DMSO (3%) and water to a final concentration of 5 mg/ml. The dose utilized for the treatment was 50 mg/Kg. Drug administration was performed by gavage (once a day) for 7 and 15 consecutive days.

The group that received only standard rodent chow (C) and high fat diet (HFD) were given only a placebo solution constituted by DMSO + water.

Assays

Leptin and adiponectin concentrations were determined by enzyme-linked immunosorbent assay (ELISA) (Linco). Serum concentrations of IL-6 and TNFα were determined using mouse IL-6 ELISA and mouse TNFα ELISA (Pierce Endogen,

Rockford, IL). Glucose values were measured from the tail venous blood of all animals with a glucose monitor (Glucometer; Bayer).

Tissue extraction

Food was withdrawn 12–14 hours before the tissue extraction. Mice were anesthetized by intraperitoneal injection of sodium thiopental and opened 10–15 min later, i.e., as soon as anesthesia was assured by the loss of pedal and corneal reflexes. The abdominal cavity was opened, the portal vein exposed and 0.2 ml of normal saline was injected with or without insulin (10^{-6} mol.l^{-1}). Thirty seconds after the insulin injection, liver was removed, and 90 seconds later, gastrocnemius muscle epididymal, retroperitoneal and mesenteric adipose tissue were extracted. Tissues were minced coarsely, and homogenized immediately in extraction buffer, as described elsewhere (12). Extracts were then centrifuged at 15,000 rpm at 4℃ for 40 min to remove insoluble material. The whole tissue extracts were subjected to SDS-PAGE and immunoblotting, as previously described (13, 14).

Protein analysis by immunoblotting

The whole-tissue extracts were treated with Laemmli sample buffer (15) containing 100 mmol of dithiothreitol and heated in a boiling water bath for 5 min, after which they were subjected to SDS-PAGE in a Bio-Rad miniature slab gel apparatus (Mini-Protean). Electro transference of proteins from the gel to nitrocellulose membranes was performed for 120 min at 120 V in a Bio-Rad Mini-Protean transfer apparatus (16). Nonspecific protein binding to the nitrocellulose was reduced by preincubating the filter for 2 h in blocking buffer (5% nonfat dry milk, 10 mm Tris, 150 mm NaCl, 0.02% Tween 20). The nitrocellulose blot was incubated

overnight at 4°C with specific antibodies. The results were visualized by autoradiography with preflashed Kodak XAR film and band intensities were quantified by optical densitometry (Hoefer Scientific Instruments, San Francisco, CA; model GS300).

Morphometry

Five µm sections from epididymal, retroperitoneal and mesenteric adipose tissue were observed with a Zeiss Axiophot light microscope using a ×20 objective and digital images were captured with a Canon PowerShot G5. The measurement of adipocytes total area (average surface area of 30 randomly sorted adipocytes, per animal) was assessed using an image analysis system, Image J (http://rsbweb.nih.gov/ij/).

Statistical Analysis

Data are expressed as means ± SEM and the number of independent experiments is indicated. The results of blots are presented as direct comparisons of bands or spots in autoradiographs and quantified by optical densitometry (UN-SCAN IT gel). For statistical analysis, the groups were compared using a 2-way ANOVA with the Bonferroni test for post hoc comparisons. The level of significance adopted was $p<0.05$.

RESULTS

Effect of different doses of AG1296 on adipogenesis of 3T3-L1 preadipocytes.

Following incubation of preadipocytes (3T3-L1) with AG1296, we examined PPARγ and C/EBPα mRNA expression, the main adipogenic transcription factors, at day 4. As could be seen at Figure 1E, PPARγ mRNA decreases with an increase in AG1296 medium concentration. This downregulation in PPARγ mRNA expression was in a dose-dependent manner, showing the effect of AG1296 in the inhibition of adipogenesis. The same occurred for C/EBPα mRNA (Figure 1 F). This inhibition was evidenced in day 12 when less lipid droplets stained with Oil Red O staining were noted with the increase of AG1296 medium concentration (Figure 1 A,B, C and D). Also, it is noteworthy the reduction in cell density with the higher dose of AG1296 (Figure 1D), marked for an increase of blank spaces.

Effect of AG1296 administration in PDGFRβ tyrosine phosphorylation in retroperitoneal, mesenteric and epididymal adipose tissue .

Tyrphostin AG1296 was first described as a specific PDGFRβ inhibitor by Kovalenko at al in 1994 (9). This drug binds to the receptor and impairs its autophosphorylation catalic reaction. To investigate the effect of AG1296 on PDGFRβ tyrosine phosphorylation in retroperitoneal, mesenteric and epididymal adipose tissue, we performed western blotting analysis of these tissues extracts. The results showed that AG1296 was able to reduce PDGFRβ tyrosine phosphorylation in the three tissues studied, in both Control+AG1296 and HFD+AG1296 groups (Figure 2 A-C, upper panels). No changes in protein expression were observed among the groups (Figure 2 A-C, lower panels).

Effect of AG1296 on body weight and fat pads of high fat diet fed-mice and standard chow fed-mice.

Six week-old Swiss mice were placed on HFD, or not, and submitted to treatment with AG1296 for 7 and 15 days before the experiments.

Weight gain after 7 days of treatment was similar in all groups (fig. 3A) and after 15 days of treatment it was higher in the groups that received high fat diet although there was no significant difference between the group that received AG1296 (HFD+AG1296) and its control (HFD) (fig. 3B). Accumulated food intake was similar among the groups that received the same diet after 7 days of treatment or 15 days of treatment (fig. 3E and F). As expected, epididymal, retroperitoneal and mesenteric fat pads were higher in HFD groups. After the AG1296 7-day treatment, there was a slight reduction, especially in epididymal fat pad in HFD+AG1296 group (Fig 3C). But, after the 15-day treatment, this reduction was not observed at HFD+ AG1296 group, but was notable at C+AG1296, when compared with the control (C) (Fig.3D).

Effect of AG1296 on metabolic parameters of high fat diet and standard chow fed-mice.

Fasting plasma glucose was higher on HFD group and treatment with AG1296 was capable of reducing it after 7 days of treatment (Fig 4A). Also, after 15 days of treatment , C+AG1296 mice exhibited lower plasma glucose levels when compared with the control (Fig. 4B). During glucose tolerance test, plasma glucose was significantly higher in HFD compared with the control and control plus AG1296, and AG1296 administration, even for only 7 days, improved glucose tolerance (Fig 4C and 4D) and after the 15-day treatment, the same occurred to the control plus AG1296 when compared to the control, as could be seen throw the area under the

curve (Fig 4D). Also, after 15 days receiving high fat diet, the group HFD presented lower glucose disappearance rate compared to the control and the treatment with AG1296 was capable of reversing this alteration (Fig. 4E).

Effect of AG1296 on C/EBPα, PPARγ and SREBP-1c protein levels in epididymal, retroperitoneal and mesenteric adipose tissue.

To investigate the effect of PDGFRβ inhibition on adipogenesis, we assessed the expression profile of the main proteins related to its activation by western blot.

After a 7-day treatment, there was a significant reduction in PPARγ, SREBP-1c and C/EBPα protein levels in epididymal adipose tissue of HFD+AG1296 compared to HFD mice (Fig. 5C, F and I). The same was not observed for C and C+AG1296 groups that showed no difference on the expression profile of these proteins (Fig. 5C, F and I). Similar results were found in retroperitoneal adipose tissue (Fig 5A, D and G). For mesenteric adipose tissue, PPARγ and SREBP-1c showed the same pattern (Fig 5B and E) but, surprisingly, C/EBPα protein expression was very similar between the groups C, C+AG1296 and HFD, although it was significantly reduced at HFD+AG1296 (Fig 5H). Next, we proceed with a 15-day treatment with AG1296 and, for C+AG1296 group, we could notice a reduction in PPARγ and SREBP-1c in retroperitoneal and epididymal fat pads. No changes in tissue protein levels were observed among the groups (Fig 5A-I and 6A-I).

Effect of AG1296 on insulin signaling in liver, muscle and epididymal adipose tissue.

To assess whether the reduction in adipogenesis would have an effect on insulin signaling, we analyzed the phosphorylation levels of the main proteins involved in this signaling pathway.

In liver (Fig. 7A, D and G), epididymal adipose tissue (Fig 7C, F and I) and muscle (Fig 7B, E and H), insulin-induced IR and IRS-1 tyrosine phosphorylation and AKT serine phosphorylation were diminished in HFD group compared with the control group. The 7 day-treatment with AG1296 was capable of improving insulin signaling in HFD+AG1296 group and in some cases the phosphorylation returned to the level of the control (Fig 7A-I). No changes in basal phosphorylation or tissue protein levels were observed among the groups (Fig 7A - I).

We also evaluated insulin signaling pathway after a 15-day treatment with AG1296 and in this case we could notice that C+AG1296 group presented an increase in the phosphorylation levels of IR, IRS-1 and Akt, especially in liver and muscle (Fig. 8A - I).

Effect of AG1296 in serum levels of pro-inflammatory cytokines (IL-6, TNFα), leptin and adiponectin.

It is known that pro-inflammatory cytokines IL-6 and TNFα are increased in mice fed a HFD (17). So, we evaluated whether serum levels of these cytokines would be affected by AG1296 treatment.

In both, 7-day treatment and 15-day treatment, as was expected, levels of IL-6 (Fig. 9G and H) and TNFα (Fig 9E and F) were increased in the HFD groups, but were not reversed in HFD+AG1296 groups. Similar results were obtained for leptin

(Fig 9A and B), although it was detected a slight reduction on leptin levels for HFD+AG1296 after 15 days of treatment (Fig. 9B). Adiponectin, in its turn, showed no alterations in its serum levels between the groups after the 7-day treatment (Fig 9C) and after 15 days of treatment, its serum level was reduced in the groups HFD and HFD+AG1296 equally (Fig 9D).

Effect of AG1296 on epidydimal, retroperitoneal and mesenteric adipose tissue morphology.

To access whether treatment with AG1296 would have any effect in adipocyte morphology, we performed a histological analysis of the three fractions of adipose tissues (epididymal, retroperitoneal and mesenteric).

Morphometric analysis revealed that adipocytes were larger in high-fat diet mice when compared with the control group for all three adipose tissues (Fig 10A-D and Fig 11A-D). The 7-day treatment with AG1296 was capable of reducing adipocyte size of HFD+AG1296 group only in epididymal adipose tissue (Fig 10A-D). On the other hand, after the 15-day treatment there was a reduction in adipocyte size in HFD+AG1296 group when compared with HFD, in all the three tissues studied (Fig 11A-D).

DISCUSSION

The increased storage of triglycerides in the adipose cells can be accomplished in two different ways: a) by expanding the available cells (hypertrophy) or b) by recruiting new fat cells (hyperplasia). In adults, hypertrophy of the fat cells is the most common form of accommodating the lipids, whereas hyperplasia predominates in the prepuberal age. Hypertrophic obesity is also more strongly associated with insulin resistance and the metabolic complications than hyperplastic obesity. Recruitment of new fat cells is less common in adults.

A recent study has shown that there is also a continuous turnover of fat cells in adult man of 10%/year (18). It is reasonable to assume that the new adipocytes are formed from mesenchymal stem cells or other precursor cells that become committed to preadipocytes (4). Recently, it was demonstrated that the adipocytes descend from a pool of proliferating progenitors that are already committed, either prenatally or early in postnatal life. These progenitors reside in the mural cell compartment of the adipose vasculature. In addition, this previous study demonstrated that PDGFRβ marks mural cells and is required for their development (8). In the present study, we take advantage of these mural cells characteristics and investigated the effect of a PDGFRβ inhibitor on fat pad and adipocyte differentiation and cell morphology, and also on insulin sensitivity and signaling in prepuberal mice on chow or high fat diet.

The results of the present study show that administration of AG1296 (PDGFRβ tyrosine kinase inhibitor) to control mice for 15 days reduce adipose fat pad, without changes in adipocyte morphology, associated with a reduction in proteins of differentiation (especially PPARγ) and, interestingly, an increase in insulin signaling in liver and muscle and a reduction in fasting plasma glucose.

Previous data showed that PDGF is an important inducer of the proliferative phase of preadipocyte differentiation, but its effect on adipogenesis is controversial. PDGF has been reported to either inhibit (19), have no effect (20) or promote (21) differentiation of preadipose cell lines. Our study was not designed to investigate the proliferative phase, but our data showed that a PDGFR inhibitor was able to modulate adipogenesis, although we cannot determine if the main effect of this drug was in the proliferative phase.

The terminal differentiation of preadipocytes involves a highly regulated and coordinated cascade of transcriptional factors. C/EBPα and PPARδ are the best-characterized transcriptional factors for adipogenesis (22, 23, 24), although SREBP-1c was also demonstrated to have a role in this event (25). In the present study we showed that use of a PDGFRβ inhibitor in control mice on regular chow, modulated these transcriptional factors in adipose tissue, at least in retroperitoneal and epididymal depots.

