GUILHERME ZWEIG ROCHA

"A Atuação da Proteína Quinase Dependente de dsRNA (PKR) no Desenvolvimento de Tumor de Cólon em Camundongos Obesos"

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"A Atuação da Proteína Quinase Dependente de dsRNA (PKR) no Desenvolvimento de Tumor de Cólon em Camundongos Obesos"

Orientador: Prof. Dr. José Barreto Campello Carvalheira

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Fisiopatologia Médica da Faculdade de Ciências Médicas da Universidade Estadual de Campinas, para obtenção do título de Doutor em Ciências.

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SUMÁRIO

PÁG.

LISTA DE FIGURAS ..... *xi*

RESUMO ..... *xii*

ABSTRACT ..... *xiv*

INTRODUÇÃO **16**

Introdução **17**

Obesidade e Câncer de Cólon **18**

Interrelaçãoentre Inflamação e Câncer **22**

a)Aspectos Epidemiológicos **22**

b) Carcinogênese e Inflamação **23**

c) Câncer de Cólon associado à Inflamação **25**

Obesidade e Inflamação Subclínica e Resistência à Insulina **26**

PKR: Características e Funções **33**

OBJETIVOS **38**

Objetivo Geral **39**

Objetivos específicos **39**

CAPÍTULO I **41**

Obesity-Induced Increase in Tumor Necrosis Factor α Leads to Development of Colon Cancer in Mice **42**

CAPÍTULO II..... 59

Absence of Double-Stranded RNA Dependent Protein Kinase Protects From Development of Colitis-Associated Cancer **..... 60**

DISCUSSÃO..... 93

Discussão 94

CONCLUSÕES 98

Conclusão Geral 99

REFERÊNCIAS BIBLIOGRÁFICAS 100

Referências Bibliog'rficas..... 101

APÊNDICES..... 104

APÊNDICE I 105

Insulin Receptor Substrate 1 Mediates Irinotecan-induced pPI3K/Akt/mTOR Pathway Activation in Colon Cancer**..... 106**

APÊNDICE II..... 133

Lista de Publicações no período..... 134

LISTA DE FIGURAS

Figura 1. Fatores de Risco comuns para Resistência à Insulina e Câncer de Cólon

Figura 2. Modelo esquemático das vias de sinalização celular relacionadas com a inibição de síntese e degradação protéica e indução de apoptose dependentes da ativação da PKR.

RESUMO

Embora a obesidade seja reconhecida como importante causa de diabetes e doença cardiovascular, a associação entre obesidade e diferentes tipos de câncer tem recebido muito menos atenção. A associação entre obesidade e o desenvolvimento de câncer de cólon representa um dos principais avanços conceituais na patogênese do câncer de cólon da última década. Recentemente a atuação da inflamação subclínica da obesidade na carcinogênese ganhou destaque. Mecanisticamente acredita-se que a obesidade atue como promotor tumoral, e seus efeitos pró-tumorigênicos dependam principalmente da resposta inflamatória de baixo grau ocasionada pela obesidade que envolve a produção de citocinas inflamatórias e pró-tumorigênicas (TNF e IL-6). Uma das principais características da inflamação induzida por obesidade é a infiltração de macrófagos no tecido adiposo, produzindo citocinas inflamatórias e outros mediadores que interferem na sinalização insulínica. Inflamação e estresse de retículo que são conectadas em diversos níveis, são sistemas adaptativos de curto período de expressão necessárias para a função e sobrevivência do organismo, e ambas são prejudiciais quando ativadas cronicamente. Neste sentido, a ativação da PKR durante a inflamação e posterior ativação de JNK pela PKR, também interfere e prejudica a via de sinalização da insulina. A relação entre o câncer de cólon e obesidade pode ser devido a ação, em nível molecular, da inflamação subclínica de baixo grau e ao estresse celular causado por essa sinalização inflamatória. Sendo a PKR responsiva à sinalização inflamatória e também à via insulínica em outros tecidos, e relacionada à carcinogênese e à progressão em diversos tipos de câncer, a investigação de sua participação é relevante a medida que propicia o entendimento da fisiopatologia molecular de tumores de cólon. Assim, o objetivo principal do estudo foi avaliar o papel da

PKR no desenvolvimento de tumores de cólon em camundongos submetido a dieta hiperlipídica. A ausência de PKR previne a formação de tumores. Além disso, aparentemente a ausência de PKR em células mielóides também confere proteção contra a resistência à insulina induzida por dieta hiperlipídica, reduzindo a inflamação induzida pela obesidade. Essas observações demostram que a PKR pode ser um ponto principal durante a carcinogênese associada à inflamação e pode representar um promissor alvo para a intervenção terapêutica.

ABSTRACT

Although obesity is recognized as a major cause of diabetes and cardiovascular disease, the association between obesity and different types of cancer has received much less attention. The association between obesity and the development of colon cancer is one of the major conceptual advances in the pathogenesis of colon cancer in the last decade. Recently the role of subclinical inflammation in obesity and in carcinogenesis gained prominence. Mechanistically it is believed that obesity acts as a tumor promoter, and their pro-tumorigenic effects depend mainly on low-grade inflammatory response caused by obesity, involving the production of inflammatory and pro-tumorigenic cytokines (TNF and IL-6). A key feature of obesity-induced inflammation is the infiltration of macrophages in adipose tissue, producing inflammatory cytokines and other mediators that interfere with insulin signaling. Reticulum stress and inflammation are connected on many levels and work as short period adaptive systems required for the function and survival of the organism, and both are detrimental when chronically activated. In this regard, the activation of PKR during inflammation and subsequent activation of JNK by PKR also interferes and impairs insulin signaling pathway. Thus, PKR can form a metabolically active inflammatory complex which then becomes part of the of insulin pathway and of the pathogens response pathway and control of translation sensible to nutrients. The relationship between colon cancer and obesity may be due to action at the molecular level, subclinical low-grade inflammation and cellular stress caused by this inflammatory signaling. PKR is responsive to inflammatory signaling and also to the insulin pathway in other tissues, and related to carcinogenesis and progression in several types of cancer. Thus, investigation of it`s participation is relevant as it provides the understanding of the molecular pathophysiology

of colon tumors. Thus, the main objective of the study was to evaluate the role of PKR in the development of colon tumors in mice subjected to a high-fat diet. The absence of PKR prevents the formation of tumors. Moreover, apparently the absence of PKR in myeloid cells also confers protection against resistance to insulin induced by a high-fat diet, reducing inflammation induced by obesity. These observations demonstrate that PKR can be a primary point during carcinogenesis associated with inflammation and may represent a promising target for therapeutic intervention.

INTRODUÇÃO

Estudos epidemiológicos da ultima década apontam a obesidade como importante condição predisponente a maior morbidade e mortalidade. A prevalência da obesidade vem aumentando em curtos períodos de tempo em todos os países desenvolvidos, com raras exceções, bem como nos países em desenvolvimento [1]. Atualmente, mais de 1 bilhão de adultos tem sobrepeso – e pelo menos 300 milhões desses são clinicamente obesos. Os atuais níveis de obesidade variam de menos de 5% na China, no Japão e em alguns países africanos, para mais de 75% na Samoa urbana. Mas, mesmo em países com baixa prevalência como a China, as taxas são de quase 20% em algumas cidades. Nos Estados Unidos cerca de 20% dos homens e 25% das mulheres são obesas [2]. No Brasil, em 2003 o excesso de peso afetava 41,1% dos homens e 40% das mulheres, sendo que obesidade afetava 8,9% dos homens e 13,1% das mulheres adultas do país. Sendo assim, os obesos representavam 20% do total de homens e um terço das mulheres com excesso de peso.

Embora a obesidade seja reconhecida como importante causa de diabetes e doença cardiovascular, a associação entre obesidade e diferentes tipos de câncer tem recebido muito menos atenção. Em um grande estudo, envolvendo mais de 900.000 indivíduos acompanhados prospectivamente por 16 anos, Calle *et al.* em 2003 [3] observaram que a taxa de mortalidade combinada por cânceres de diferentes localizações, em indivíduos com índice de massa corporal (IMC) maior que 40, foi 52% (homens) e 62% (mulheres) superior a de pessoas com peso normal. Tal tendência mostrou-se verdadeira também para tumores diretamente relacionados ao tabagismo (pulmão, esôfago), mesmo quando comparada entre indivíduos não fumantes. Com efeito, acredita-se que a obesidade atualmente seja a causa de 15-20% de todos os cânceres nos

Estados Unidos, constituindo-se dessa maneira no principal fator de risco para o desenvolvimento de câncer em indivíduos não fumantes.

OBESIDADE E CÂNCER DE CÓLON

A obesidade foi associada ao aumento no risco de câncer de cólon. No entanto, existem diferenças importantes quanto à associação por gênero e sítio. A relação entre IMC e risco de câncer de cólon foi positiva no sexo masculino, RR=1,24, embora as evidências no sexo feminino sejam menos claras, RR=1,09 [4].

Existem algumas hipóteses para explicar estas diferenças entre os gêneros. Uma delas, reforçada por evidências epidemiológicas, é de que a adiposidade central, mais frequentemente observada entre os homens, pode desempenhar um importante papel fisiopatológico, levando à associação entre obesidade abdominal e aumento no risco de câncer de cólon. Esta associação poderia ser explicada devido à maior implicação do aumento da circunferência abdominal com alterações metabólicas quando comparado à obesidade gluteofemoral. Neste sentido, existem consistentes resultados que associam resistência à insulina e hiperinsulinemia ao risco de câncer de cólon.

A associação entre obesidade e o desenvolvimento de câncer de cólon representa um dos principais avanços conceituais na patogênese do câncer de cólon da última década [5-6]. A incidência de sobrepeso e obesidade, condições freqüentemente associadas à *diabetes melittus* tipo 2, é crescente e já alcançou níveis alarmantes no ocidente [7-9]. Por outro lado, o câncer de cólon é a segunda causa mais freqüente de câncer no mundo ocidental e sua incidência aumenta marcadamente em indivíduos idosos. Portanto, a magnitude desse problema de saúde pública

tende a crescer, particularmente porque pessoas idosas são mais resistentes à insulina e espera-se que o número de pessoas idosas aumente significativamente nas próximas décadas.

A obesidade tem sido consistentemente associada ao aumento do risco de desenvolver câncer de cólon em homens (RR aproximadamente 1,3) e em mulheres (RR aproximadamente 1,1) [10]. Há vários anos sabe-se, através de estudos de carcinogênese em animais, que a restrição alimentar e exercício físico inibem marcadamente o desenvolvimento de câncer de cólon [11-12], e que dietas hipercalóricas e ricas em gordura promovem a carcinogênese colônica [13]. Mc-Keown-Eyessen [6] e Giovannucci [5] notaram que os fatores de riscos de câncer de cólon são muitos semelhantes aos da resistência à insulina (Figura 1). Isso levou os autores a sugerirem que o estilo de vida e os hábitos alimentares seriam causa da resistência à insulina e hiperinsulinemia, e que esta agiria estimulando o crescimento das células do câncer de cólon. Atualmente muitas evidências sustentam a associação entre câncer de cólon e resistência à insulina. Estas incluem: (*a*) estudos de coorte de pacientes que têm evidência de resistência à insulina ou diabetes que subseqüentemente reportaram maior incidência de câncer de cólon [14-17], (*b*) estudos de caso-controle de pacientes com polipose colônica e câncer de cólon que mostram maior nível de insulina, triglicérides, VLDL, maior obesidade abdominal ou tolerância anormal à glicose nos pacientes afetados que no grupo controle [18-19] e (*c*) estudos prospectivos que mostram maior mortalidade por câncer de cólon em indivíduos obesos [3].

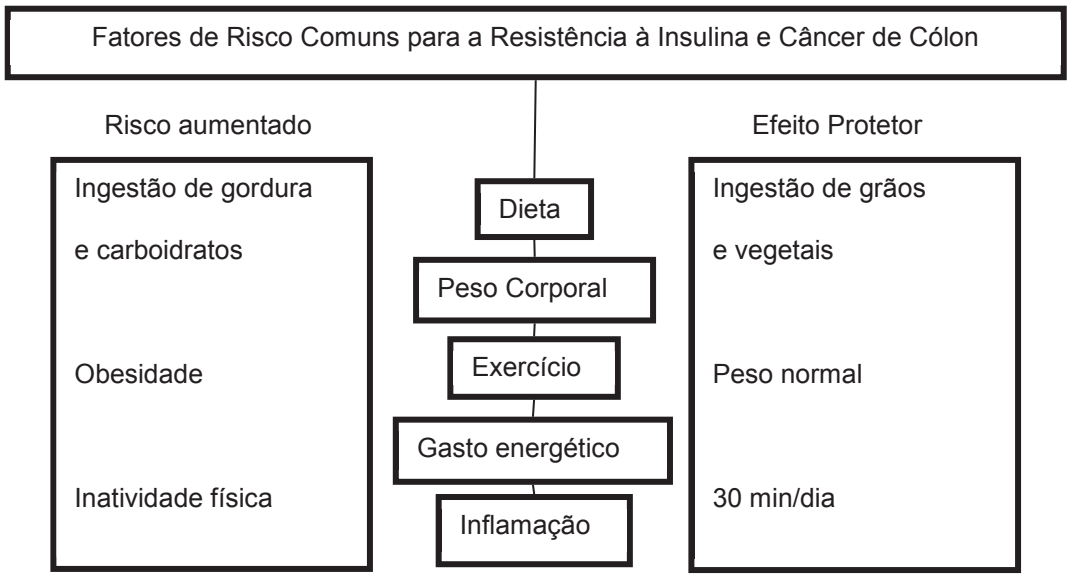

Figura 1: Fatores de Risco comuns para Resistência à Insulina e Câncer de Cólon

Atualmente, os mecanismos que conectam a obesidade com o risco aumentado de desenvolver câncer de cólon envolvem os efeitos endócrinos e metabólicos da obesidade. Nesse contexto, destacam-se a hiperinsulinemia crônica e a resistência à insulina [20].

A hiperinsulinemia crônica está associada com a patogênese do câncer de cólon [21]. Esses efeitos podem ser mediados diretamente pela presença de receptores de insulina nas células pré-neoplásicas estimulando o crescimento [22], ou ter a sua gênese mediada por mecanismos comuns que ocasionam a resistência à insulina como, por exemplo, a inflamação crônica subclínica com o aumento do TNFα que agiria como agente promotor do crescimento tumoral [23].

A insulina é o principal hormônio na regulação da glicemia em mamíferos, que age estimulando a captação de glicose em tecidos muscular e adiposo e inibindo a gliconeogênese no tecido hepático, como também modificando a expressão ou atividades de uma série de enzimas e sistemas de transporte [24]. Desde a descoberta desse hormônio, muito tem sido feito no sentido de

se entender os mecanismos moleculares de sua ação. Além disso, dados pré-clínicos e clínicos implicam o eixo do fator análogo à insulina (IGF) à carcinogênese coloretal. A insulina exerce diversas influências sobre este eixo, como aumento nos níveis de IGF-1, bem como redução da síntese de IGFBP. Dessa forma, as implicações da hiperinsulinemia sobre o eixo do IGF podem ser um dos mecanismos responsáveis pelo aumento no risco de câncer de cólon associado à obesidade.

Em relação às citocinas e hormônios derivados do tecido adiposo, resultados recentes associam estas adipocinas à carcinogênese do cólon. Quanto à leptina, cujos níveis circulantes são intimamente relacionados ao volume de tecido adiposo e à resistência à insulina, dados pré-clínicos a implicam na progressão desta neoplasia. É importante ressaltar que os níveis de leptina encontram-se elevados em situações de alto consumo alimentar e estoque energético, por outro lado quando há restrição calórica estes diminuem. Neste sentido, destacam-se consistentes dados que associam restrição calórica à proteção ao câncer. Estas evidências sugerem que a leptina possa influenciar a associação entre obesidade e câncer de cólon. Dois estudos de caso-controle encontraram relação significativa para esta associação, reforçando a hipótese. Por outro lado, a adiponectina é inversamente relacionada ao desenvolvimento de resistência à insulina, além de apresentar propriedades antiinflamatórias. Seus níveis séricos costumam ser baixos em indivíduos acima do peso. Até o momento, os dados que relacionam baixos níveis de adiponectina ao aumento no risco de câncer coloretal são controversos e há necessidade de novos estudos prospectivos para investigar esta hipótese.

Entretanto, recentemente a atuação da inflamação subclínica da obesidade na gênese do câncer ganhou destaque. Em um estudo relacionando obesidade e hepatocarcinogênese, a

obesidade atua como promotor tumoral, e seus efeitos pró-tumorigênicos dependem principalmente da resposta inflamatória de baixo grau ocasionada pela obesidade que envolve a produção de citocinas inflamatóriase pró-tumorigênicas TNF e IL-6 e ativação de STAT3 [25].

INTER-RELAÇÃO ENTRE INFLAMAÇÃO E CÂNCER

a) Aspectos epidemiológicos

Estados de inflamação crônica provocados por vírus ou bactérias são responsáveis por aproximadamente 1,2 milhões de casos novos de neoplasia ao ano [26-27]. Diversos vírus e bactérias como papilomavírus, vírus das hepatites B e C , Epstein-Barr vírus e *Helicobacter pylori* são fatores de risco importantes para câncer cervical, hepatocarcinoma, doenças linfoproliferativas, respectivamente. Mecanismos inflamatórios, bem como outros de inibição de proteínas de supressão tumoral através dos quais infecções crônicas podem levar à promoção e progressão tumoral são conhecidos[28]. Além disso, existem várias outras causas não-infecciosas de inflamação crônica, como doença inflamatória intestinal e agentes externos (tabaco, asbestos, sílica), que aumentam o risco de desenvolvimento de câncer. Os órgãos mais suscetíveis a estados de inflamação crônica e conseqüente desenvolvimento neoplásico são: pulmões, bexiga, e mais freqüentemente o trato gastrointestinal.

Além dos dados epidemiológicos, existem outras evidências que associam inflamação crônica ao câncer. Polimorfismos no gene do fator de necrose tumoral (TNF) com promoção de níveis aumentados de TNF estão associados com pior prognóstico em pacientes com Linfoma não-Hodgkin [29]. Ainda mais, o uso prolongado de anti-inflamatórios não-esteroidais, como a aspirina, reduz a incidência de câncer de cólon, pulmão, estômago, esôfago, ovário, bem como Linfoma de Hodgkin [30-33]. Embora existam controvérsias quanto a esta relação devido ao relato

do aumento do risco de desenvolvimento de câncer pancreático ou Linfoma não-Hodgkin, através de mecanismos ainda não muito bem compreendidos.

b) Carcinogênese e inflamação

O processo de carcinogênese pode ser, convenientemente, dividido em três fases. Na primeira delas, iniciação, o DNA da célula sofre mutações pela ação de carcinógenos físicos ou químicos, promovendo ativação de oncogenes e/ou inativação de genes supressores tumorais. A segunda fase da carcinogênese, promoção, é caracterizada por expansão clonal das células geneticamente alteradas, através do aumento da proliferação celular e/ou redução de morte celular. Finalmente, a terceira fase da carcinogênese (progresão) é caracterizada por invasão e metástases, bem como crescimento no tamanho do tumor. Durante essa última fase, aquisições de novas mutações que favoreçam um fenótipo maligno podem acontecer.

De forma geral, inflamação e imunidade podem afetar cada uma dessas fases de diferentes maneiras. Comumente inflamação e imunidade inata costumam exercer efeitos pró-tumorigênicos, enquanto que imunidade adaptativa apresenta ação potencialmente anti-tumorigênica [34-36]. Esses efeitos são mediados por diversos tipos de leucócitos, incluindo macrófagos, macrófagos associados ao tumor, células dendríticas, neutrófilos, mastócitos e células T, recrutados ao microambiente tumoral através de interações com células do estroma local e células malignas. Esses leucócitos produzem citoquinas, fatores angiogênicos e de crescimento, bem como metaloproteinases e seus inibidores, que possibilitam proliferação das células malignas, invasão e disseminação à distância.

A expressão de diversas citoquinas pró-inflamatórias, como TNF, IL-1 e IL-8 é regulada por genes alvo da via de ativação do NF-κB dependente do IKKβ. Ainda mais, muitos oncogenes

e carcinógenos provocam ativação do NF-κB, enquanto que substâncias com conhecidas propriedades quimiopreventivas podem interferir com sua ativação. Recentes estudos em modelo animal trouxeram fortes e diretas evidências genéticas de que a via de ativação do NF-κB dependente do IKKβ é um mediador crucial na promoção tumoral [37-38].

Existem duas vias de ativação do NF-κB [39]. A via clássica é ativada por estímulos pró-inflamatórios, dentre os quais: citoquinas (TNF, IL-1), proteínas da membrana celular bacteriana (lipopolissacárides), vírus. Estas substâncias promovem fosforilação do IκB dependente do IKKβ e do IKKγ, resultando em ubiquitinação proteossomal e conseqüente liberação dos dímeros do NF-κB (geralmente p50-REL-A) para migração nuclear e transcrição de genes alvo. A via alternativa é ativada por membros da família do TNF e, de forma independente do IKKβ e IKKγ, promove fosforilação do p100 através do IKKα, resultando na translocação nuclear dos dímeros p52-REL-B. A ativação da via clássica, que envolve os dímeros p50-REL-A, promove aumento na transcrição de genes de três classes funcionais, todos eles contribuintes para promoção e progressão tumoral. Os genes transcritos expressam proteínas envolvidas na regulação do ciclo proliferação/morte celular como Bcl-X_L (linfoma de célula B X_L), cIAPs (inibidores celulares de apoptose), GADD45β (bloqueador de proliferação e indutor de dano ao DNA 45β), BFL1 (proteína 2 relacionada ao linfoma de célula B), SOD2 (superoxido dismutase 2); proteínas do sistema imune inato como citoquinas, proteases e moléculas de adesão, ou mediadores inflamatórios como COX2, iNOS, TNF, IL-6.

A contribuição da via clássica de ativação do NF-κB na inflamação e proliferação celular é bem aceita e a ativação sustentada do NF-κB foi descrita em diversas neoplasias [40]. Devido à variedade de genes alvo da via clássica, incluindo aqueles que expressam mediadores inflamatórios, foi proposto que a via clássica de ativação do NF-κB seja determinante na

associação entre inflamação e promoção e progressão tumoral [40]. A utilização de camundongos em estudos sobre câncer hepático e colônico associados à inflamação apóiam esta hipótese e explicam como a inflamação impulsiona a promoção e progressão tumoral [37-38].

c) Câncer de cólon associado à inflamação

Em modelo animal de câncer de cólon associado à inflamação (CCAI), a deleção do IKKβ (promovendo diminuição da atividade do NF-κB) nos enterócitos revelou que a atividade de promoção tumoral do NF-κB resultou de sua propriedade anti-apoptótica sobre as células pré-malignas. Neste modelo, camundongos eram expostos ao pró-carcinógeno azoximetano, que sofre metabolização nos enterócitos, seguido por administração oral de dextran-sulfato de sódio, indutor de colite crônica através da ruptura da barreira intestinal e exposição dos macrófagos da lâmina própria às bactérias entéricas. A exposição dos macrófagos às bactérias resulta em ativação do NF-κB nessas células através da sinalização de toll-like receptors, provocando, assim, produção e secreção de citoquinas pró-inflamatórias que ativam NF-κB nas células epiteliais intestinais. Ablação específica do IKKβ nos enterócitos mostrou diminuição na incidência tumoral, embora não tenha afetado o tamanho e composição tumoral (progressão) ou indução nas mutações em oncogenes (iniciação), o que indica que a ativação da via de ativação do NF-κB dependente do IKKβ atua desde o início do processo de promoção tumoral [37].

Um segundo mecanismo pelo qual NF-κB pode afetar a promoção tumoral no CCAI resulta de sua propriedade em induzir à produção de mediadores pró-inflamatórios pelas células mielóides. Este mecanismo foi identificado através da ablação específica do IKKβ nas células mielóides. Esta deleção diminuiu tanto a quantidade como o tamanho dos tumores, resultado não observado quando foi realizada ablação enterócito específica [37]. A diminuição no tamanho tumoral teria acontecido como conseqüência da menor proliferação das células epiteliais

malignas, uma vez que elas necessitam de fatores de crescimento produzidos pelas células mielóides. Um desses fatores parece ser a IL-6, pois em estudo de CCAI em modelo animal a administração de anticorpos específicos para o receptor da IL-6 também provocou diminuição na quantidade e tamanho tumoral [41], resultado semelhante aquele da ablação mielóide específica do IKKβ. Além disso, em modelo de carcinoma hepático com camundongos knockout Mdr2 o estroma tumoral também é importante fonte de citoquinas, neste caso o TNF, que favorecem a proliferação das células malignas[38]. Dessa forma, em ambos os modelos animais, o NF-κB promove proliferação de células tumorais através da oferta de fatores de crescimento pelo estroma tumoral ou células inflamatórias associadas ao tumor.