The reduction in adipose fat pad was accompanied by a reduction in fasting plasma glucose and in an increase in insulin signaling in liver and muscle. Taken together these data indicate that the reduction in adipose mass increased insulin-induced Akt phosphorylation in liver and muscle. This improvement in insulin signaling in liver may have a role in hepatic glucose production and can account for the decrease in fasting plasma glucose. It is possible that some hormone or cytokine produced in adipose tissue might have an influence on this process. However, the main candidates that we expected to be reduced as IL-6 or TNFα did not show differences in circulating levels. We cannot exclude the possibility that the method that we used to determine these cytokines was not sensitive enough to detect small differences. Moreover, an increase in adiponectin levels could also contribute to

explain these results, but we also did not observe changes in the circulating levels of this hormone.

In order to investigate the effect of PDGFR inhibitor in a situation of excess of nutrients to adipose tissue, we used mice on HFD. Our data showed that the use of this drug in mice on HFD reduced the adipogenic protein expression in fat pad associated with changes in adipocyte morphology and improvement in insulin signaling, insulin sensitivity and in glucose tolerance.

It is important to mention that this effect was observed early (7 days) compared to mice on regular chow, associated with a more marked reduction in adipogenic protein expression and a reduction in epididymal fat pad. These early changes after 7 days of HFD and the use of an inhibitor of PDGFRβ were accompanied by an improvement in insulin signaling pathway and reduction in fasting plasma glucose. There was a clear increase in insulin-induced insulin receptor and IRS-1 tyrosine phosphorylation levels, and in insulin-induced Akt phosphorylation in liver, muscle and adipose tissue.

However, after 15 days of HFD plus AG1296 some changes that were observed at 7 days now were less evident or disappeared. No changes in epididymal, mesenteric or retroperitoneal fat pads were observed, and the reductions in transcription factors also were less evident. There was only a clear decrease in PPARγ in adipose tissue. The improvement in insulin signaling in liver disappeared. However, some relevant modifications were still present: there was a clear change in the adipocyte area, and the improvement in insulin signaling in muscle was maintained. It is now well established that an improvement in insulin action in muscle may reduce insulin resistance and improve glucose metabolism (26, 27). The improvement in insulin-induced IR and IRS-1 tyrosine phosphorylation levels and in

Akt phosphorylation induced by AG1296 in muscle of mice on HFD was associated with an increase in glucose disappearance rate determined during the insulin tolerance test, indicating a clear reduction in insulin resistance.

In summary, our results showed that the use of AG1296 and the consequent inhibition of PDGFRβ, has an effect in adipogenesis, promoting the decrease of the main adipogenic proteins and in parallel improves insulin signaling in liver, muscle and epididymal adipose tissue. We, therefore, suggest that inhibition of PDGFRβ receptor by AG1296 may presents an interesting alternative in preventing obesity and insulin resistance.

AUTHORS' CONTRIBUTIONS

A.C.P.: researched data, contributed to discussion, wrote/reviewed/edited manuscript; F.M.: researched data; K.L.C.: researched data; B.M.C: researched data; A.O.G.: researched data; D.G.: researched data; J.M.: researched data; L.V.: researched data; J.V.: researched data; J.B.C.C.: contributed to discussion, reviewed/edited manuscript; M.J.A.S.: contributed to discussion, wrote/ reviewed/edited manuscript.

CONFLICT OF INTEREST

No potential conflicts of interest relevant to this work were reported.

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REFERENCES

1. World Health Organization. Obesity and overweight. Fact sheet nº 311. WHO Web site. http://www.who.int/mediacentre/factsheets/fs311/en/ . March 2011.

2. Spiegelman BM, Flier JS. Obesity and Regulation of Energy Balance. **Cell** 104, p.531-543, 2001.

3. Sunyer P, Xavier F. The obesity epidemic: pathophysiology and consequences of obesity. **Obes Res**. 10, p. 97S–104S, 2002

4. Gesta S, Tseng Y, Kahn R. Developmental origin of Fat: Tracking Obesity to its Source. **Cell** 13, p. 242-56, 2007

5. Rosen ED, MacDougld OA. Adipocyte differentiation from the inside out. **Nature Rev Mol. Cell Bio** 7, p.885-896, 2006

6. Rosen ED, Walkey CJ, Puigserver P., Spiegelman BM. Transcriptional regulation of adipogenesis. **Genes Dev.** 14, p. 1293-1307, 2000

7. Claesson-Welsh L. Mechanism of Action of Platelet-derived Growth Factor. **Int. J. Biochem. Cell Biol.** 28 (4), p.373-385, 1996

8. Tang W, Zeve D, Suh, J, Bosnakovski D, Kyba M, Hammer B, Tallquist MD, Graff JM. White fat progenitor cells reside in adipose vasculature. **Science** 322 (5901), p.583-586, 2008

9. Kovalenko M., Gazit A., Böhmer A., Rorsman CH.,Rönnstrand L., Heldin C.-H., Waltenberger J., Böhmer F. D. & Levitzki, A. Selective Platelet-derived Growth Factor Receptor Kinase Blockers Reverse sis-Transformation **Cancer Res.** *54*, p. 6106-6114, 1994

10. Kovalenko M., Rönnstrand L., Heldin C.-H., Loubtchenkov M., Gazit A., Levitzki A., Böhmer F. D. Phosphorylation Site-Specific Inhibition of Platelet-derived Growth Factor β-Receptor Autophosphorylation by the Receptor Blocking Tyrphostin AG1296. **Biochemistry** 36, p. 6260-6269, 1997

11. Levitzki A. PDGF receptor kinase inhibitors for the treatment of PDGF driven diseases. **Cytokine & Growth Factor Reviews** v. 15, p.229-235, 2004

12. Thirone AC, Carvalheira JB, Hirata AE, Velloso LA, Saad MJ. Regulation of Cbl-associated protein/Cbl pathway in muscle and adipose tissues of two animal models of insulin resistance. **Endocrinology** 145, p. 281-293, 1994

13. Carvalho-Filho MA, Ueno M, Hirabara SM, Seabra AB, Carvalheira JB, de Oliveira MG, Velloso LA, Curi R, Saad MJ. S-nitrosation of the insulin receptor, insulin receptor substrate 1, and protein kinase B/Akt: a novel mechanism of insulin resistance. **Diabetes** 54, p.959-967, 2005

14. Tsukumo DM, Carvalho-Filho MA, Carvalheira JB, Prada PO, Hirabara SM, Schenka AA, Araujo EP, Vassallo J, Curi R, Velloso LA, Saad MJ**.** Loss-of-function mutation in Toll-like receptor 4 prevents diet-induced obesity and insulin resistance. **Diabetes** 56, p. 1986-1998, 2007

15. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. **Nature** (Lond.) 227, p. 680-685, 1970

16. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. **Proc Natl Acad Sci U S A** 76, p. 4350-4354, 1979

17. Prada PO, Ropelle ER, Mourao RH, de Souza CT, Pauli JR, Cintra DE, Schenka A, Rocco SA, Rittner R, Franchini KG, Vassallo J, Velloso LA, Carvalheira JB, Saad MJ. EGFR tyrosine kinase inhibitor (PD153035) improves glucose tolerance and insulin action in high-fat diet-fed mice. **Diabetes** 58, p. 2910-2919, 2009

18. Spalding KL, Arner E, Westermark PO, Bernard S, Buchholz BA, Bergmann O, Blomqvist L, Hoffstedt J, Näslund E, Britton T, Concha H, Hassan M, Rydén M, Frisén J, Arner P. Dynamics of fat cell turnover in humans. **Nature** 453 (7196): 783-787, 2008

19. Navre M, Ringold GM. A Gowth Factor-repressible Gene Associated with protein Kinase C-mediated Inhibition of Adipocyte Differentiation. **J Cell Biol.** 107(1):279-86, 1998

20. Schmidt W, Pöll-Jordan G, Löffler G. Adipose conversion of 3T3-L1 cells in a serum-free culture system depends on epidermal growth factor, insulin-like growth factor I, corticosterone, and cyclic AMP. **J Biol Chem.** 265(26):15489-95, 1990

21. Bachmeier M, Löffler G. The effect of platelet-derived growth factor and adipogenic hormones on the expression of CCAAT/enhancer-binding proteins in 3T3-L1 cells in serum-free conditions. **Eur J Biochem.** 243(1-2):128-33, 1997

22. Gregoire FM, Smas CM, Sul HS. Understanding adipocyte differentiation. **Physiol Rev.** v. 78, p.783-809, 1998

23. Rosen ED., Spiegelman BM. PPARγ: a Nuclear Regulator of Metabolism, Diferentiation, and Cell Growth. **The Journal of Biological Chemistry** 276 (41), p. 37731-37734, 2001

24. Farmer S.R. Transcriptional control of adipocyte formation. **Cell Metab.** 4, p. 263-273, 2006

25. Payne VA, Au WS, Lowe CE, Rahman SM, Friedman JE, O'Rahilly S, Rochford JJ. C/EBP transcription factors regulate SREBP1c gene expression during adipogenesis. **Biochem J.** 425 (1):215-23, 2009.

26. Ropelle ER, Pauli JR, Prada PO, de Souza CT, Picardi PK, Faria MC, Cintra DE, Fernandes MF, Flores MB, Velloso LA, Saad MJ, Carvalheira JB. Reversal of diet-induced insulin resistance with a single bout of exercise in the rat: the role of PTP1B and IRS-1 serine phosphorylation. **J Physiol.** 577(3):997-1007, 2006.

27. Smith AG, Muscat GE. Skeletal muscle and nuclear hormone receptors: implications for cardiovascular and metabolic disease**. Int J Biochem Cell Biol.** 37(10):2047-63, 2005

FIGURE LEGENDS

Figure 1. Effect of different doses of AG1296 on adipogenesis of 3T3-L1 preadipocytes. *Confluent 3T3-L1 preadipocytes were differentiated into adipocytes in medium with different concentrations of AG1296 for 12 days. The fully differentiated adipocytes were stained with Oil Red O stain (for the lipid droplets). A: fully differentiated adipocytes without AG1296. B: fully differentiated adipocytes with AG1296 0,5 μM C: fully differentiated adipocytes with AG1296 2 μM D: fully differentiated adipocytes with AG1296 4 μM. Original magnification X 200. E: PPARγ mRNA levels in 3T3-L1 culture at day 4 of differentiation. F: C/EBPα mRNA levels in 3T3-L1 culture at day 4 of differentiation. Data are presented as means ± SEM *P < 0.05 vs. Control group.*

Figure 2. Effect of AG1296 administration in PDGFRβ tyrosine phosphorilation. *Representative blots show the tyrosine phosphorylation of PDGFRβ of Control, Control + AG1296, HFD and HFD + AG1296 mice in Retroperitoneal (A), Mesenteric (B) and Epididymal (C) adipose tissues (upper panels). Total protein expression of PDGFRβ (A-C, lower panels). Data are presented as means ± SEM from eight to ten mice per group, *P < 0.05 vs. Control group and #P < 0.05 vs. HFD group. IB, immunoblot.*

Figure 3. Effect of AG1296 on body weight and fat pads of high fat diet fed-mice and standard chow fed-mice. *A: Body weight (7 days of treatment). B: Body weight (15 days of treatment) C: Fat Mass (7 days of treatment). D: Fat Mass (15 days of treatment) E: Energy intake (7 days of treatment). F: Energy intake (15 days of*

*treatment). Data are presented as means ± SEM of five to eight mice per group. *P < 0.05 vs. Control group; # P < 0.05 vs. HFD group.*

Figure 4. Effect of AG1296 on metabolic parameters. *A: Fasting plasma glucose after 7 days of treatment with AG1296. B: Fasting plasma glucose after 15 days of treatment with AG1296. C: Glucose tolerance test after 7 days of treatment. D: Glucose tolerance test after 15 days of treatment. E: Glucose disappearance rate after 15 days of treatment. Data are presented as means ± SEM of five to eight mice per group. *P < 0.05 vs.Control group; #P < 0.05 vs. HFD group; **P < 0,001 vs. HFD; § P < 0,01 vs.HFD.*

Figure 5. Effect of AG1296 on adipogenic protein levels in epididymal, retroperitoneal and mesenteric adipose tissue after the 7-day treatment. *Representative blots of PPARγ (A-C), SREBP-1c (D-F) and C/EBPα (G-I) in epididymal, retroperitoneal and mesenteric adipose tissue of Control, Control+AG1296, HFD and HFD+AG1296 mice after 7 days of treatment with AG1296 (upper panels). Total protein expression of β-actin (A-I, lower panels). Data are presented as means ± SEM from eight to ten mice per group, *P < 0.05 vs. Control group and # P < 0.05 vs. HFD group. IB, immunoblot.*

Figure 6. Effect of AG1296 on adipogenic protein levels in epididymal, retroperitoneal and mesenteric adipose tissue after the 15-day treatment. *Representative blots of PPARγ (A-C), SREBP-1c (D-F) and C/EBPα (G-I) in epididymal, retroperitoneal and mesenteric adipose tissue of Control, Control+AG1296, HFD and HFD+AG1296 mice after 15 days of treatment with*

*AG1296 (upper panels). Total protein expression of β-actin (A-I, lower panels). Data are presented as means ± SEM from five mice per group, *P < 0.05 vs. Control group and # P < 0.05 vs. HFD. IB, immunoblot.*

Figure 7. Effects of AG1296 administration on insulin signaling in liver, muscle and epididymal adipose tissue after the 7-day treatment. *Representative blots show tyrosine phosphorylation of IRβ (A-C), IRS-1 (D-F) and serine phosphorylation of Akt (G-I) of Control, Control+AG1296, HFD and HFD+AG1296 mice in liver, muscle and epididymal adipose tissue after 7 days of treatment with AG1296 (upper panels). Total protein expression of IRβ (A–C), IRS-1 (D-F) and AKT (G-I) (lower panels). Data are presented as means ± SEM from eight to ten mice per group, *P < 0.05 vs. control group and #P < 0.05 vs. HFD group. IB, immunoblot.*

Figure 8. . Effects of AG1296 administration on insulin signaling in liver, muscle and epididymal adipose tissue after the 15-day treatment. *Representative blots show tyrosine phosphorylation of IRβ (A-C), IRS-1 (D-F) and serine phosphorylation of Akt (G-I) of Control, Control+AG1296, HFD and HFD+AG1296 mice in liver, muscle and epididymal adipose tissue after 15 days of treatment with AG1296 (upper panels). Total protein expression of IRβ (A–C), IRS-1 (D-F) and AKT (G-I) (lower panels). Data are presented as means ± SEM from five mice per group, *P < 0.05 vs. Control group and #P < 0.05 vs. HFD group. IB, immunoblot.*

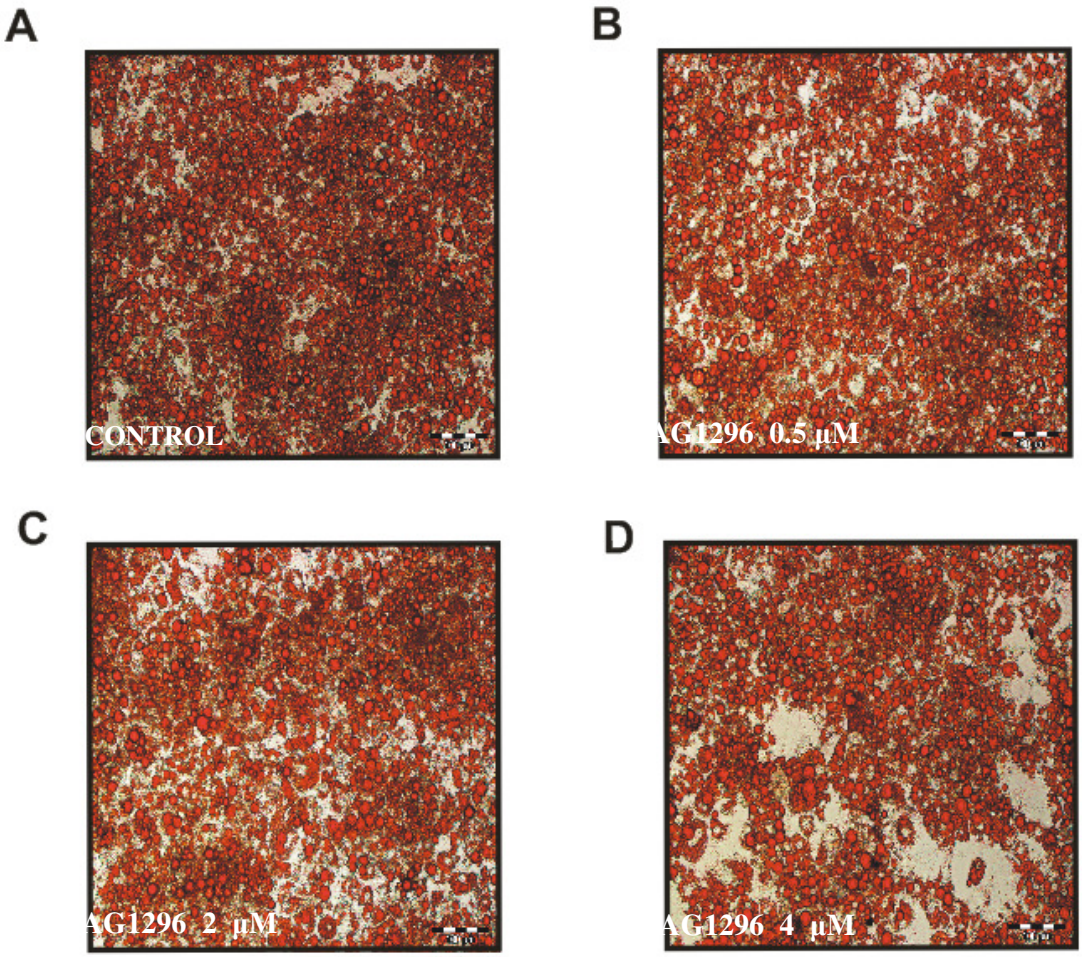
Figure 9. Effect of AG1296 in serum levels of pro-inflammatory cytokines (IL-6, TNFα), leptin and adiponectin. *Serum levels of Leptin (A and B), Adiponectin (C*

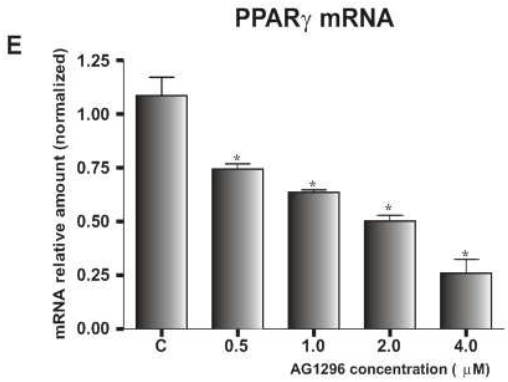
*and D), TNFα (E and F) and IL-6 (G and H) after 7 and 15 days of treatment with AG1296 were obtained using ELISA assay. Data are presented as means ± SEM of six to eight mice per group. *P < 0.05 vs. control group; #P < 0.05 vs. HFD group.*

Figure 10. Morphologic characterization of epididymal, retroperitoneal and mesenteric adipose tissue after the 7-day treatment with AG1296. *A-C: Hematoxylin & Eosin staining of 5 µm histological sections of epididymal (A), retroperitoneal (B) and mesenteric (C) fat pads from Control, Control + AG1296, HFD and HFD+AG1296 mice after 7 days of treatment. 100µm scale bar for all pictures. D: Quantification of adipocytes area (square micrometers). About 30 cells were measured in each group, and the average adipocyte area was calculated. Data are presented as means ± SEM of eight to ten mice per group. *P < 0.05 vs. Control group; #P < 0.05 vs. HFD group. Original magnification X 200.*

Figure 11. Morphologic characterization of epididymal, retroperitoneal and mesenteric adipose tissue after the 15-day treatment with AG1296. *A-C: Hematoxylin & Eosin staining of 5 µm histological sections of epididymal (A), retroperitoneal (B) and mesenteric (C) fat pads from Control, Control + AG1296, HFD and HFD+AG1296 mice after 15 days of treatment. 100µm scale bar for all pictures. D: Quantification of adipocytes area (square micrometers). About 30 cells were measured in each group, and the average adipocyte area was calculated. Data are presented as means ± SEM of five mice per group. *P < 0.05 vs. Control group; #P < 0.05 vs. HFD group. Original magnification X 200.*

FIGURE 1


A
CONTROL
B
AG1296 0.5 μM
C
AG1296 2 μM
D
AG1296 4 μM


E
PPARγ mRNA
mRNA relative amount (normalized)
1.25
1.00
0.75
0.50
0.25
0.00
C
0.5
1.0
2.0
4.0
AG1296 concentration (μM)

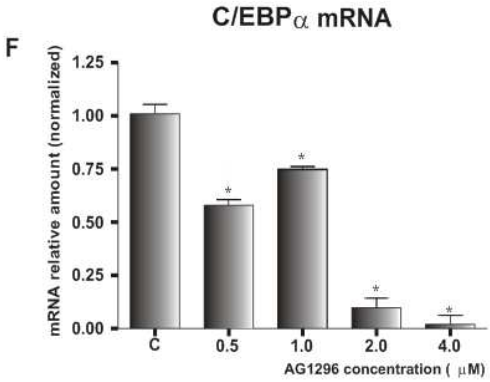

F
C/EBPα mRNA
mRNA relative amount (normalized)
1.25
1.00
0.75
0.50
0.25
0.00
C
0.5
1.0
2.0
4.0
AG1296 concentration (μM)

FIGURE 2

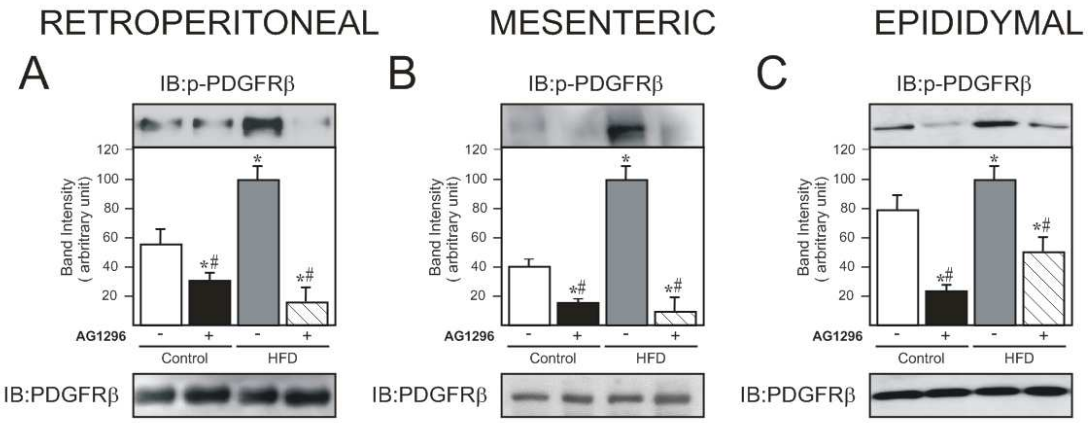

RETROPERITONEAL
MESENTERIC
EPIDIDYMAL
A
B
C
IB:p-PDGFRβ
Band Intensity (arbitrary unit)
120
100
80
60
40
20
AG1296
-
+
Control
HFD
IB:PDGFRβ