OBESIDADE E INFLAMAÇÃO SUBCLÍNICA E RESISTÊNCIA À INSULINA

Acredita-se que a inflamação seja um mecanismo chave na patogenicidade de algumas doenças como artrite reumatóide, doença de Crohn e aterosclerose, bem como câncer de fígado, estômago e cólon [42]. Hoje em dia já é bem estabelecido que obesidade é associada a um estado de inflamação crônica subclínica (inflamação metabólica) e que possui um importante papel na patogênese de diversas desordens metabólicas, incluindo diabetes tipo2 e a síndrome metabólica [43-44].As proteínas quinases ativadas por estresse, c-Jun N-terminal quinase 1 (JNK1) e quinase β inibidora de IκB (IKKβ) são sinalizadoras centrais na imunidade inata e resposta a estresse que controlam a expressão de diversos genes pró-inflamatórios. De maneira geral, JNK e o complexo IKK estão situados em posições centrais de diferentes vias sinalizadoras envolvidas na inflamação da imunidade inata e estresse, cuja principal função é a de ativar a defesa do hospedeiro e manter a homeostase [45-47]. Recentemente ficou evidente que a interferência na atividade de JNK1 ou IKKβ melhora a sinalização de insulina em modelos murinos de obesidade. Como quase todos os estressores metabólicos que causam resistência à insulina ou

disfunção na função de ilhota ativam JNK1 e/ou IKKβ, estas proteínas quinases ocupam papéis chave na relação entre obesidade e resistência à insulina, diabetes tipo 2 e síndrome metabólica.

A primeira evidência de uma função da inflamação em diabetes tipo 2 é datada de mais de um século quando foi mostrado que a droga antiinflamatória salicilato causava efeitos benéficos em pacientes diabéticos [48-49]. A hipótese de inflamação metabólica foi revivida e ganhou nova popularidade com a demonstração de que a interferência na sinalização normal de TNFα protegia contra a resistência à insulina causada pela obesidade em modelos de roedores[50-51]. Além de TNFα, outras citocinas pró-inflamatórias, como IL-6 e IL-1, são induzidas durante a obesidade e foram mostradas ocasionando resistência à insulina em alguns modelos [52-53].

Uma das principais características da inflamação induzida por obesidade é a infiltração de macrófagos no tecido adiposo [54-55]. Na obesidade, os macrófagos do tecido adiposo (ATM) são polarizados para a inflamação de fenótipo M1 e expressam o marcador CD11c, e a maioria deles estão localizadas em torno de adipócitos necróticos ou próximos a gotículas de lipídio residuais deixadas por adipócitos mortos e formam estruturas crown-like típicas [56-58]. Acredita-se que os macrófagos tenham uma importante função na resistência à insulina induzida por obesidade pelo reconhecimento de acumulação de lipídios ou dano celular e produzindo citocinas e outros mediadores que interferem na sinalização insulínica [43]. A importância dos macrófagos e de células hematopoiéticas em geral é apoiada por diversos estudos em camundongos portadores de diferentes mutações genéticas em células hematopoiéticas (geradas pelo transplante de medula óssea) ou em células mielóides (obtidas por combinação Cre-lox condicional). Estes estudos mostraram que a interrupção do gene *Ikkβ* em células mielóides ou do gene *Jnk1*em células hematopoiéticas reduz a inflamação metabólica e melhora a sensibilidade à insulina em modelos

de obesidade induzida por dieta [59-61]. Também foi mostrado que o recrutamento de macrófagos para o tecido adiposo por superexpressão da quimiocina MCP-1 em tecido adiposo de camundongos magros é suficiente para induzir resistência à insulina [62].

O mecanismo principal pelo qual os sinais inflamatórios interferem com a ação da insulina envolvem modificações pós-traducionais das moléculas dos substratos do receptor de insulina, principalmente através da fosforilação em serina. Essa modificação é essencialmente universal para todas as formas de resistência à insulina sejam elas genética ou quimicamente induzidas nas células, modelos animais ou doenças humanas[42]. Em esforços para identificar os mecanismos que levam à resistência à insulina em geral e a fosforilaçãoem serina do substrato do receptor de insulina, identificou-se JNK como um mediador central da resistência à insulina em cultura de células e modelos de animais obesos [45]. Esses estudos mostraram que a obesidade resulta em ativação de JNK em tecidos sensíveis à insulina, como tecido adiposo e fígado. Além disso, o bloqueio da atividade de JNK utilizando estratégias químicas, bioquímicas ou moleculares em camundongos obesos resultou em aumento da sensibilidade à insulina e correção da hiperglicemia, indicando um potencial para a utilização da inibição de JNK como estratégia terapêutica contra diabetes tipo 2. Estudos recentes examinaram o papel da atividade de JNK em células derivadas de medula óssea e como elas se relacionam com a inflamação do tecido adiposo e hepático e a sensibilidade sistêmica à insulina [46, 63-64]. Estes estudos mostraram que enquanto a atividade de JNK das células derivadas da medula óssea contribui para a homeostase metabólica, este efeito foi menor quando comparado com o de células do parênquima [46]. Estes resultados apóiam a hipótese de que a liberação de citocinas inflamatórias de células metabólicas são reguladores dominantes das alterações inflamatórias, resistência à insulina e diabetes tipo 2 induzidas por dieta [46, 63]. Como JNK representa um mecanismo central levando a doença

metabólica, existe um aumento no interesse nas vias que levam a ativação de JNK na obesidade e diabetes tipo 2, particularmente em tecido adiposo e hepático. Esses esforços levaram recentemente à descoberta de disfunções de organelas, particularmente o estresse de retículo endoplasmático como potencial mecanismo levando a ativação de JNK e resistência à insulina em modelos de obesidade animal [65].

O retículo endoplasmático é uma rede membranosa responsável pelo tráfego de um grande número de proteínas. O retículo endoplasmático é o principal sítio de síntese de proteínas, maturação destas proteínas e, junto com o complexo de Golgi, o responsável pelo transporte e liberação de proteínas corretamente montadas. Como o retículo endoplasmático possui um papel central na integração de múltiplos sinais metabólicos críticos na homeostase celular, é de grande importância para a célula a manutenção de um retículo endoplasmático com função normal [47]. Assim, em condições que desafiem o bom funcionamento do retículo endoplasmático, principalmente sua capacidade de montagem de proteínas, a organela apresentou um sistema de resposta adaptativa conhecida como resposta de proteínas mal-formadas (UPR). Condições que podem ativar UPR incluem aumento da síntese de proteína, a presença de proteínas mutantes ou mal-formadas, inibição da glicosilação de proteínas, desbalanceamento dos níveis de cálcio, deprivação energética e de glicose, hipóxia, patógenos ou componentes ou toxinas associadas a patógenos [47]. Os três braços de resposta de UPR (PERK (PKR-like eukaryotic initiation factor 2a kinase), IRE1 (inositol requiring enzyme 1), e ATF6 (activating transcription factor-6)) intersectam-se com uma variedade de sistemas de sinalização de estresse e de inflamação, incluindo as vias NFκB/IKK e JNK-AP1, bem como vias ativadas por estresse oxidativo, que podem influenciar o metabolismo. Inflamação e estresse de retículo que são conectadas em diversos níveis, são sistemas adaptativos de curto período necessárias para a função e

sobrevivência do organismo, e ambas são prejudiciais quando ativadas cronicamente. Interessantemente, durante a UPR, também existe ativação de JNK. Dado que UPR é integrada com sinalização de estresse, inflamação e ativação de JNK, bem como o fato de a obesidade apresentar muitas condições que desafiem o bom funcionamento do retículo endoplasmático (de aumento na demanda de síntese a disponibilidade energética e fluxos), hipotetiza-se que a obesidade pode levar a uma condição de estresse de retículo em tecidos e órgãos metabolicamente ativos. Em uma tentativa de se comprovar esta hipótese, mostrou-se que a indução de estresse de retículo em células hepáticas utilizando tunicamicina ou tapsigargina, agentes usados comumente para estimular o estresse de retículo, aumentou a fosforilação em serina do substrato do receptor de insulina-1 de maneira dependente de IRE1α e de JNK e bloqueou a ação da insulina [66]. Em contraste, aumentando a capacidade de montagem de proteínas pelo retículo endoplasmático por estratégias transgênicas protegem os camundongos contra a resistência à insulina induzida por dieta.

Ativação de PERK resulta na fosforilação de eIF2α (eukaryotic translational initiation factor 2a) em serina 51, que converte eIF2α em um competidor de eIF2B e reduz a taxa de formação do complexo ternário, resultando em redução global da síntese protéica e subseqüente redução no trabalho do retículo endoplasmático [67-68]. PERK é uma das quatro proteínas que podem mediar a fosforilação de eIF2α, as outras três quinases são PKR (doublestranded RNA-activated protein kinase), GCN2 (general control non-derepressible kinase 2), e HRI (heme-regulated inhibitor kinase). Apesar de estudos recentes mostrarem que PKR é ativada durante o estresse de retículo e influenciar UPR e eventos sinalizatórios relacionados à inflamação [69]. Além da redução geral na síntese protéica este braço da UPR também está relacionado a regulação ampla da transcrição através de mecanismos distintos, incluindo regulação transcricional de

RNA ribossomal [70]. Isto resulta em ativação de ATF4 (activating transcription factor-4), Nrf2 (nuclear erythroid 2 p45-related factor 2), e NF-κB (nuclear factor kappa b), um fator de transcrição importante com inúmeras funções, incluindo regulação da resposta inflamatória.. Estudos recentes mostraram que NFκB pode ser ativado através desta via metabólica pela supressão traducional de IκB, resultando na regulação de mediadores da inflamação como IL-6 e TNFα. [71-72].

Se o retículo endoplamático funciona como uma organela sensora de nutrientes, outras vias sensoras de nutrientes poderiam estar ligadas de maneira próxima a resposta gerada pelo retículo endoplasmático. Ativação da via de sinalização da mTOR, que é uma via sensível a nutrientes, estimula a síntese de proteínas e remodelamento no retículo endoplasmático [73]. Assim, é possível que na presença de excesso de nutrientes, esta via é estimulada adicionando à demanda traducional e contribuindo para o estresse de retículo e disfunção metabólica. Esclerose tuberosa é uma condição caracterizada por ativação constitutiva da via da mTOR devido a mutações nos genes TSC-1 e TSC-2 [74-76]. Interessantemente, a esclerose tuberosa é associada com severa resistência à insulina celular. Em estudos recentes, foi demonstrado que esta hiperatividade da via da mTOR promove estresse de retículo e contribui para inibição da sinalização do receptor de insulina, pelo menos em parte , através da fosforilação em serina do IRS-1 mediada por JNK [77]. Interessantemente, a atividade da mTOR é aumentada na obesidade [78], e este aumento na sinalização pode ter papel na ação anormal da insulina associada com o estado de obesidade. Previamente, a prórpia mTOR e a ativação de S6K1 dependente de mTOR, foram relacionadas como potenciais mecanismos para a inibição da ação da insulina através da fosforilação direta de IRS1 [79-80]. Trabalhos recentes também mostraram um papel para o estresse de retículo e a ativação de JNK na regulação da ação da insulina, através da via da mTOR. É possível que o

aumento da atividade da mTOR causado pela obesidade possa contribuir para a resposta do estresse de retículo observada nesta condições, apesar de ser improvável que esta atividade por si só seja um evento causal. Um estudo de angiogênese tumoral revelou que a proteína TSC-1 é um alvo direto da quinase IKKβ, levando a sua degradação e conseqüente ativação da via da mTOR [81]. Este achado levanta a possibilidade de que a ativação de quinases inflamatórias podem estar relacionadas à via da mTOR e do estresse de retículo. De fato, inibição da via da mTOR em vários tecidos resulta em um arranjo de anormalidades metabólicas, apesar de ainda ser necessário mais estudos para estabelecer uma relação entre estas doenças e sua ligação com a homeostase do retículo endoplasmático ou a alterações na resposta inflamatória [73]. Assim, a falta de nutrientes (e energia), bem como o excesso de nutrientes podem ser percebidos pelo retículo endoplasmático de diferentes maneiras levando a montagem de diferentes respostas adaptativas. Estas respostas adaptativas também estimulam diferentes sinais de estresse e vias inflamatórias.

Entretanto, a relação entre inflamação e estresse de retículo pode depender do tipo celular, e muito trabalho ainda é necessário para o entendimento dos principais parâmetros que determinam a susceptibilidade da célula a sinais inflamatórios e subseqüente efeitos na homeostasia do retículo. Na verdade, muito do trabalho realizado até o presente momento é baseado em experimentos in vitro. Entretanto, estudos recentes no cérebro proporcionam evidências in vivo apoiando o modelo de que tanto estresse de retículo e inflamação são capazes de ativar um ao outro e inibir o metabolismo normal da célula [82]. Ativação de IKKβ pode levar a estresse de retículo, e ativação de estresse de retículo pode estimular IKKβ. Dependendo do tipo celular, estas respostas podem ser diferentes. No adipócito, por exemplo, sinais inflamatórios podem não causar ativação de UPR (ou podem apenas ativá-la quando as células se encontram em estresse metabólico), mas ativação de UPR pode permitir uma resposta inflamatória.