FIGURE 3

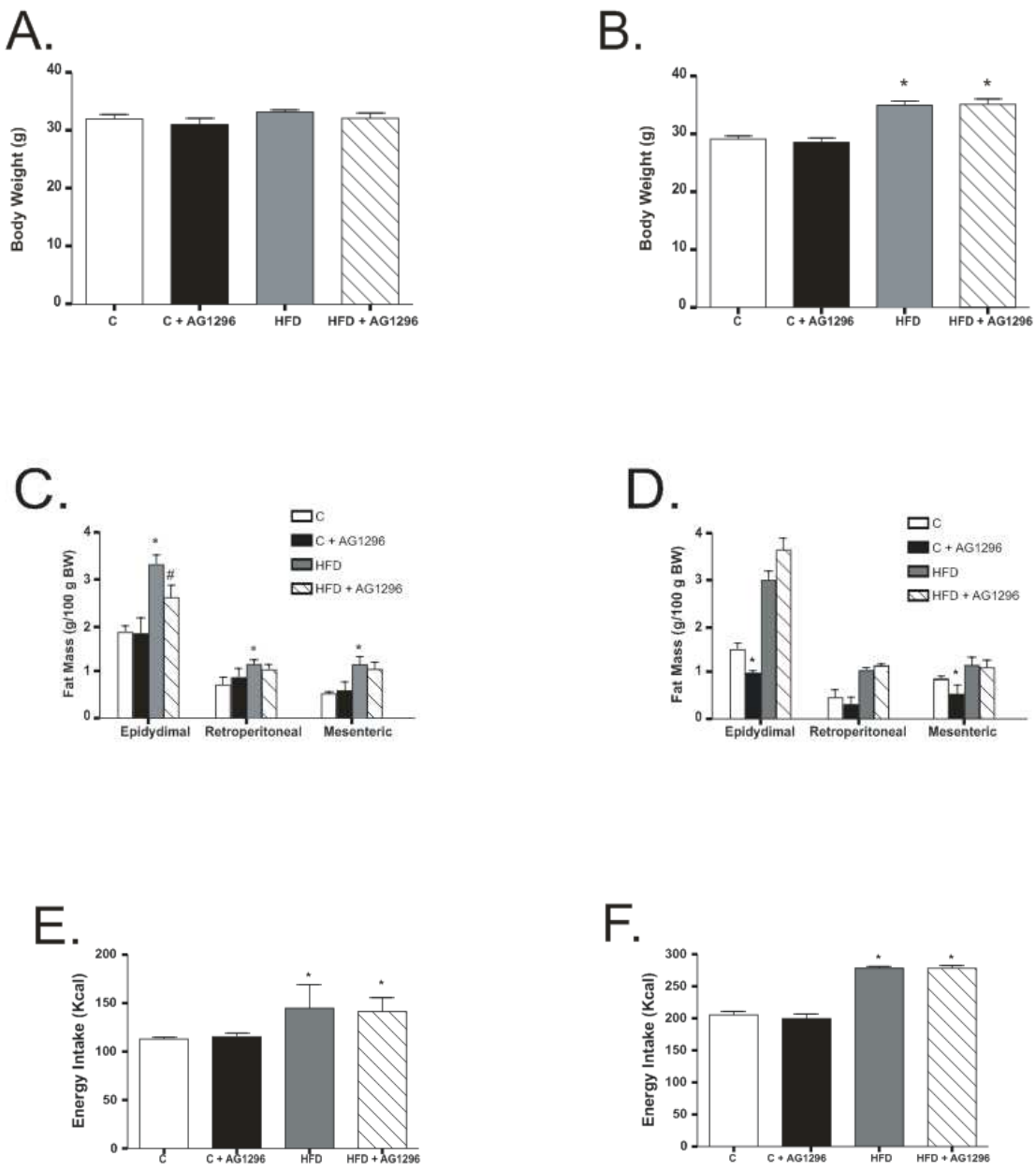

A.
Body Weight (g)
40
30
20
10
0
C
C + AG1296
HFD
HFD + AG1296
B.
Body Weight (g)
*
*
C
C + AG1296
HFD
HFD + AG1296
C.
Fat Mass (g/100 g BW)
C
C + AG1296
HFD
HFD + AG1296
*
#
*
*
Epidydimal
Retroperitoneal
Mesenteric
D.
Fat Mass (g/100 g BW)
C
C + AG1296
HFD
HFD + AG1296
*
*
Epidydimal
Retroperitoneal
Mesenteric
E.
Energy Intake (Kcal)
*
*
C
C + AG1296
HFD
HFD + AG1296
F.
Energy Intake (Kcal)
*
*
C
C + AG1296
HFD
HFD + AG1296

FIGURE 4

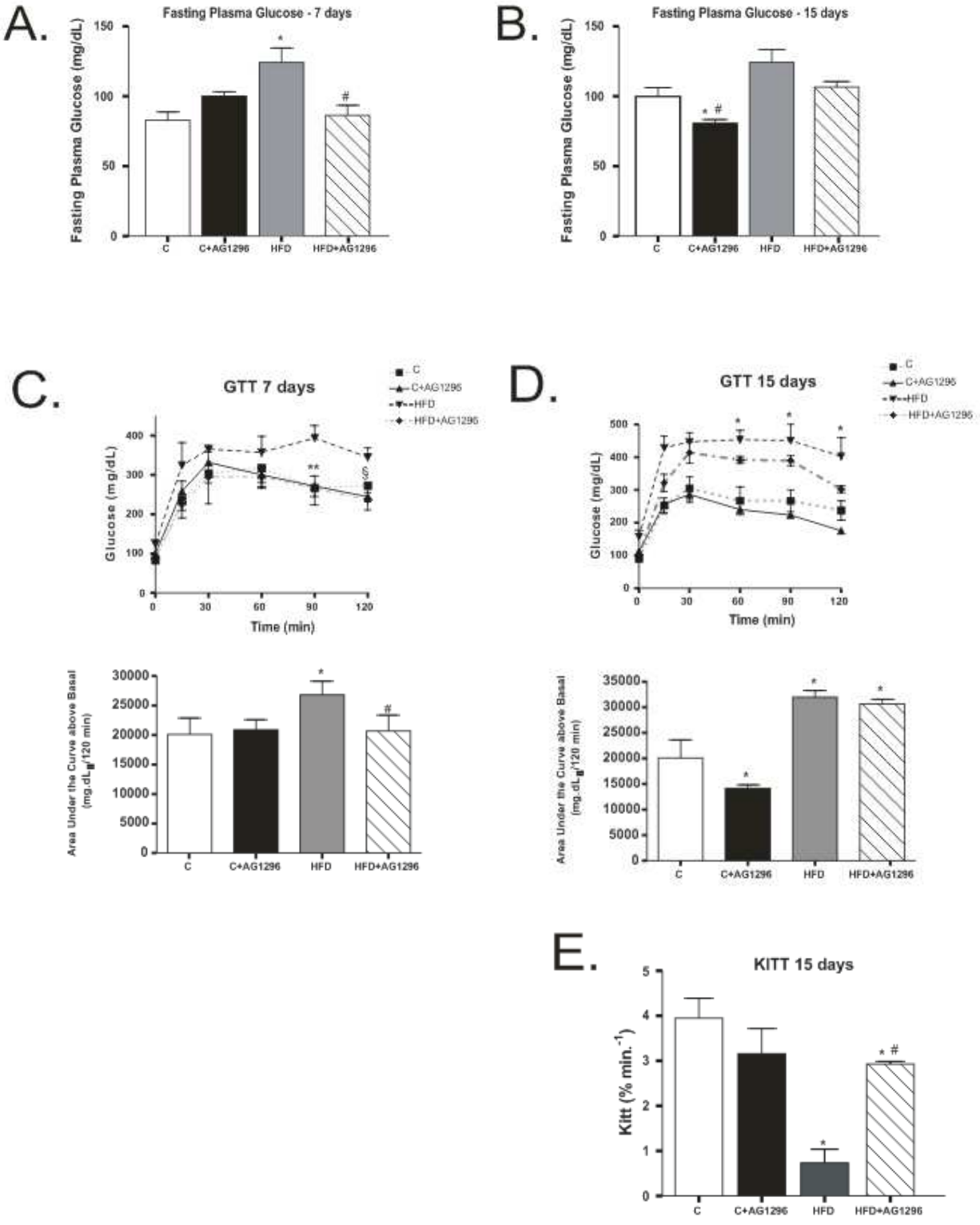

A.
Fasting Plasma Glucose - 7 days
Fasting Plasma Glucose (mg/dL)
C
C+AG1296
HFD
HFD+AG1296
B.
Fasting Plasma Glucose - 15 days
C.
GTT 7 days
Glucose (mg/dL)
Time (min)
Area Under the Curve above Basal (mg.dL-1.120 min)
D.
GTT 15 days
E.
KITT 15 days
Kitt (% min.-1)

FIGURE 5

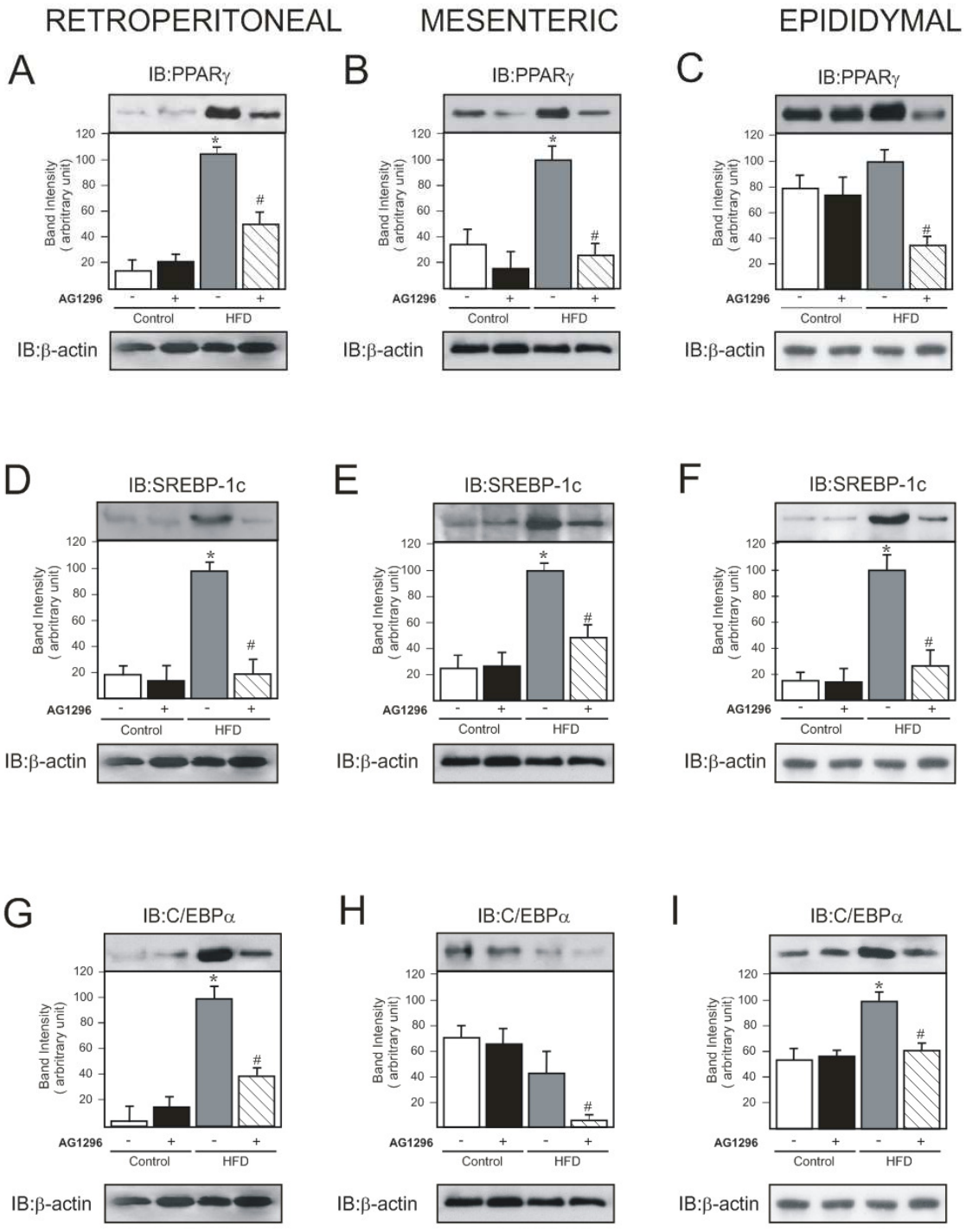

RETROPERITONEAL
MESENTERIC
EPIDIDYMAL
A
B
C
D
E
F
G
H
I
IB:PPARγ
IB:SREBP-1c
IB:C/EBPα
IB:β-actin
Band Intensity (arbitrary unit)
AG1296
Control
HFD

FIGURE 6

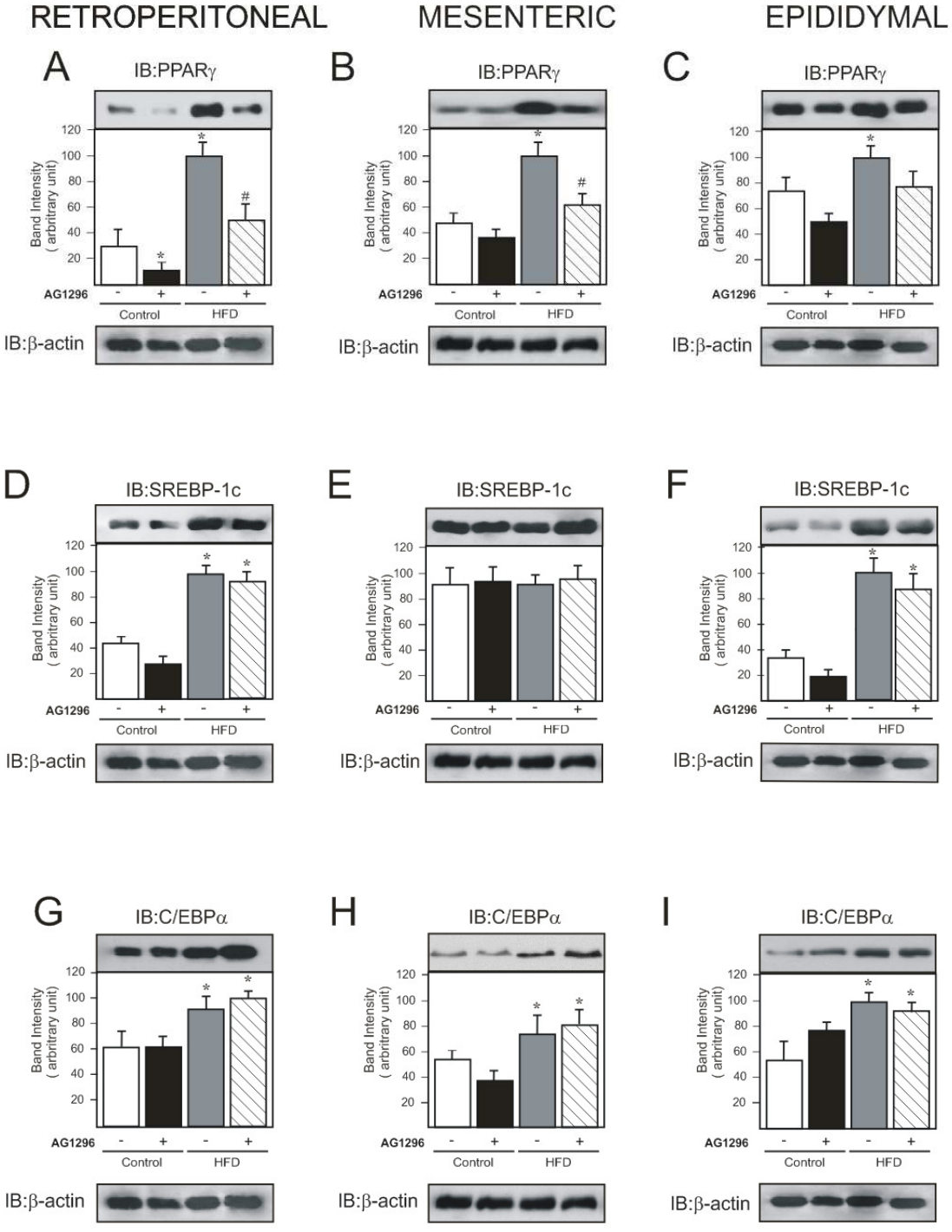

RETROPERITONEAL
MESENTERIC
EPIDIDYMAL
A
B
C
D
E
F
G
H
I
IB:PPARγ
IB:SREBP-1c
IB:C/EBPα
IB:β-actin
Band Intensity (arbitrary unit)
AG1296
Control
HFD