Recentemente, descobriu-se uma função única para a PKR, uma quinase de eIF2α, na ligação de nutrientes e estresse de retículo com inflamação e regulação metabólica [69]. A PKR é ativada por lipídeos e durante a obesidade tem um importante papel na ativação de JNK e na resposta inflamatória. Interessantemente, PKR também interfere diretamente na ação da insulina pela interação com IRS1. Assim, PKR pode formar um complexo infamatório metabolicamente ativo – chamado inflamassomo ou metaflamassomo – que passa então a integrar a ação da insulina, resposta a patógenos, controle da tradução com sensibilidade a nutrientes e estresse de retículo. A formação de um metaflamossomo e a sua ativação por nutrientes e estresse de retículo pode explicar a sobreposição funcional entre múltiplas vias de sinalização, como JNK, IKK e outras na modulação do metabolismo.

PKR: CARACTERÍSTICAS E FUNÇÕES

A PKR é uma proteína formada por 551 aminoácidos (68 KDa) e possui dois domínios funcionalmente distintos: domínio regulatório N-terminal para ligação de dsRNA e o domínio catalítico C-terminal com atividade quinase. O domínio regulatório (dsRBD – *ds RNA binding domain*) possui duas regiões para ligação de dsRNA com aproximadamente 70 resíduos de aminoácidos cada. A homologia das seqüências de aminoácidos entre os dois dsRBDs da PKR é de 49%, com 29% dos resíduos idênticos. O domínio quinase contém subdomínios conservados de proteínas quinases e a homologia entre esses subdomínios identifica a PKR como uma serina/treonina quinase

Esta proteína é constitutivamente expressa em baixos níveis na forma desfosforilada no citoplasma celular, associada ao ribossomo. Uma pequena fração pode ser encontrada também no núcleo. A PKR foi inicialmente descrita como um mediador da atividade antiviral e

antiproliferativa de interferons [83]. Sua ativação e/ou inibição podem ocorrer em resposta a uma série de agentes que inclui dsRNA, compostos polianiônicos como a heparina e proteínas celulares e virais [84]. Assim, na presença destes compostos, a PKR sofre dimerização e autofosforilação devido a um rearranjo estrutural do domínio catalítico C-terminal [85]. Neste domínio, foram identificados cinco possíveis sítios de fosforilação, sendo eles, Thr-451, Thr-446, Thr-258, Thr-255 Ser-242. Uma vez ativada, esta enzima fosforila e ativa seu principal substrato direto, o fator de inicialização da tradução eucariótica eIF2α, inibindo assim a atividade viral (Figura 2).

O eIF2α é um fator essencial para a síntese protéica [86], formando um complexo terciário com GTP e o iniciador Met-tRNA. Este complexo liga-se à subunidade ribossomal 40S que, juntamente com o mRNA e fatores da mesma família como o eIF1, eIF1A, eIF3, eIF4 e eIF5, formam um novo complexo denominado 48S. O início da tradução ocorre a partir da hidrólise do GTP pelo fator eIF5 (GTPase) e liberação do eIF2-GDP do complexo 48S. Com isso ocorre o recrutamento da subunidade ribossomal 60S e formação do complexo iniciador de tradução, 80S. Finalmente, o GDP do eIF2α é reciclado a GTP com a ajuda do fator trocador de nucleotídeo de guanina, eIF2B (GEF), com formação de um novo complexo terciário eIF2α-GTP-Met-tRNA, necessário para uma nova etapa da tradução protéica. A subunidade α do eIF2 (eIF2α) contém um sítio de fosforilação na Serina 51 e é considerada como subunidade regulatória desse fator. Sua fosforilação aumenta a afinidade pelo GEF. No entanto, este último só é capaz de atuar como trocador se o eIF2α estiver desfosforilado. Deste modo, o eIF2α fosforilado, apesar de sua alta afinidade, atua como um inibidor do eIF2B. Com o trocador inibido, o eIF2α não retorna ao seu estado ativo (ligado ao GTP) e, consequentemente, é bloqueado o processo de tradução protéica em função da ausência de formação do complexo terciário.

Sendo o eIF2α essencial para a inicialização da tradução protéica, sua alteração funcional mais ampla torna a existência da vida inviável. Devido a isto é que sua estrutura permanece conservada ao longo do processo evolucionário das espécies. É raro alguma doença estar relacionada diretamente com mutações desta proteína, sendo mais comum uma disfunção de proteínas quinases que regulam sua atividade, como, por exemplo, a PKR. Deste modo, a PKR se torna um importante alvo de estudos para a descoberta de novos mecanismos de desenvolvimento de várias doenças e potencial emprego terapêutico de drogas que possam modular sua atividade.

A PKR também é ativada por elementos diferentes de dsRNA, como fatores de crescimento, citocinas, estímulos pró-inflamatórios e estresse oxidativo [87-88]. Além disso, existe ampla constatação experimental de que esta proteína integra e transmite sinais às vias que envolvem uma série de fatores como STAT, IRF1, p53, JNK e p38 [89-90]. De maneira geral, a ativação da PKR provoca inibição da síntese e aumento da degradação de proteínas. A inibição da síntese protéica se dá em função de sua atividade modulatória sobre o eIF2α, descrita anteriormente. O aumento da degradação protéica, por sua vez, é induzido pelo aumento da expressão e atividade da via ubiquitina-proteassoma mediado pelo NF-κB [91-92]. Através de adaptadores, a PKR é capaz de ativar o complexo de quinases IKKα/IKKβ, que por sua vez, fosforila o inibidor do NF-κB, IκB. Este, fosforilado, é degradado, liberando o fator NF-κB, o qual, no núcleo, irá ativar vários genes relacionados com eventos da resposta inflamatória e degradação protéica (Figura 2). Outro papel importante da PKR é o de indução de apoptose, dependente ou não de infecção viral [93]. A partir de sua ativação, a PKR atua sobre as vias de caspases, com liberação de citocromo-*c* mitocondrial e consequente indução de apoptose celular [88].

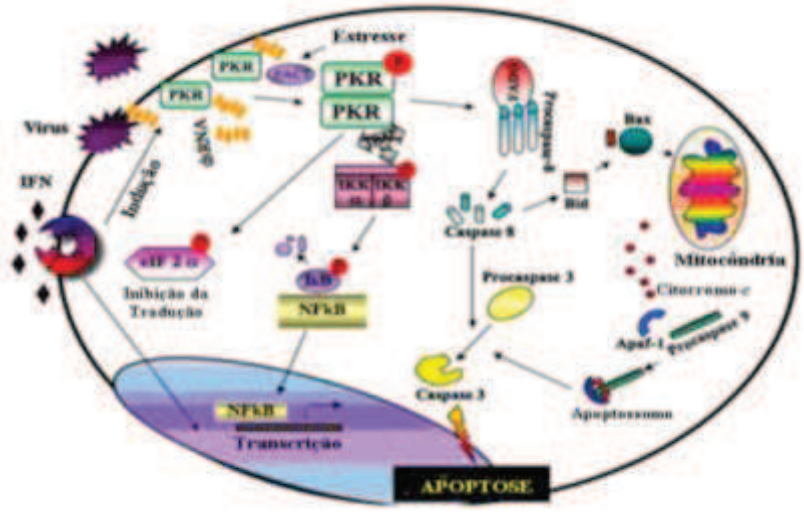

Figura 2. Modelo esquemático das vias de sinalização celular relacionadas com a inibição de síntese e degradação protéica e indução de apoptose dependentes da ativação da PKR.

Os efeitos da PKR sobre a tradução e apoptose exercem grande impacto no crescimento celular [94]. Neste sentido, é sugerido que a PKR atue como um componente de supressão tumoral através da ativação de múltiplos sinais que levam a inibição do crescimento celular e indução de apoptose [95-97]. No entanto, animais *knockout* para PKR não apresentam aumento espontâneo da taxa de crescimento e desenvolvimento tumoral. Adicionalmente, muitos estudos demonstraram que a expressão e atividade da PKR estão aumentadas em tumores humanos [98-100]. Por exemplo, experimentos conduzidos em câncer de colo de útero, mama, fígado e de cabeça e pescoço mostraram uma correlação positiva entre aumento de expressão da PKR e aumento de células tumorais diferenciadas [101-102]. Paradoxalmente, tecidos normais tendem a ter baixos níveis desta proteína [103]. Essas observações sugerem que a proteína quinase dependente de ds-RNA não possui papel de supressor tumoral e sim o de manter as propriedades tumorais de células [104].

Outro estudo mostra, neste sentido, que o tratamento, *in vitro*, de células tumorais com o inibidor específico da atividade da PKR (PKRi), {8-(imidazol-4-ilmetileno)-6H-azolidino[5,4-e] benzotiazol-7-ona}, atenua a atrofia muscular e o crescimento tumoral em modelo de caquexia em câncer [105]. Até o presente momento, os dados acumulados a respeito do papel da PKR no mecanismo de desenvolvimento e manutenção de células tumorais ainda são bastante contraditórios e devem ser mais caracterizados.

Foi observado que a insulina e o IGF-I (*Insulin-Like Growth Factor*) atenuam a indução da degradação protéica pelo PIF através da diminuição da atividade da PKR e que a utilização do inibidor da fosfatase do eIF2α, salubrinal, reverte este efeito [105]. Neste trabalho, sugere-se que insulina e IGF-I exerçam papel modulatório sobre a atividade da proteína PKR, podendo, desta forma, estar envolvida em doenças relacionadas com disfunções metabólicas. Portanto, as vias de sinalização descritas atualmente como indutoras de estresse celular envolvendo, por exemplo, IL-1, TNF, dsRNA, LPS (Lipopolissacárides) e IFNs são mediadas em grande parte pela PKR. Vias da resposta inflamatória e apoptótica estão estreitamente conectadas e mudanças sutis em algum fator determinante destas vias podem balançar o equilíbrio para um lado ou para outro.

OBJETIVOS

Objetivo Geral

O objetivo principal do estudo será avaliar o papel da proteína quinase PKR no desenvolvimento de tumores de cólon em camundongos submetido à dieta hiperlipídica.

Objetivos Específicos

- Caracterizar a importância da PKR no desenvolvimento de tumores cólon induzido por azoximetano em camundongos C57 e *knockout* para PKR.
- Avaliar o efeito da inflamação induzida por dieta hiperlipídica na ativação da proteína quinase PKR em cólon de camundongos C57 tratados ou não com azoximetano e dextran-sulfato de sódio.
- Avaliar o efeito da inibição farmacológica da PKR no desenvolvimento de tumores de cólon em camundongos C57 tratados com azoximentano e dextran-sulfato de sódio.
- Comparar o número de tumores, o índice de proliferação e de apoptose entre camundongos C57 e *knockout* para PKR em dieta padrão após tratamento com azoximetano e dextran-sulfato de sódio.
- Comparar o número de tumores, o índice de proliferação e de apoptose entre camundongos C57 e *knockout* para PKR obesos induzidos por dieta hiperlipídica após tratamento com azoximetano e dextran-sulfato de sódio.
- Avaliar a função da PKR de macrófagos no desenvolvimento de tumores de cólon em animais *knockout* para PKR, através do transplante de medula óssea.
- Avaliar o desenvolvimento de tumores de cólon em animais C57 com macrófagos *knockout* para PKR , através de transplante de medula óssea.

- Caracterizar a resistência à insulina e avaliar a sinalização basal da via da insulina em animais C57 com macrófagos *knockout* para PKR, através de transplante de medula óssea.
- Caracterizar a resistência à insulina e avaliar a sinalização basal da via da insulina em animais *knockout* para PKR com macrófagos de C57, através de transplante de medula óssea.
- Caracterizar a via de inflamação (JNK e IKKβ/IκBα/NFκB) e avaliar a sinalização basal da via inflamatória em animais C57 e com macrófagos *knockout* para PKR, através de transplante de medula óssea.
- Caracterizar a via de inflamação (JNK e IKKβ/IκBα/NFκB) e avaliar a sinalização basal da via inflamatória em animais *knockout* para PKR e com macrófagos de C57, através de transplante de medula óssea.