FIGURE 7

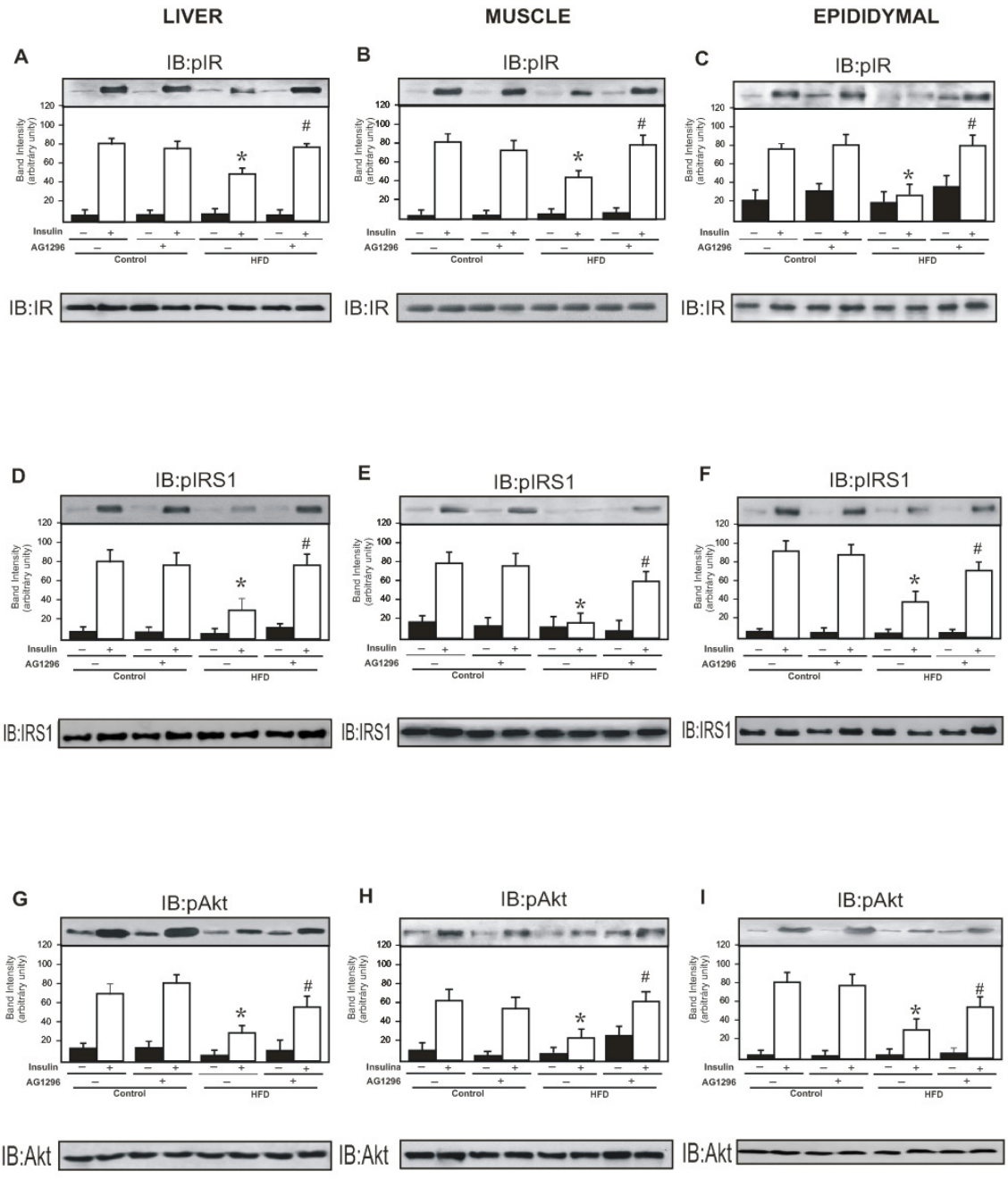

LIVER
MUSCLE
EPIDIDYMAL
A
B
C
IB:pIR
IB:pIR
IB:pIR
Band Intensity (arbitrary unity)
Insulin
AG1296
Control
HFD
IB:IR
IB:IR
IB:IR
D
E
F
IB:pIRS1
IB:pIRS1
IB:pIRS1
IB:IRS1
IB:IRS1
IB:IRS1
G
H
I
IB:pAkt
IB:pAkt
IB:pAkt
IB:Akt
IB:Akt
IB:Akt

FIGURE 8

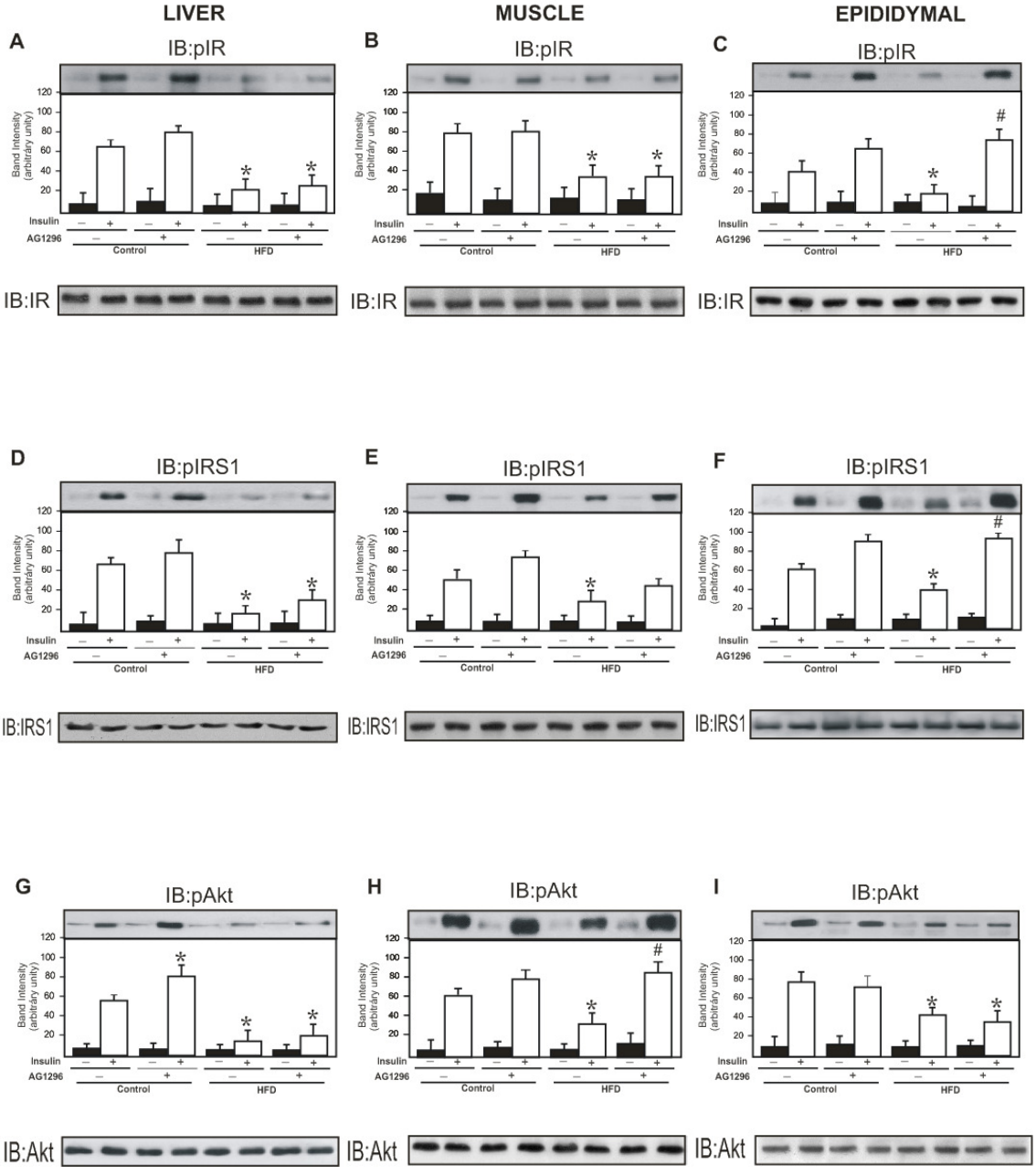

LIVER
MUSCLE
EPIDIDYMAL
A
B
C
IB:pIR
Band Intensity (arbitrary unity)
Insulin
AG1296
Control
HFD
IB:IR
D
E
F
IB:pIRS1
IB:IRS1
G
H
I
IB:pAkt
IB:Akt

FIGURE 9

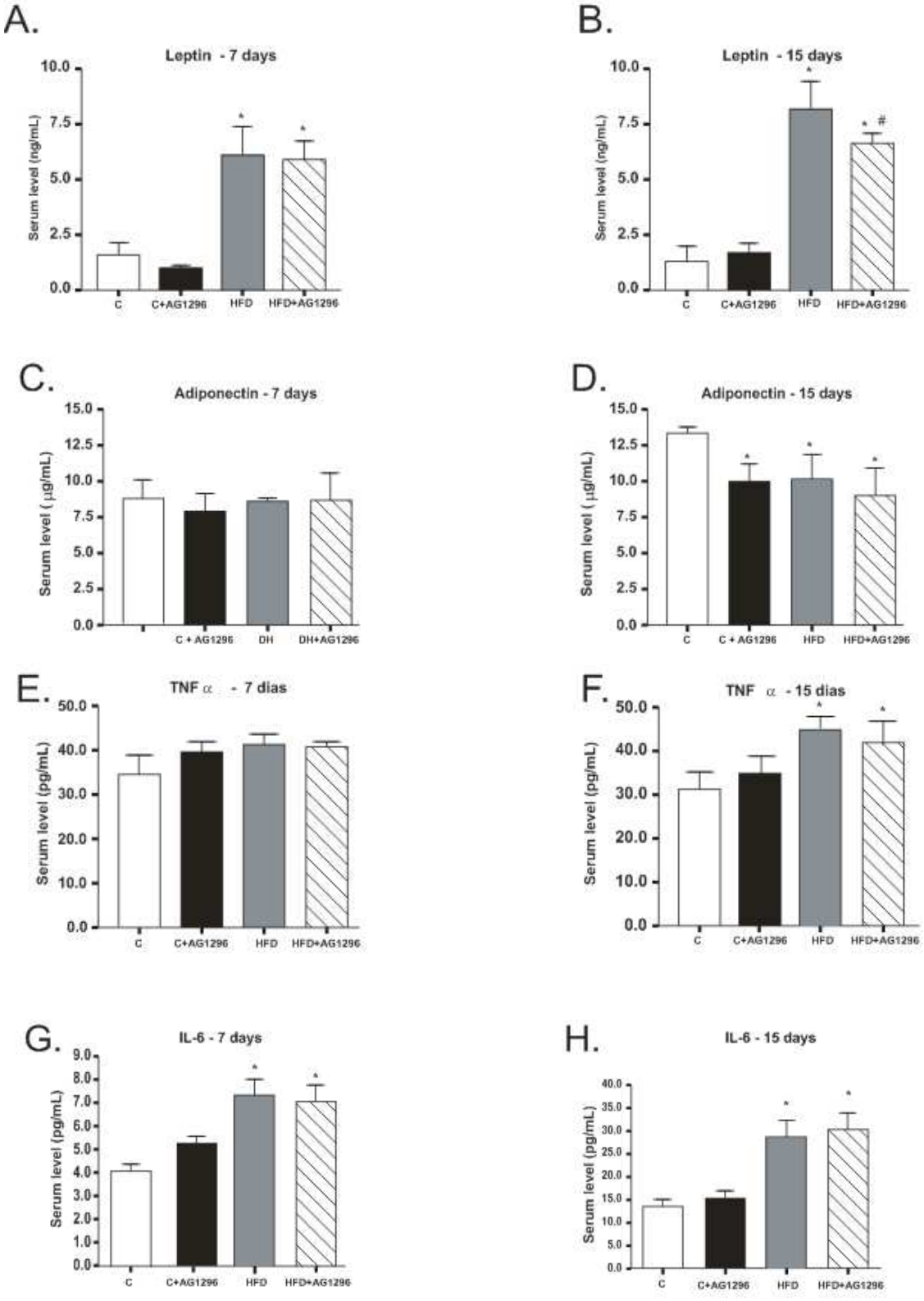
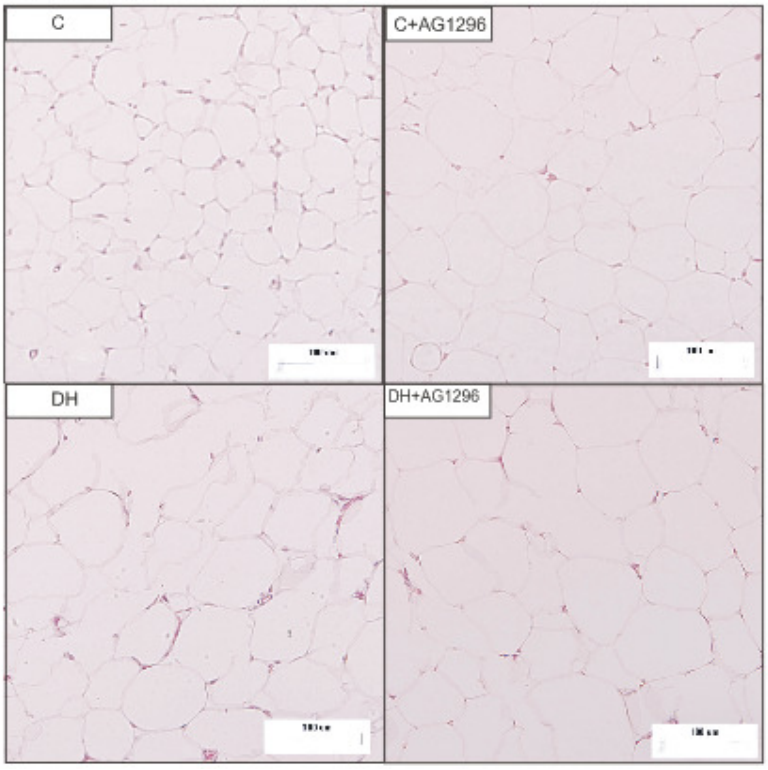

A.
Leptin - 7 days
Serum level (ng/mL)
10.0
7.5
5.0
2.5
0.0
C
C+AG1296
HFD
HFD+AG1296
B.
Leptin - 15 days
Serum level (ng/mL)
C
C+AG1296
HFD
HFD+AG1296
C.
Adiponectin - 7 days
Serum level (µg/mL)
15.0
12.5
10.0
7.5
5.0
2.5
0.0
C + AG1296
DH
DH+AG1296
D.
Adiponectin - 15 days
Serum level (µg/mL)
C
C + AG1296
HFD
HFD+AG1296
E.
TNF α - 7 dias
Serum level (pg/mL)
50.0
40.0
30.0
20.0
10.0
0.0
C
C+AG1296
HFD
HFD+AG1296
F.
TNF α - 15 dias
Serum level (pg/mL)
C
C+AG1296
HFD
HFD+AG1296
G.
IL-6 - 7 days
Serum level (pg/mL)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
C
C+AG1296
HFD
HFD+AG1296
H.
IL-6 - 15 days
Serum level (pg/mL)
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
C
C+AG1296
HFD
HFD+AG1296

FIGURE 10

A.

Epididymal Adipose Tissue - 7 days



B.

Retroperitoneal adipose tissuel- 7 days

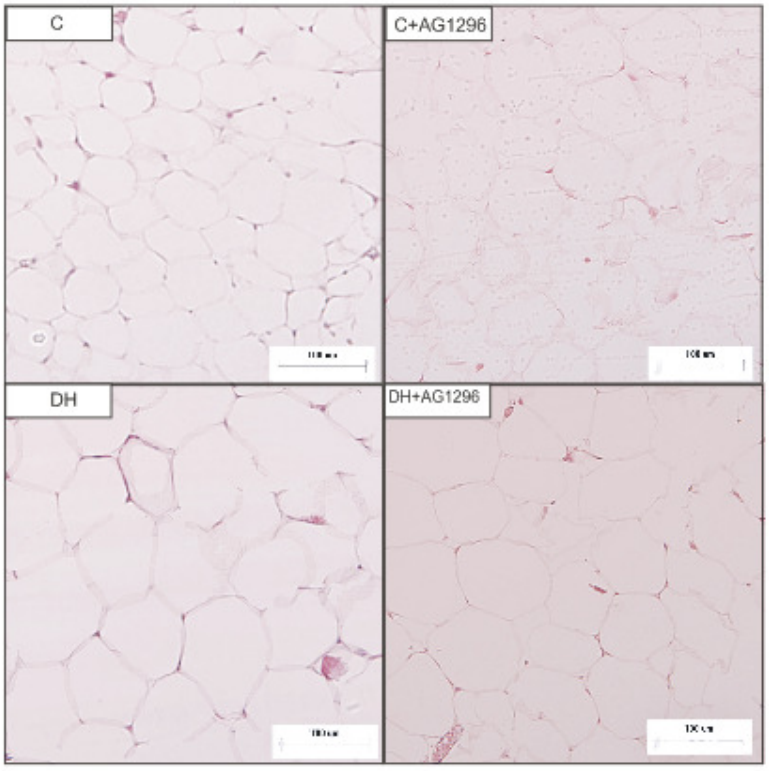
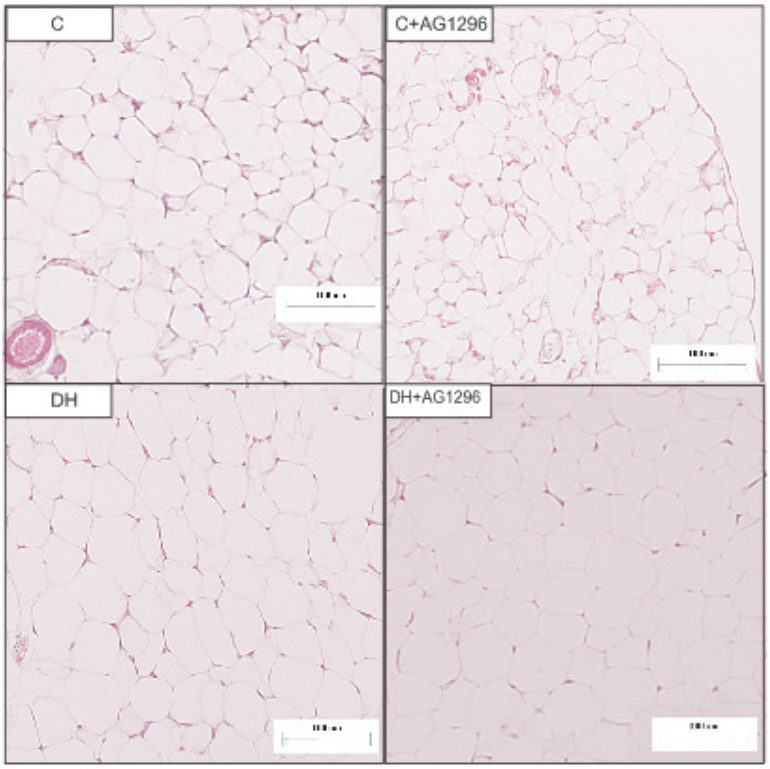


FIGURE 10

C. Mesenteric adipose tissue- 7 days



D.

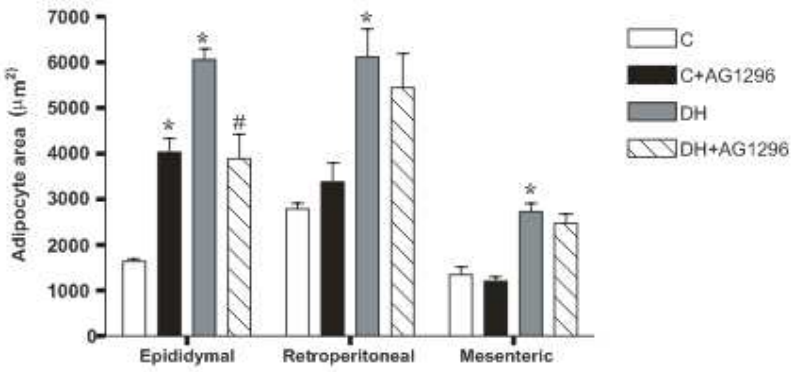
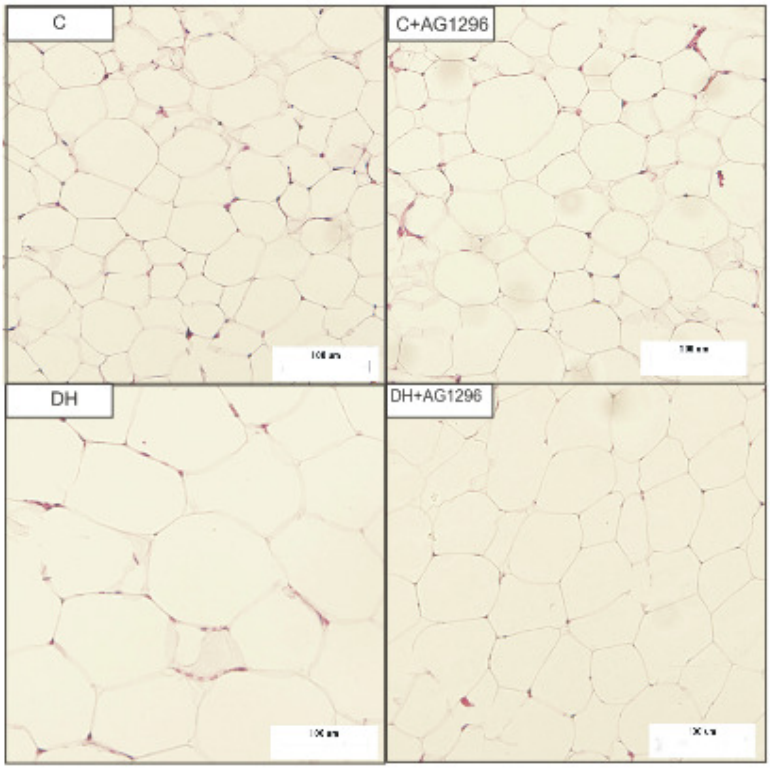