CAPÍTULO I

Obesity-Induced Increase in Tumor Necrosis Factor-α Leads to Development of Colon Cancer in Mice

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BACKGROUND & AIMS: Epidemiology studies have shown that obesity increases risk for colorectal cancer (CRC). We investigated the contribution of obesity-induced increases in levels of tumor necrosis factor (TNF)-α and hyperinsulinemia to the development of CRC in mice. **METHODS:** Lean and obese mice (C57BL6/J and *ob/ob*) were given a combination of azoxymethane and dextran sulfate sodium, which led to the development of CRC; lean and obese severe combined immunodeficient mice were injected with HT-29 cells. We analyzed the roles of TNF-α and insulin in the development of obesity-mediated CRC using immunoblot, immunohistochemical, and apoptosis assays. **RESULTS:** Genetic- and diet-induced obesity increased the incidence and size of tumors that developed after administration of azoxymethane and dextran sulfate sodium, compared with lean mice. HT-29 xenograft tumors grew more rapidly in obese than lean mice. Neutralization of TNF-α reduced activation of c-Jun N-terminal kinase, IκB kinase, and the phosphatidylinositol 3-kinase–Akt–mammalian target of rapamycin signaling pathway; it also reduced the growth and development of tumors in obese mice. Reducing levels of insulin levels had no effect on tumor growth in obese mice. **CONCLUSIONS: TNF-α contributes to colon tumor growth in obese mice. Reagents that inhibit TNF-α might prevent the development or progression of CRC in obese individuals.**

Keywords: JNK; Inflammation; Insulin Resistance; Mouse Model.

Colorectal cancer (CRC) remains a major health burden with more than 1 million cases worldwide and a disease-specific mortality of approximately 33% in the developed world.[1,2] The association between obesity and the risk for CRC development is observed in both men and women (relative risk, 1.2–2.0).[3,4] In addition to its association with obesity, inflammation and hyperinsulinemia also primarily may contribute to the risk for development of CRC.[5–8]

Among the major mediators of the inflammatory response is tumor necrosis factor (TNF)-α, whose overexpression in adipose tissue is a common feature in human and animal models of obesity.[9,10] TNF-α contributes to the deregulation of the insulin-signaling pathway, including serine phosphorylation of insulin-receptor substrate (IRS) proteins by kinases such as c-jun N terminal kinase (JNK)[11] and inhibitor of nuclear factor-κB kinase (IKK).[12] JNK and IKK activation induce inhibitory serine 307 (Ser^{307}) phosphorylation of IRS-1,[11,13,14] which decreases insulin-mediated phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway activation.[13]

TNF-α, first identified as an antitumor agent, now also is recognized as a tumor-promoting cytokine that links inflammation and cancer.[15] The importance of TNF-α and its intracellular mediators, such as JNK and IKK, as colonic tumorigenic promoters is strengthened by knockout and pharmacologic studies, using azoxymethane (AOM) or AOM combined with dextran sulfate sodium (DSS) as inducers of colorectal carcinogenesis.[16–18] Furthermore, the increased TNF-α levels associated with obesity are a potent liver tumor promoter in mice.[19]

Although the evidence for the potential involvement of inflammation and hyperinsulinemia in the development of obesity-mediated cancer is quite extensive, a systematic evaluation of the independent contribution of these factors to CRC development is lacking. Here, we examined whether obesity modulates insulin signaling and inflammation in the colon and CRC. We show that the obesity-induced abnormal inflammatory response strongly promotes CRC.

Materials and Methods

Antibodies, Chemicals, and Buffers

All the reagents were from Sigma-Aldrich (St Louis, MO), unless otherwise specified. Octreotide was from Novartis PharmaStein AG (Stein, Switzerland), pioglitazone was from Takeda Pharmaceutical (Osaka, Japan), infliximab was from Centocor (Horsham, PA), and DSS was from MP Biochemicals (Solon, OH). Anti–phospho-mTOR, anti-mTOR, anti–phospho-

Abbreviations used in this paper: AOM, azoxymethane; CRC, colorectal cancer; DSS, dextran sulfate sodium; HFD, high-fat diet; IKK, inhibitor of nuclear factor-κB kinase; IP, intraperitoneally; IRS, insulin receptor substrate; ITT, insulin tolerance test; JNK, c-jun N terminal kinase; mTOR, mammalian target of rapamycin; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; SCID, severe combined immune deficiency; TNF, tumor necrosis factor.

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p70S6K, anti-p70S6K, anti–phospho-Akt, anti-Akt, and anti–β-tubulin antibodies were from Cell Signaling Technology (Beverly, MA). Anti–phospho-IR, anti-IR, anti–phospho-IRS-1 Tyr971, anti–phospho-IRS-1 Ser307, anti–IRS-1, anti–phospho-IKKβ, anti-IKKβ, anti-IκBα, anti–phospho-JNK, anti-JNK, anti–phospho-c-jun, anti–c-jun, anti–TNF-α, and anti-TNF receptor 1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Animals

The Ethics Committee of the State University of Campinas approved all experiments. Mice were provided from the Central Breeding Center of the State University of Campinas and were divided randomly into 2 groups: control and high-fat diet (HFD). The diet composition is described in Supplementary Table 1.

Insulin Tolerance Test, Serum Insulin, Leptin, and TNF-α Quantification

The mice were given an insulin tolerance test (ITT; 1.5 IU insulin/kg body weight) as described previously.[20] Plasma was separated by centrifugation (1100 × g) for 15 minutes at 4°C and stored at −80°C until the assay. Serum insulin and leptin were measured by using a mouse enzyme-linked immunosorbent assay kit (Linco, St. Charles, MO). Serum TNF-α was measured using a mouse enzyme-linked immunosorbent assay kit (Thermo Scientific, Rockford, IL).

Tumor Induction and Analysis

Four-week-old male mice (C57BL6/J Unib and *ob/ob*) were placed on standard chow or on a high-fat diet for 1 week and then injected intraperitoneally (IP) with 12.5 mg/kg AOM. After 5 days, 2.5% DSS (molecular weight, 36–50 kilodaltons) was given in the drinking water for 5 days, followed by 14 days of regular water. This cycle was repeated twice and mice were killed 10 days after the last cycle, at 16 weeks of age. Colons were removed, flushed with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde, and paraffin-embedded. Sections (5 μm) were cut and stained with H&E. Tumor counts were performed in a blinded fashion. Tumor sizes were measured with calipers. Assessment of colitis disease scores was performed as previously described.[21]

Cell Culture

The human colon cancer cell line, HT-29, was purchased from the Rio de Janeiro Cell Bank (Rio de Janeiro, Brazil), and cells were cultured in McCoy's medium containing 10% fetal bovine serum with the addition of antibiotics and fungicides. Cells were maintained at 37°C in a humid atmosphere and 5% CO_2.

Human Tumor Xenograft Models

Four-week-old male severe combined immune deficiency (SCID) mice (n = 10 per group) were inoculated subcutaneously in the dorsal region with 1×10^6 HT-29 cells. Tumor volume (V) was calculated daily by measuring length (L) and width (W) of the tumor with calipers and using the following formula: $V = \{W \times L \times [(W + L)/2]\} \times 0.52$.

Treatments with octreotide, pioglitazone, or infliximab began with cell inoculation. Octreotide was given subcutaneously twice a day for a total dosage of 0.01 mg/animal/day. Pioglitazone was given orally for a total dosage of 50 mg/kg/day. Infliximab was given daily IP for a total dosage of 5 mg/kg body weight.

Tissue Extracts

Mice were anesthetized with sodium amobarbital (15 mg/kg body weight, IP). Tumors were removed, minced coarsely, and homogenized in extraction buffer (1% Triton-X 100 [Sigma-Aldrich], 100 mmol/L Tris, pH 7.4, containing 100 mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, 10 mmol/L EDTA, 10 mmol/L sodium vanadate, 2 mmol/L phenylmethylsulfonyl fluoride, and 0.1 mg of aprotinin/mL). The extracts were centrifuged at 11,000 rpm and 4°C, and the supernatants of these tissues were used.

Isolation of the Stromal Vascular Fraction and Adipocyte of Adipose Tissue

Epididymal fat pads from mice were excised and minced in PBS with calcium chloride and 0.5% bovine serum albumin. Collagenase type II was added at 1 mg/mL and incubated at 37°C for 20 minutes with shaking. The cell suspension was filtered through a 100-μm filter and then centrifuged at 300 × g for 5 minutes to separate floating adipocytes from the stromal vascular fraction pellet. Samples were digested until adipocyte fractions were free of adherent cells by these 2 quality control methods to ensure recovery of the majority of the stromal vascular fraction population. Afterward, the 2 fractions were used for protein analysis by immunoblotting.

Colonic Macrophage Isolation

The colons of mice were removed and washed in PBS. The intestines were opened longitudinally, washed in Hank's balanced salt solution, and cut into 0.5-cm sections. Tissue was placed in 10 mL fresh calcium/magnesium-free Hank's balanced salt solution containing 1 mmol/L EDTA and 1 mmol/L dithiothreitol, the tube was placed in a shaking water bath for 15 minutes at 37°C and shaken vigorously, and the supernatant separated. The remaining tissue was digested with 0.02% collagenase V and 0.05% DNase (Roche Diagnostic, Indianapolis, IN) in complete RPMI 1640 for 30 minutes at 37°C in a shaking water bath until complete digestion of the tissue. At the start of and every 5–10 minutes during the incubation, the tube was shaken vigorously, and the final supernatant was passed through a 100-μm filter. Afterward, the fraction was used for protein analysis by immunoblotting.

Protein Analysis by Immunoblotting

Whole-tissue extracts were homogenized in extraction buffer, treated with Laemmli sample buffer containing 100 mmol/L dithiothreitol, and heated in a boiling water bath. For total extracts, similar-sized aliquots (50 μg protein) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were resolved in 8%–15% sodium dodecyl sulfate gels and blotted onto nitrocellulose membranes (Bio-Rad, Hercules, CA). Band intensities were quantified by optical densitometry of developed autoradiographs using Scion Image software (Scion Corporation, Frederick, MD).

Immunohistochemistry and Terminal Deoxynucleotidyl Transferase–Mediated Deoxyuridine Triphosphate Nick-End Labeling Assay

Ki67 staining was performed as described previously.[22] Terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling staining was performed using a commercial apoptosis detection kit (Roche Diagnostics GmbH,

Mannheim, Germany), according to the manufacturer's recommendations. Analysis and documentation of results were performed as described previously.[22]

Statistical Analysis

Data are presented as means ± standard error of the mean (SEM) of at least 3 independent experiments. Statistical analysis was performed by using the analysis of variance test with the Bonferroni post test. Significance was established at the $P < .05$ level.

Results

Diet-Induced Obesity Engenders Colonic Inflammation and Insulin Signaling Impairment

Four-week-old male mice received a single dose of AOM and 3 cycles of DSS (AOM+DSS), as described in the Materials and Methods section. Mice exposed to HFD presented increased body weight, epididymal fat, serum insulin, leptin, and TNF-α, compared with the controls (Supplementary Table 2). We observed decreased insulin sensitivity in the obese animals, as measured with the ITT (Supplementary Table 2).

Colon sections from the obese mice showed higher amounts of macrophages and leukocytes associated with epithelial cell damage and adenoma formation, leading to an inflammatory state in the colon (Figure 1*A*). To verify the source of the increased TNF-α leading to the inflammatory state observed in the colon, we evaluated the adipose tissue, with its two major components (the adipocytes and the stromal vascular fractions), or the colonic tissue. As evidenced by the dosage of serum and tissue TNF-α, we observed that the majority of the TNF-α, is derived from the adipocytes and from the macrophages present in the adipose tissue (Figure 1*B*). The alterations in colon tissue were accompanied by an increase in Ki67 staining and diminished apoptosis (Figure 1*C*) when compared with control animals. In agreement with these results, we observed an increase in the activation of proinflammatory proteins in the obese mice. As shown in Figure 1*D*, HFD increases TNF-α expression, IKKβ phosphorylation, decreases IκBα expression, and also increases JNK and c-jun phosphorylation in colonic tissues.

We observed a slightly higher activation of insulin receptor β and IRS-1 with increased tyrosine phosphorylation, accompanied by increased IRS-1 Ser^{307} phosphorylation as well as higher levels of phosphorylated Akt, mTOR, and p70S6K in the colons of the obese mice, as compared with control mice (Figure 1*E*). Next, we examined the response of colonic tissue to insulin in control mice. Insulin increased Akt phosphorylation in a time-dependent manner, with maximum phosphorylation at 10 minutes (Supplementary Figure 1*A*). The HFD impaired insulin-induced tyrosine phosphorylation of IRβ and IRS-1 (Figure 1*F*). As observed in Figure 1*F*, phosphorylated Akt levels are similar in the insulin-stimulated and nonstimulated colons of obese (HFD-fed) mice.

The Diet-Induced Obesity-Mediated Inflammatory Microenvironment Promotes Colon Cancer

Mice kept on an HFD showed increased tumor incidence and size compared with control mice (Figure 2*A* and *B*). Similar to the colonic tissues, colon tumors show increased TNF-α expression, IKKβ phosphorylation, decreased IκBα expression, and increased levels of phosphorylated JNK and c-jun (Figure 2*C*).