FIGURE 11

A. Epididymal Adipose Tissue - 15 days



B. Retroperitoneal adipose tissuel- 15 days

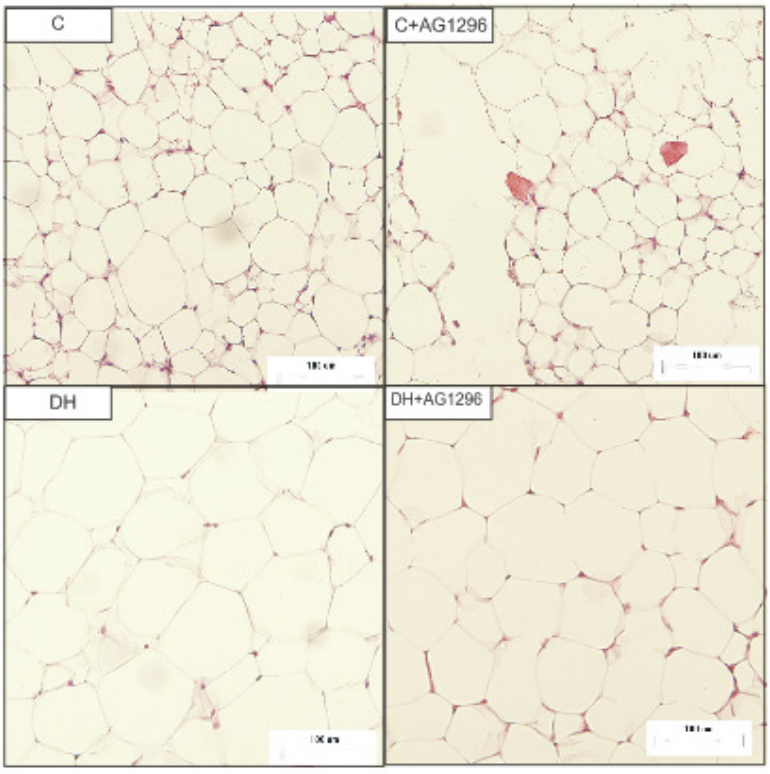
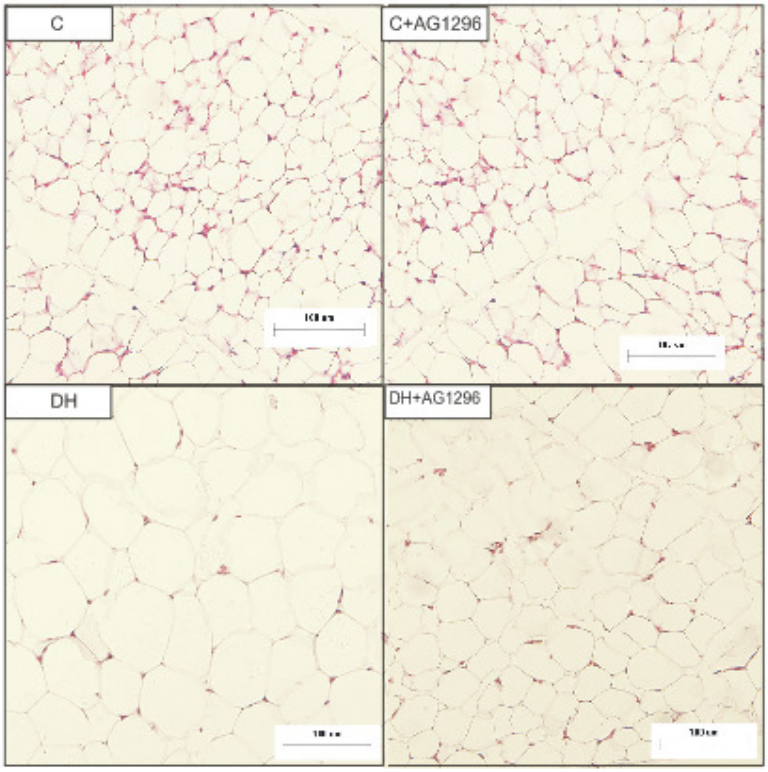
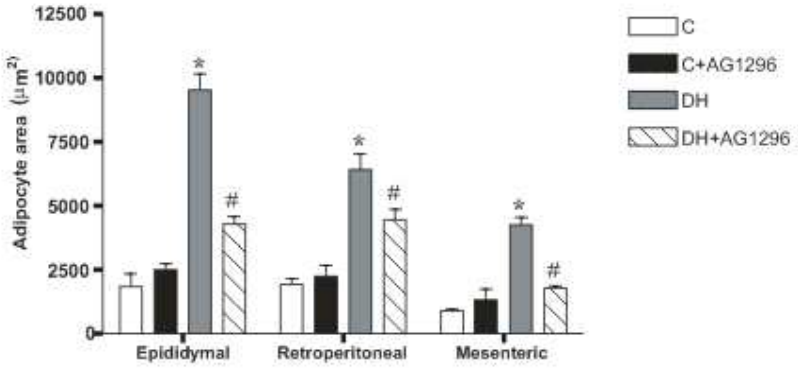


FIGURE 11

C. Mesenteric adipose tissue- 15 days



D.



Discussão

4-Discussão

O aumento no armazenamento de triglicerídeos nas células adiposas pode ser realizado de duas maneiras: a) pela expansão das células existentes (hipertrofia) ou b) pelo recrutamento de novos adipócitos (hiperplasia). Em adultos, a hipertrofia dos adipócitos é a maneira mais comum de acomodar o excesso de lipídeos, enquanto a hiperplasia predomina na fase jovem do indivíduo. A obesidade hipertrófica é também mais fortemente associada com resistência à insulina e complicações metabólicas do que a obesidade hiperplásica. O recrutamento de novas células adiposas é menos comum em adultos.

Um estudo recente mostrou que existe uma renovação continua de adipócitos em adultos numa taxa de aproximadamente 10% por ano (Spaldin et al, 2008). Esses novos adipócitos são formados a partir de células tronco mesenquimais ou outros precursores comprometidos com a linhagem dos pré-adipócitos (Gesta et al, 2007). Recentemente, demonstrou-se que os adipócitos descendem de células progenitoras já comprometidas com sua linhagem, na fase pré-natal ou jovem. Esses progenitores residem nas paredes dos vasos que irrigam o tecido adiposo. Além disso, esse estudo demonstrou que essas células expressam PDGFRβ e que este receptor é necessário para seu desenvolvimento (Tang et al, 2008).

Neste estudo, levando-se em consideração essas características das células precursoras dos adipócitos, nós investigamos o efeito de um inibidor do receptor PDGFβ no tecido adiposo e na diferenciação do adipócito e sua morfologia e também na sensibilidade à insulina e sinalização insulínica em camundongos jovens submetidos à dieta padrão e também à dieta hiperlipídica.

Existem muitas maneiras de relacionar dieta com adipogênese. Sabe-se que a insulina (o principal hormônio liberado após alimentação) promove a ação adipogênica do PPARγ, já que a diferenciação das células que expressam esse receptor aumenta (Hu et al). Outra ligação entre adipogênese e insulina é a proteína SREBP-1c, que é influenciada por esse hormônio (Streicher et al, 1996). Juntos, esses fatores podem explicar, pelo menos em parte, a conexão entre o aumento da adipogênese e excessos alimentares.

Os resultados demonstraram que a administração de AG1296 (um inibidor do receptor tirosina quinase PDGFRβ) a camundongos controle (submetidos à dieta padrão) reduziu tecido adiposo, sem alterações na morfologia dos adipócitos, associado a uma redução nas proteínas adipogênicas (principalmente PPARγ) e surpreendentemente melhorou a sinalização insulínica no fígado e no músculo, além de causar uma redução na glicemia de jejum desses animais.

Dados preliminares demonstraram que o PDGF é um importante indutor da fase proliferativa da diferenciação do pré-adipócito, porém seu efeito sobre a adipogênese é controverso. Estudos reportam que esse efeito pode ser inibitório (Navre et al, 1998), nulo (Schimidt, W, 1990) ou promotor (Bachmeier,M, 1995) da diferenciação de linhagens de pré-adipócitos.

A diferenciação terminal dos pré-adipócitos em adipócitos envolve uma cascata de fatores de transcrição altamente regulada e coordenada. C/EBPα e PPARγ são os fatores de transcrição pró-adipogênicos melhores caracterizados (Gregoire et al, 1998; Rosen and Spiegelman, 2001; Farmer, 2006), embora tenha sido demonstrado que a proteina SREBP-1c também tenha um papel importante neste evento (Payne et al, 2009). Nosso estudo demonstrou que o uso do inibidor do

PDGFRβ em camundongos controle sob dieta padrão modulou esses fatores de transcrição nas frações retroperitoneal e epididimal do tecido adiposo.

A redução no tecido adiposo foi acompanhada pela redução na glicemia de jejum e de uma melhora na sinalização insulínica no fígado e músculo dos animais controle. Juntos, esses dados indicam que a redução na massa adiposa aumentou a fosforilação da Akt, estimulada por insulina, no fígado e músculo e essa melhora na sinalização insulínica no fígado pode ter um papel na produção de glicose hepática, o que pode ter contribuído para a redução da glicemia de jejum. É possível que algum hormônio ou citocina produzida no tecido adiposo possa ter influenciado nesse processo. Contudo, os principais candidatos, que esperávamos estarem reduzidos, como IL-6 ou TNFα, não mostraram diferenças em seus níveis circulantes. Não podemos excluir a possibilidade de que os métodos utilizados para determinação dessas citocinas não tenham sido sensíveis o suficiente para detectar pequenas alterações. Além disso, um aumento nos níveis de adiponectina poderiam também explicar esses resultados, porém essas alterações também não foram detectadas.

Para investigar o efeito sobre o tecido adiposo do inibidor do receptor PDGFβ em situação de excesso de nutrientes, foram utilizados camundongos em dieta hiperlipídica. Nossos dados demonstraram que o uso desta droga nesses animais reduziu a expressão das proteínas adipogênicas no tecido adiposo, associado a mudanças na morfologia do adipócito e melhora na sinalização insulínica e na sensiblidade à insulina e tolerância à glicose.

Com 7 dias de dieta hiperlipídica, não foi possível detectar diferenças no peso corpóreo entre os grupos estudados, devido ao curto período deste regime alimentar. Porém, a distribuição da gordura corporal modificou-se. Como esperado,

os animais em dieta hiperlipídica tiveram maiores quantidades de gordura que aqueles alimentados com dieta padrão. O mesmo resultado foi obtido foi obtido em outros estudos utilizando o mesmo tipo de dieta (Prada et al, 2009; Jo et al, 2009). Contudo, como pode ser visto no tecido adiposo epididimal após 7 dias de tratamento, este aumento não foi observado no grupo em dieta hiperlipídica que recebeu o AG1296, o que sugere que este inibidor teve um efeito sobre a formação dessa fração de tecido adiposo. Não houve diferença entre a quantidade de energia ingerida através da dieta entre o grupo que recebeu apenas dieta hiperlipídica e o que recebeu também o tratamento com AG1296.

É importante mencionar que este efeito foi observado precocemente (7 dias) se comparado com os camundongos em dieta padrão, acompanhado a uma redução mais pronunciada na expressão das proteínas adipogênicas e uma redução na fração epididimal do tecido adiposo. Essas alterações precoces após 7 dias de dieta hiperlípídica e uso do AG1296 foram acompanhadas por uma melhora na sinalização insulínica. Houve um claro aumento na fosforilação do IR , IRS-1 e Akt no fígado, músculo e tecido adiposo.

Contudo, após 15 dias de dieta hiperlipídica e tratamento com AG1296, algumas dessas alterações, observadas aos 7 dias de tratamento, tornaram-se menos evidentes ou desapareceram. Nenhuma alteração nas frações epididimal, mesentérica ou retroperitoneal foi observada e a redução nos fatores de transcrição pró-adipogênicos também tornaram-se menos evidentes.Houve somente uma clara diminuição nos níveis de PPARγ no tecido adiposo. A melhora na sinalização insulínica no fígado desapareceu. Contudo, algumas modificações relevantes ainda estavam presentes: houve uma clara redução na área dos adipócitos e a melhora na sinalização insulínica no músculo manteve-se.

É bem estabelecido, atualmente, que uma melhora na sinalização insulínica no músculo pode reduzir a resistência à insulina e melhorar o metabolismo da glicose (Ropelle et al, 2006; Smith and Muscat, 2005). A melhora na fosforilação do IR, IRS-1 e da Akt induzida por insulina produzida pelo tratamento com AG1296 no músculo dos camundongos tratados com dieta hiperlipídica estava associada a um aumento da taxa de desaparecimento da glicose determinada durante o teste de teste de tolerância a insulina (ITT), indicando uma clara redução na resistência à insulina.

Em resumo, nossos resultados mostram que o uso do AG1296 e a conseqüente inibição do PDGFRβ tiveram um efeito sobre a adipogênese, promovendo a diminuição das principais proteínas adipogênicas em paralelo com uma melhora na sinalização insulínica em fígado, músculo e tecido adiposo. Portanto, sugerimos que a inibição do PDGFRβ pelo AG1296 pode representar uma alternativa interessante na prevenção da obesidade e resistência à insulina.

Conclusão

5-Conclusões

- ✓ As proteínas pró-adipogênicas foram afetadas pelo tratamento com AG1296.

- ✓ O tratamento com AG1296 por 7 dias foi capaz de reduzir a expressão das proteínas adipogênicas (PPARγ, C/EBPα e SREBP-1c) nos tecidos adiposos dos animais tratados com dieta hiperlipídica.

- ✓ O tratamento com AG1296 por 7 dias melhorou a sinalização insulínica nos tecidos adiposo epididimal, fígado e músculo dos animais tratados com dieta hiperlipídica e também a tolerância à glicose.

- ✓ De forma geral, o tratamento com AG1296 não causou alteração significativa nos níveis séricos das adipocinas e citocinas pró-inflamatórias estudadas.

- ✓ Após tratamento por 15 dias com AG1296, os camundongos controle tiveram tecido adiposo reduzido, sem alterações na morfologia dos adipócitos, associado a uma redução nas proteínas adipogênicas (principalmente PPARγ) e melhora na sinalização insulínica no fígado e no músculo, além de uma redução na glicemia de jejum.

✓ Conclusão Geral

O uso do AG1296 e a conseqüente inibição do PDGFRβ tiveram um efeito sobre a adipogênese, promovendo a diminuição das principais proteínas adipogênicas em paralelo com uma melhora na sinalização insulínica em fígado, músculo e tecido adiposo e pode representar uma alternativa interessante na prevenção da obesidade e resistência à insulina.