Next, we examined the effects of obesity on several kinases involved in insulin signaling in carcinomas. We observed a slightly higher activation of IRβ and IRS-1 with increased tyrosine phosphorylation, accompanied by increased IRS-1 Ser^{307} phosphorylation, as well as higher levels of phosphorylated Akt, mTOR, and p70S6K on the tumors in the obese mice, when compared with the control mice (Figure 2*D*). The HFD impaired insulin-induced tyrosine phosphorylation of IRβ and IRS-1 (Figure 2*E*). As observed in Figure 2*E*, phosphorylated Akt levels are similar in insulin-stimulated and nonstimulated colon tumors from HFD-fed obese mice.

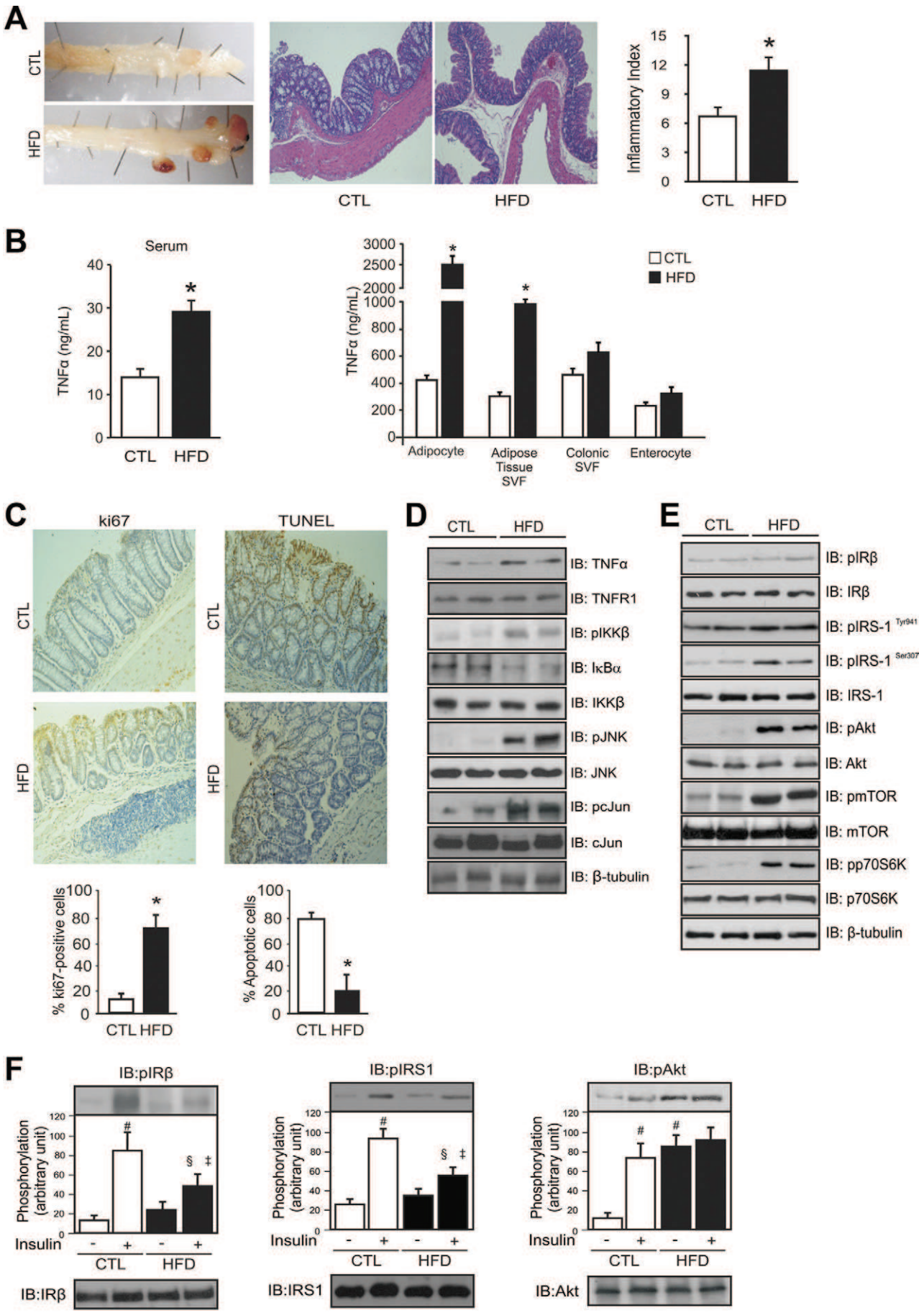
To rule out the effect of the inflammation caused by DSS in the colon, we performed an experiment in which mice were placed on an HFD or on a control diet, and a single dose of AOM (12.5 mg/kg) was given IP. Twenty weeks after the AOM treatment, the mice were killed, and the colon was examined for the presence of tumors. As observed previously, obese mice presented a higher inflammatory state in the colon (Supplementary Figure 2*A*), owing to an obesity-related increase in circulating levels of inflammatory cytokines, such as TNF-α, resulting in increased tumor incidence and size compared with control mice (Supplementary Figure 2*B*).

Genetic Obesity Promotes Colonic Inflammation and Insulin Signaling Impairment

We submitted 4-week-old male *ob/ob* and WT mice to the same AOM+DSS protocol. As expected, *ob/ob* mice presented increased body weight, epididymal fat, serum insulin, and leptin when compared with controls (Supplementary Table 2). We observed decreased insulin sensitivity in the obese animals, measured with the ITT (Supplementary Table 2).

During the protocol, *ob/ob* mice showed significantly more clinical signs of inflammatory response than controls, including rectal bleeding and prolapse (Figure 3*A*). Consistent with these signs, we observed a significantly higher level of histologic damage (Figure 3*A*). These alterations were accompanied by an increase in Ki67 staining and diminished apoptosis (Figure 3*B*) when compared with lean animals.

We next examined proinflammatory protein expression and observed increased TNF-α expression, IKKβ phosphorylation, and decreased IκBα expression, associated with increased levels of phosphorylated JNK and c-jun in the *ob/ob* colon, when compared with control mice (Figure 3*C*). We examined the effects of obesity on several kinases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IB: TNFR1
IB: pIKKβ
IB: IκBα
IB: IKKβ
IB: pJNK
IB: JNK
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IB: IRS-1
IB: pAkt
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IB: pmTOR
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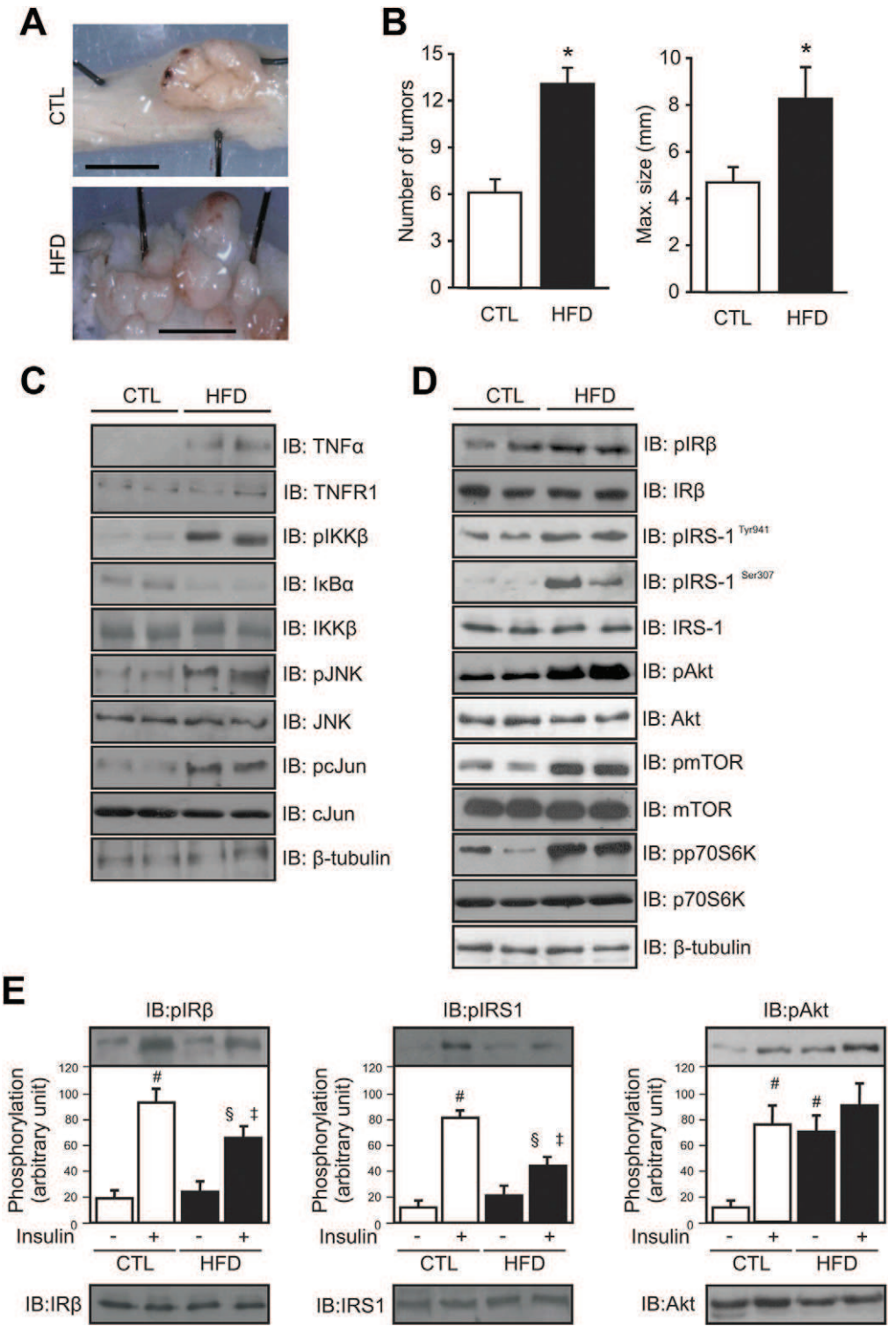

Figure 2. Diet-induced obesity promotes colon carcinogenesis. (*A*) Macroscopic changes in colonic tissues. *Scale bars*, 5 mm. (*B*) Tumor incidence and size of colons removed at the end of protocol from CTL and HFD-fed mice. Results are means ± SEM (n = 8). Western blots showing colonic tumor lysates from C57BL6/J control and HFD-fed mice. (*C*) Colonic tumor TNF-α, TNFR1, IKKβ, IκBα, JNK, and c-Jun expression and phosphorylation. (*D*) Colonic tumor IRβ, IRS-1, IRS-1 Ser^{307}, Akt, mTOR, and p70S6K expression and phosphorylation. (*E*) Insulin-induced IRβ, IRS-1, and Akt phosphorylation in colon tumors. Data are the means ± SEM. $^{*}P < .05$ vs respective control group. $^{\#}P < .05$ vs CTL−. $^{\ddagger}P < .05$ vs HFD−. §vs CTL+. CTL, *white bars*; HFD, *black bars*.

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Figure 1. Diet-induced obesity promotes colonic inflammation and insulin signaling impairment. (*A*) Macroscopic changes in epithelial tissues, H&E staining of histologic sections, and inflammatory index of colons from control (CTL) and HFD-fed mice. Results are means ± SEM (n = 8). (*B*) TNF-α dosage of serum, isolated adipocytes, adipose tissue stromal vascular fraction (SVF), enterocytes, and colonic SVF from CTL and HFD-fed mice. (*C*) Representative microphotographs and quantification of Ki67 and apoptotic (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling [TUNEL]) staining on colonic tissue sections of CTL and HFD-fed mice, 4 fields per tumor section, mean ± SEM. (*D*) Western blots showing colonic tumor lysates from C57BL6 mice. Colonic TNF-α, IKKβ, JNK, and c-Jun expression and phosphorylation, and colonic expression of IκBα TNF receptor 1 (TNFR1) and β-tubulin. (*E*) Colonic IRβ, IRS-1, IRS-1 Ser^{307}, Akt, mTOR, and p70S6K expression and phosphorylation. (*F*) Insulin-induced IRβ, IRS-1, and Akt phosphorylation in the colon. Data are the means ± SEM. $^{*}P < .05$ vs respective lean group. $^{\#}P < .05$ vs CTL−, $^{\ddagger}P < .05$ vs HFD−, §vs CTL +. CTL, *white bars*; HFD, *black bars*.