Referências Bibliográficas

6- Referências Bibliográficas

Ahima RS, Flier JS. Adipose tissue as an endocrine organ. **Trends Endocrinol Metab**. 11 (8): 327-332, 2000

Bachmeier M, Löffler G. The effect of platelet-derived growth factor and adipogenic hormones on the expression of CCAAT/enhancer-binding proteins in 3T3-L1 cells in serum-free conditions. **Eur J Biochem.** 243(1-2):128-33, 1997

Bastard JP, Maachi M, Lagathu C, Kim MJ, Caron M, Vidal H, Capeau J, Feve B. Recent advances in the relationship between obesity, inflammation, and insulin resistance. **Eur Cytokine Netw.** 17 (1): 4-12, 2006

Camp HS, Ren D, Leff T. Adipogenesis and fat-cell function in obesity and diabetes. **Trends in Molecular Medicine** 8 (9): 442-447, 2002

Carvalho-Filho MA, Ueno M, Hirabara SM, Seabra AB, Carvalheira JB, de Oliveira MG, Velloso LA, Curi R, Saad MJ. S-nitrosation of the insulin receptor, insulin receptor substrate 1, and protein kinase B/Akt: a novel mechanism of insulin resistance. **Diabetes** 54 (4): 959-967, 2005

Claesson-Welsh L. Mechanism of Action of Platelet-derived Growth Factor. **Int. J. Biochem. Cell Biol.** 28 (4): 373-385, 1996

Cooper JA, kazlauskas A. Signal transduction via PDGFbeta-receptor. In Biology of Platelet-derived Growth Factor. **Cytokines** (Edited by Westermark B. and Sorg C.) Vol 5, pp. 44-73. Karger, S. AG, Basel, 1993.

Farmer S.R. Transcriptional control of adipocyte formation. **Cell Metab.** 4 (4): 263-273, 2006

Fonseca-Alaniz, M. H., Takada, J., Alonso-Vale, M. I. C., Lima, F. B. O tecido adiposo como órgão endócrino: da teoria à prática. ***J. Pediatr.*** 83(5 suppl): S192-303, 2007

Frayn KN, Karpe F, Fielding BA, Macdonald IA, Coppack SW. Integrative physiology of human adipose tissue. **International Journal of Obesity** 27 (8): 875–888, 2003

Gesta S, Tseng Y, Kahn R. Developmental origin of Fat: Tracking Obesity to its Source. **Cell** 131 (2): 242-56, 2007

Gregoire FM, Smas CM, Sul HS. Understanding adipocyte differentiation. **Physiol Rev.** 78 (3):.783-809, 1998

Grundy SM. Obesity, metabolic syndrome, and cardiovascular disease. **J Clin Endocrinol Metab** 89 (6): 2595–2600, 2004

Heldin C-H. Dimerization of cell surface receptors in signal transduction. **Cell** 80 (2): 213-223, 1995

Hirsch J, and Batchelor B. Adipose tissue cellularity in human obesity. **Clin. Endocrinol. Metab**. 5 (2): 299-311, 1976

Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. **Science** 259 (5091): 87-9, 1993

Hotamisligil GS. Inflammatory pathways and insulin action. **Int. J. of Obesity** 27 (suppl 3): S53-S55, 2003

Hotamisligil, G. S., P. Arner, et al. Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. **J Clin Invest** 95(5): 2409-2415, 1995

Hu E, Tontonoz P and Spiegelman BM. Transdifferentiation of myoblasts by the adipogenic transcription factors PPARγ and C/EBPα. ***Proc. Natl. Acad. Sci. USA*** 92 (21): 9856–9860, 1995.

Jiang Y, Jahagirdar BN , Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM. Pluripotency of mesenchymal stem cells derived from adult marrow. **Nature** 418 (6893): 41-49, 2002

Jo J., Gavrilova O., Pack S., Jou W., Mullen S., et al. Hypertrophy and/or Hyperplasia: Dynamics of Adipose Tissue Growth. **PLoS Comput Biol** 5 (3): e1000324 , 2009

Kern PA, Di Gregorio GB, Lu T, Rassouli N, Ranganathan G. Adiponectin Expression From Human Adipose Tissue Relation to Obesity, Insulin Resistance, and Tumor Necrosis Factor-α Expression. **Diabetes** 52 (7): 1779-1785, 2003

Kershaw EE, Flier JS. Adipose tissue as an endocrine organ. **J Clin Endocrinol Metab** 89 (6): 2548-56, 2004

Konieczny SF, Emerson CP. 5-azacytidine induction of stable mesodermal stem cell lineages from 10T1/2 cells: evidence for regulatory genes controlling determination. **Cell** 38 (3): 791-800, 1984.

Kopelman PG. Obesity as a medical problem. **Nature** 404 (6778): 635-43, 2000.

Kovalenko M., Gazit A., Böhmer A., Rorsman CH.,Rönnstrand L., Heldin C.-H., Waltenberger J., Böhmer F. D. & Levitzki, A. Selective Platelet-derived Growth Factor Receptor Kinase Blockers Reverse sis-Transformation **Cancer Res.** *54 (23):* 6106-6114, 1994

Kovalenko M., Rönnstrand L., Heldin C.-H., Loubtchenkov M., Gazit A., Levitzki A., Böhmer F. D. Phosphorylation Site-Specific Inhibition of Platelet-derived Growth

Factor β-Receptor Autophosphorylation by the Receptor Blocking Tyrphostin AG1296. **Biochemistry** 36 (21): 6260-6269, 1997

Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. ***Nature*** 227 (5259):680-685, 1970

Lane MD, and Tang QQ From multipotent stem cell to adipocyte. **Birth Defects Res. A. Clin. Mol. Teratol**. 73 (7): 476-477, 2005

Lane MD, Tang Q, Jiang M. Role of the CCAAT Enhancer Binding Proteins (C/EBPs) in Adipocyte Differentiation **Biochem Biophys Res Commun** 266 (3): 677-83, 1999

Lefterova MI and Lazar MA. New developments in adipogenesis **Trends in Endocrinology and Metabolism** 20 (3): 107-114, 2009

Levitzki A. PDGF receptor kinase inhibitors for the treatment of PDGF driven diseases. **Cytokine & Growth Factor Reviews** 15 (4): 229-235, 2004

Linhart HG, Ishimura-Oka K, DeMayo F, Kibe T, Repka D, Poindexter B, Bick RJ, Darlington GJ. C/EBPalpha is required for differentiation of white and brown adipose tissue. **Proc Natl. Acad. Sci.** 98 (22): 12532-12537, 2001

Mundaliar S, Henry RR. New oral therapies for type 2 diabetes mellitus: The glitazones or insulin sensitizers. **Annu. Rev. Med.** 52: 239-257, 2001

Navre M, Ringold GM. A Gowth Factor-repressible Gene Associated with protein Kinase C-mediated Inhibition of Adipocyte Differentiation. **J Cell Biol.** 107(1):279-86, 1998

Otto TC, Lane MD. Adipose development: from stem cell to adipocyte. **Crit. Rev. Biochem. Mol. Biol.** 40 (4): 229-242, 2005

Payne VA, Au WS, Lowe CE, Rahman SM, Friedman JE, O'Rahilly S, Rochford JJ. C/EBP transcription factors regulate SREBP1c gene expression during adipogenesis. **Biochem J**. 425(1): 215-23, 2009.

Pietras K., Sjoblom T, Rubin K, Heldin CH, Ostman A. PDGF receptors as cancer drug targets. **Cancer Cell** 3 (5): 439-43, 2003

Prada PO, Ropelle ER, Mourão RH, de Souza CT, Pauli JR, Cintra DE, Schenka A, Rocco SA, Rittner R, Franchini KG, Vassallo J, Velloso LA, Carvalheira JB, Saad MJ. EGFR tyrosine kinase inhibitor (PD153035) improves glucose tolerance and insulin action in high-fat diet-fed mice. **Diabetes** 58 (12): 2910-9, 2009

Prins JB, and O'Rahilly S. Regulation of adipose cell number in man. **Clin. Sci. (Lond.)** 92(1): 3-11, 1997.

Raines EW, Ross R. Platelet-derived growth factor in vivo. Em **Biology of Platelet-Derived Growth Factor Cytocines** (Editado por Westernmark B. e Sorg C.) 5: 74-114, 1993.

Rodeheffer M, Birsoy K, Friedman, JM. Identification of white adipocyte progenitor cells in vivo. **Cell** 135(2):.240-249, 2008

Ropelle ER, Pauli JR, Prada PO, de Souza CT, Picardi PK, Faria MC, Cintra DE, Fernandes MF, Flores MB, Velloso LA, Saad MJ, Carvalheira JB. Reversal of diet-induced insulin resistance with a single bout of exercise in the rat: the role of PTP1B and IRS-1 serine phosphorylation. **J Physiol.** 577(3):997-1007, 2006.

Rosen ED, MacDougld OA. Adipocyte differentiation from the inside out. **Nature Rev Mol. Cell Bio** 7 (12): 885-896, 2006

Rosen ED, Walkey CJ, Puigserver P., Spiegelman BM. Transcriptional regulation of adipogenesis. **Genes Dev.** 14 (11): 1293-1307, 2000

Schmidt W, Pöll-Jordan G, Löffler G. Adipose conversion of 3T3-L1 cells in a serum-free culture system depends on epidermal growth factor, insulin-like growth factor I, corticosterone, and cyclic AMP. **J Biol Chem.** 265(26):15489-95, 1990

Sharma AM, Staels B. Review: Peroxisome proliferator-Activated Receptor γ and Adipose Tissue- Understanding Obesity-Related Changes in regulation of Lipid and Glucose Metabolism. **J Clin Endocrinol Metab.** 92 (2): 386-395, 2007

Smith AG, Muscat GE. Skeletal muscle and nuclear hormone receptors: implications for cardiovascular and metabolic disease**. Int J Biochem Cell Biol.** 37(10):2047-63, 2005

Spalding KL, Arner E, Westermark PO, Bernard S, Buchholz BA, Bergmann O, Blomqvist L, Hoffstedt J, Näslund E, Britton T, Concha H, Hassan M, Rydén M, Frisén J, Arner P. Dynamics of fat cell turnover in humans. **Nature** 453 (7196): 783-787, 2008

Streicher R, Kotzka J, Muller-Wieland D, Siemeister G, Munck M, Avci H and Krone W. SREBP-1 mediates activation of the low density lipoprotein receptor promoter by insulin and insulin-like growth factor-1. ***J. Biol. Chem.*** 271 (12): 7128–7133, 1996.

Sunyer P, Xavier F. The obesity epidemic: pathophysiology and consequences of obesity. **Obes Res**. 10 (Suppl 2): 97S–104S, 2002

Spiegelman BM, Flier JS. Obesity and Regulation of Energy Balance. **Cell** 104 (4): 531-543, 2001.

Tang W, Zeve D, Suh, J, Bosnakovski D, Kyba M, Hammer B, Tallquist MD, Graff JM. White fat progenitor cells reside in adipose vasculature. **Science** 322 (5901): 583-586, 2008.

Thirone AC, Carvalheira JB, Hirata AE, Velloso LA, Saad MJ. Regulation of Cbl-associated protein/Cbl pathway in muscle and adipose tissues of two animal models of insulin resistance. **Endocrinology** 145 (1): 281-293, 1994

Tontonoz P, Hu E, Spiegelman BM. Regulation of adipocyte gene expression and differentiation by peroxisome proliferator activated receptor gamma. **Curr Opin Genet Dev.** 5 (5) : 571-6, 1995

Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. **Proc Natl Acad Sci U S A** 76(9): 4350-4354, 1979

Tsukumo DM, Carvalho-Filho MA, Carvalheira JB, Prada PO, Hirabara SM, Schenka AA, Araujo EP, Vassallo J, Curi R, Velloso LA, Saad MJ**.** Loss-of-function mutation in Toll-like receptor 4 prevents diet-induced obesity and insulin resistance. **Diabetes** 56 (8): 1986-1998, 2007

UIlrich A, Schlessinger J. Signal transduction by receptors with tyrosine kinase activity. **Cell** 61 (2):203-212, 1990

Xu H., Barnes G. T., Yang Q., Tan G., Yang D., Chou C.J., Sole J, Nichols A., Ross J. S, Tartaglia L.A., Chen H. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. **J. Clin. Invest.** 112 (12): 1821–1830, 2003.

Warne JP. Tumour necrosis factor alpha: a key regulator of adipose tissue mass. **J Endocrinol** 177 (3): 351-5, 2003

Wellen, K. E. and Hotamisligil GS. Inflammation, stress, and diabetes. **J Clin Invest** 115(5): 1111-1119, 2005.

Wisse BE. The inflammatory syndrome: the role of adipose tissue cytokines in metabolic disorders linked to obesity. **J Am Soc Nephrol**. 15 (11): 2792–2800, 2004

World Health Organization. Obesity and overweight. Fact sheet nº 311. WHO Web site. http://www.who.int/mediacentre/factsheets/fs311/en/ . March 2011.

Wu Z, Rosen ED, Brun R, Hauser S, Adelmant G, Troy AE, McKeon C, Darlington GJ, Spiegelman BM. Cross-Regulation of C/EBPα and PPARγ Controls the Transcriptional Pathway of Adipogenesis and Insulin Sensitivity. **Molecular Cell** 3 (2): 151-158, 1999