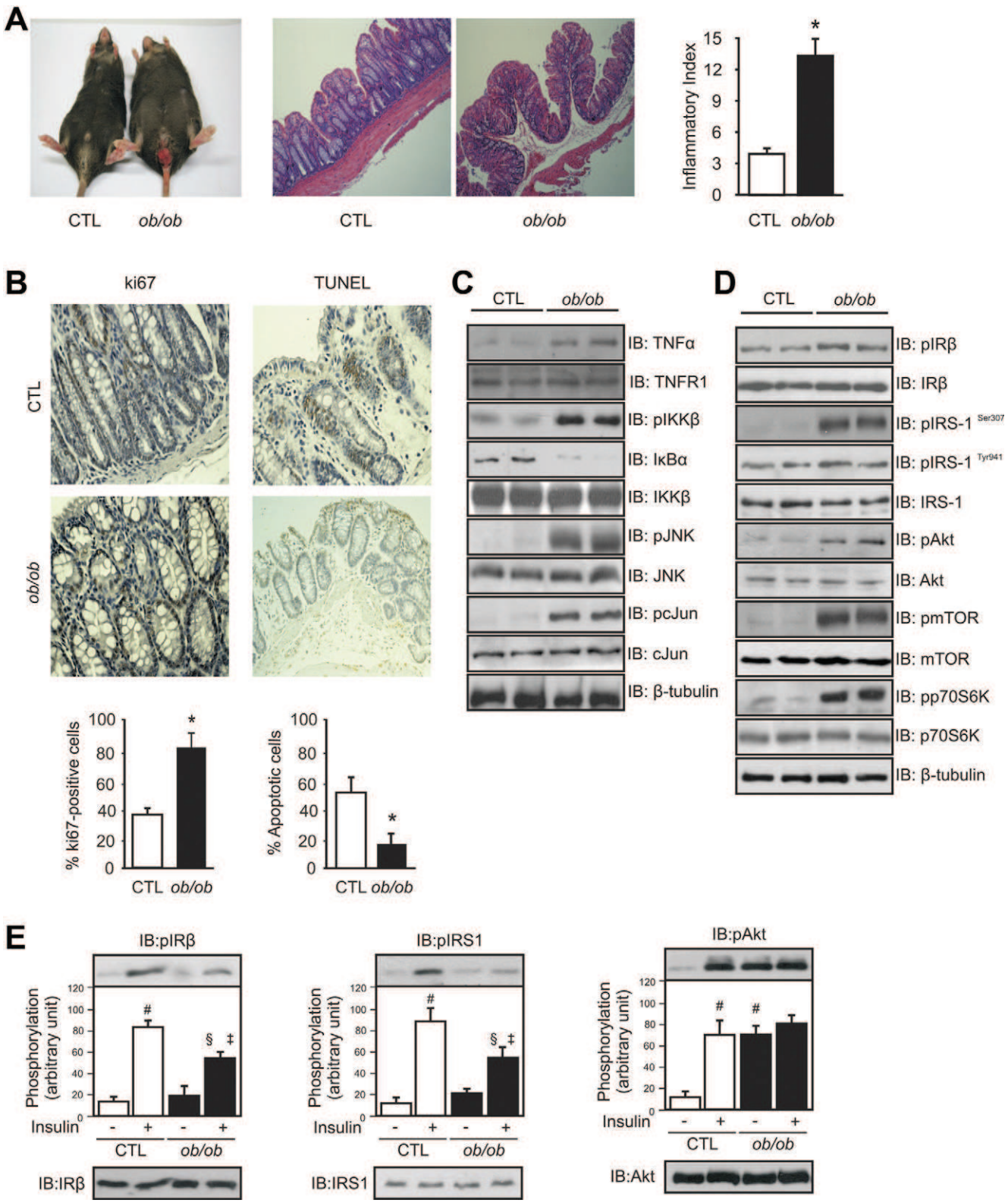

Figure 3. Genetic obesity promotes colonic inflammation and insulin signaling impairment. (*A*) Rectal prolapse presented in *ob/ob* mice during protocol treatment, H&E staining histologic sections, and inflammatory index of colons from CTL and *ob/ob* mice. Results are means ± SEM (n = 10). (*B*) Representative microphotographs and quantification of Ki67 and apoptotic (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling [TUNEL]) staining on colonic tissue sections of CTL and *ob/ob* mice, 4 fields per tumor section, mean ± SEM. Western blots of 6 independent experiments showing colonic lysates from control or *ob/ob* mice. (*C*) Colonic TNF-α, TNFR1, IKKβ, IκBα, JNK, and c-Jun expression and phosphorylation. (*D*) Colonic IRβ, IRS-1, IRS-1 Ser^{307}, Akt, mTOR, and p70S6K expression and phosphorylation. (*E*) Insulin-induced IRβ, IRS-1, and Akt phosphorylation in the colon. Data are the means ± SEM. $^{*}P < .05$ vs respective control group. $^{\#}P < .05$ vs CTL−. $^{\ddagger}P < .05$ vs HFD−. §vs CTL+. CTL, *white bars*; *ob/ob*, *black bars*.

involved in insulin signaling in the *ob/ob* colon. A slightly higher activation of IRβ and IRS-1 was observed with increased tyrosine phosphorylation, accompanied by increased IRS-1 Ser^{307} phosphorylation, as well as higher levels of phosphorylated Akt, mTOR, and p70S6K on the colons of obese mice compared with the colons of control mice (Figure 3*D*). Colons from *ob/ob* mice presented insulin resistance, with a lower activation of IRβ and IRS-1 after insulin stimulation (Figure 3*E*). As observed in Figure 3*E*, phosphorylated Akt levels are similar in insulin-stimulated and nonstimulated colons in *ob/ob* mice.

Genetic Obesity Promotes Colon Carcinogenicity Associated With Inflammation and Colonic Insulin Signaling Impairment

After the AOM+DSS protocol, we found that *ob/ob* mice also showed enhanced tumor incidence and size compared with control mice (Figure 4*A* and *B*). Next, we examined the inflammatory pathway in tumor tissue of control and *ob/ob* mice. As shown in Figure 4C, *ob/ob* mice present increased TNF-α expression and IKKβ phosphorylation, decreased IκBα expression, and increased levels of phosphorylated JNK and c-jun. These results indicate that *ob/ob* mice present a higher inflammatory state than control mice. In addition, we also examined the insulin signaling pathway in the tumors from control and *ob/ob* mice. As shown in Figure 4*D*, *ob/ob* mice present a slightly higher activation of IRβ and IRS-1 with increased tyrosine phosphorylation and increased IRS-1 Ser^{307} phosphorylation. The *ob/ob* mice also presented higher levels of phosphorylated Akt, mTOR, and p70S6K when compared with control animals.

Next, we examined the response of tumor tissue to insulin in control and *ob/ob* mice. Tumors from *ob/ob* mice presented lower activation of IRβ and IRS-1 after insulin stimulation (Figure 4*E*). As observed in Figure 4*E*, phosphorylated Akt levels are similar in insulin-stimulated and nonstimulated colon tumors from *ob/ob* mice.

High-Fat Diet Enhances HT-29 Xenograft Tumor Growth in SCID Mice

The SCID mice submitted to HFD showed increased body weight, epididymal fat, serum insulin, leptin, and TNF-α levels as well as decreased insulin sensitivity (Supplementary Table 2), compared with the controls. The greater the degree of host obesity, the faster the HT-29 xenografts grew, which reached the largest size in the HFD mice (Figure 5*A*).

As previously observed in the AOM+DSS–induced colonic tumors, the xenografts also presented increased TNF-α expression, IKKβ phosphorylation, and decreased IκBα expression, with higher phosphorylation of JNK and c-jun, when compared with control mice (Figure 5*B*). When we analyzed the insulin signaling pathway of these tumors, we observed the discrete activation of IRβ and IRS-1, represented by tyrosine phosphorylation and the higher activation of Akt, mTOR, and p70S6K; we also observed an increase in IRS-1 Ser^{307} phosphorylation (Figure 5*C*).

Next, we examined the phosphorylation of IRβ, IRS-1, and Akt of the control and obese SCID mice after insulin stimulation (Figure 5*D*). As observed, IRβ and IRS-1 lead to impaired insulin signaling in the HFD mice, whereas despite increased steady-state Akt serine phosphorylation, Akt also presents impaired activation after insulin stimulus.

The enhanced tumor growth in the HFD mice was caused by the increased proliferation of tumor cells, as shown by Ki67 staining (Figure 5*E*), and diminished apoptosis (Figure 5*F*) relative to the control animals. Thus, the effect of obesity on HT-29 xenografts is characterized by enhanced cell proliferation and decreased cell death.

Neutralization of TNF-α Reverses Obesity-Induced Effects on Tumor Growth

Infliximab treatment exerted a stronger inhibitory effect on tumor growth in HFD mice when compared with nontreated mice, nearly restoring tumor growth to the levels observed in lean animals (Figure 6*A*). The reduced tumor growth of the infliximab-treated mice, when compared with the HFD mice, is owing to decreased proliferation, shown by Ki67 staining (Figure 6*B*), and enhanced apoptosis of tumor cells (Figure 6*C*). Thus, TNF-α plays a significant role in the proliferation of tumor cells in obese mice, and its ablation is capable of reducing tumor growth to levels observed in the control group.

We observed that infliximab treatment induced a decrease in IRS-1 Ser^{307} phosphorylation and, interestingly, a decrease in the phosphorylation of Akt, mTOR, and p70S6K (Figure 6*D*). Infliximab was effective in reducing the increased proinflammatory effect mediated by obesity. Thus, infliximab treatment decreased the IKKβ phosphorylation, increased IκBα expression, and reduced the phosphorylation of JNK and c-jun, when compared with HFD mice (Figure 6*E*).

The octreotide treatment was effective in reducing insulin levels in HFD mice (Supplementary Figure 3*A*), in improving insulin resistance in peripheral tissues (Supplementary Figure 3*B*), but was not effective in altering tumor growth (Supplementary Figure 3*C*), or decreasing Akt phosphorylation (Supplementary Figure 3*D*), proliferation, or apoptosis (Supplementary Figure 3*E* and *F*) when compared with untreated HFD mice. The pioglitazone treatment also was effective in improving insulin sensitivity, although it was not effective in altering tumor growth, Akt phosphorylation, proliferation, or apoptosis when compared with untreated mice (Supplementary Figure 4).

TNF-α Neutralization Protects From the Effects of Obesity on Tumor Formation

We designed a pharmacologic approach to determine the effects of TNF-α on tumor formation during

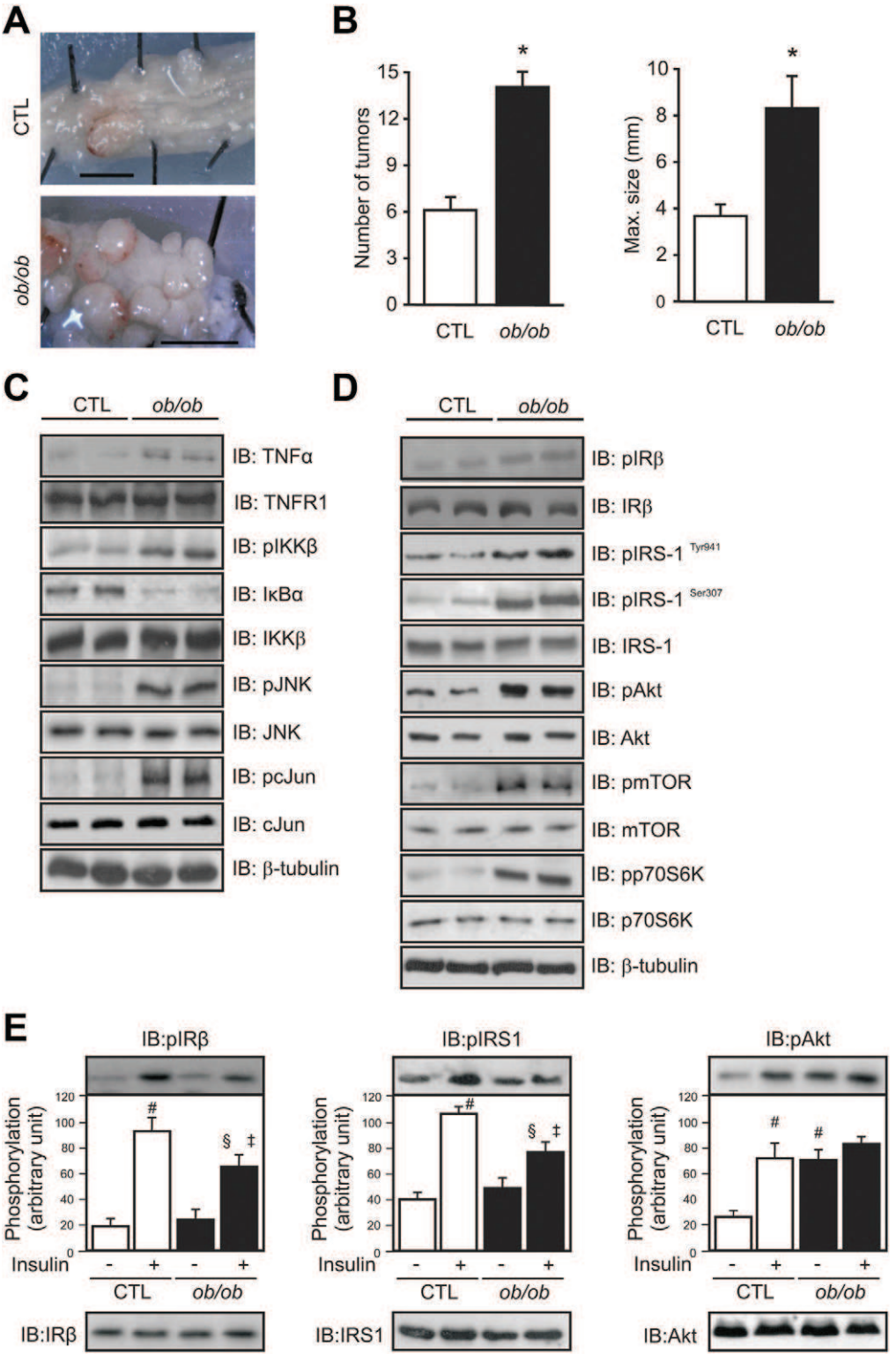

Figure 4. Genetic obesity enhances tumor formation. (*A*) Macroscopic changes in epithelial tissues. *Scale bars*, 5 mm. (*B*) Tumor incidence and size from colons removed at the end of the protocol from CTL and *ob/ob* mice. Results are means ± SEM (n = 8). Western blots of 5 independent experiments showing colonic tumor lysates from control and *ob/ob* mice. (*C*) Colonic tumor TNF-α, TNFR1, IKKβ, IκBα, JNK, and c-Jun expression and phosphorylation. (*D*) Colonic tumor IRβ, IRS-1, IRS-1 Ser^{307}, Akt, mTOR, and p70S6K expression and phosphorylation. (*E*) Insulin-induced IRβ, IRS-1, and Akt phosphorylation in the colon tumor. Data are the means ± SEM. $^{*}P < .05$ vs the respective control group. $^{\#}P < .05$ vs CTL−. $^{\ddagger}P < .05$ vs HFD−. §vs CTL+. CTL, *white bars*; *ob/ob*, *black bars*.

obesity. Concomitantly with the AOM+DSS protocol with the *ob/ob* mice, we started treatment with infliximab and observed that the infliximab treatment resulted in decreased levels of histologic damage in the colon sections (Figure 7*A*). Consistent with these signs, we observed that infliximab treatment decreased colon tumor incidence and size in *ob/ob* mice (Figure 7*B*), an effect that was not observed with pioglitazone (data not shown). In addition, infliximab treatment exerted an inhibitory effect on tumor growth in the *ob/ob* mice, reducing proliferation, shown by Ki67 staining (Figure 7*C*), and enhancing apoptosis (Figure 7*D*). Furthermore, consistent with our previous studies, infliximab treatment, besides neutralizing TNF-α, increased insulin sensitivity as measured by the rate constant for glucose disappearance in the insulin tolerance test[9,23] (Supplementary Table 3).

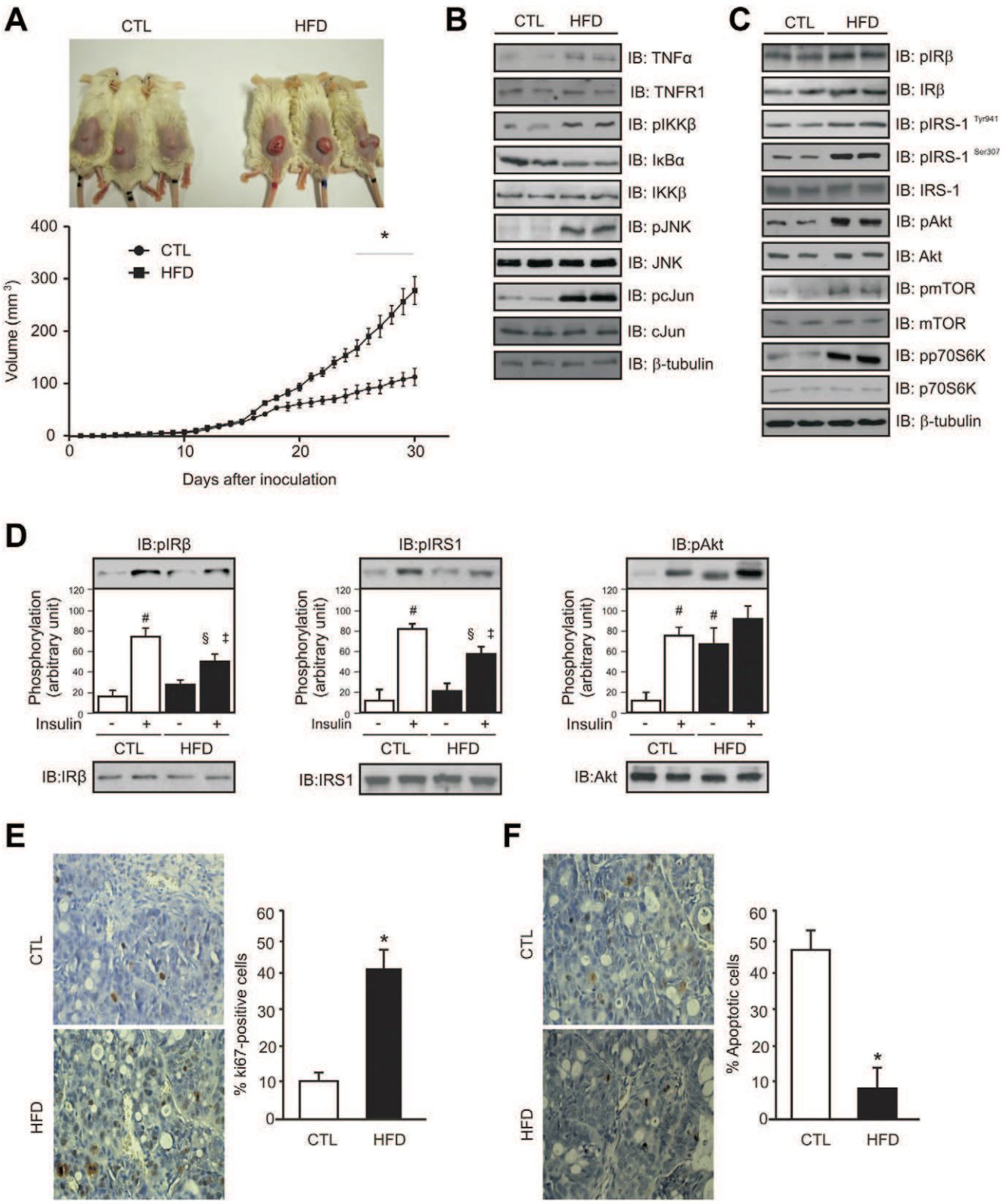

Figure 5. High-fat diet enhances HT-29 xenograft tumor growth in SCID mice. (*A*) HT-29 tumor xenografts from normal chow and HFD-fed SCID mice and tumor growth were measured for 30 days. The results are means ± SD of 5–8 mice per group. Western blots of 6 independent experiments showing xenografts lysates from control and high-fat diet–fed mice. (*B*) HT-29 xenograft TNF-α, TNFR1, IKKβ, IκBα, JNK, and c-Jun expression and phosphorylation. (*C*) HT-29 xenograft IRβ, IRS-1, IRS-1 Ser^{307}, Akt, mTOR, and p70S6K expression and phosphorylation. (*D*) Insulin-induced IRβ, IRS-1, and Akt phosphorylation in the xenograft tumor. Data are the means ± SEM. $^{*}P < .05$ vs the respective lean group. Representative microphotographs and quantification of (*E*) Ki67 and (*F*) apoptotic staining on HT-29 xenograft sections of control and HFD mice, 4 fields per tumor section, mean ± SEM. $^{*}P < .05$ vs control. $^{\#}P < .05$ vs CTL−. $^{\ddagger}P < .05$ vs HFD−. §vs CTL+. CTL, *white bars*; HFD, *black bars*.

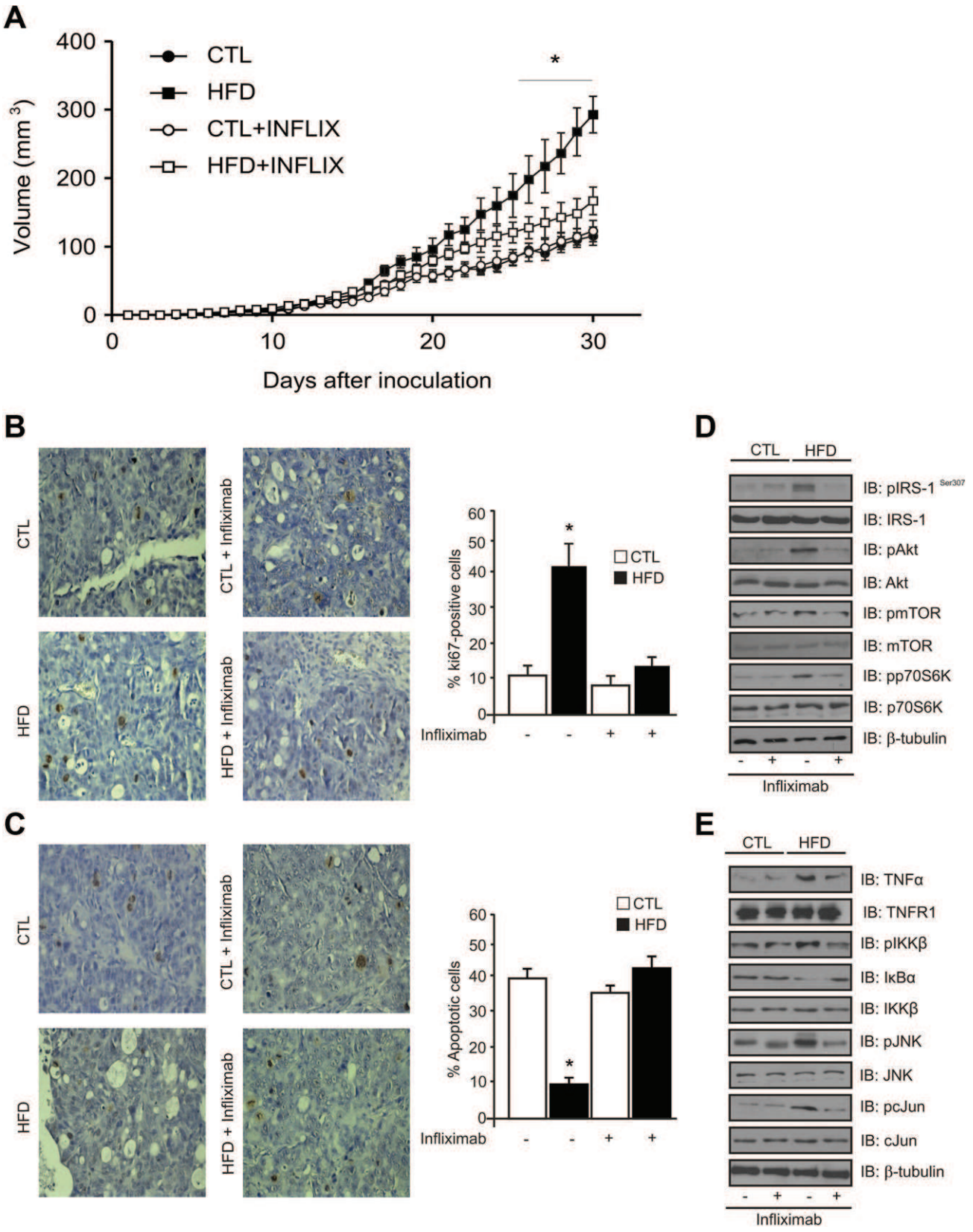

Figure 6. Neutralization of TNF-α reverses obesity-induced effects on tumor growth. (*A*) Tumor growth was measured for 30 days in SCID mice maintained on normal chow or HFD for 8 weeks before tumor inoculation with HT-29 cells (1×10^6 cells/mouse) and kept on normal chow or HFD for 30 days after inoculation and with the indicated treatment. The results are means ± SD from 5–8 mice per group. (*B* and *C*) Representative microphotographs and quantification of (*B*) Ki67 and (*C*) apoptotic staining on HT-29 xenograft sections, 4 fields per tumor section, mean ± SEM. *$P < .05$ vs control. Western blots showing HT-29 xenograft lysates from tumor-bearing SCID mice. (*D*) HT-29 xenograft IRS-1, IRS-1 Ser^{307}, Akt, mTOR, and p70S6K expression and phosphorylation. (*E*) HT-29 xenograft TNF-α, TNFR1, IKKβ, IκBα, JNK, and c-Jun expression and phosphorylation. INFLIX, infliximab. CTL, *white bars*; HFD, *black bars*.

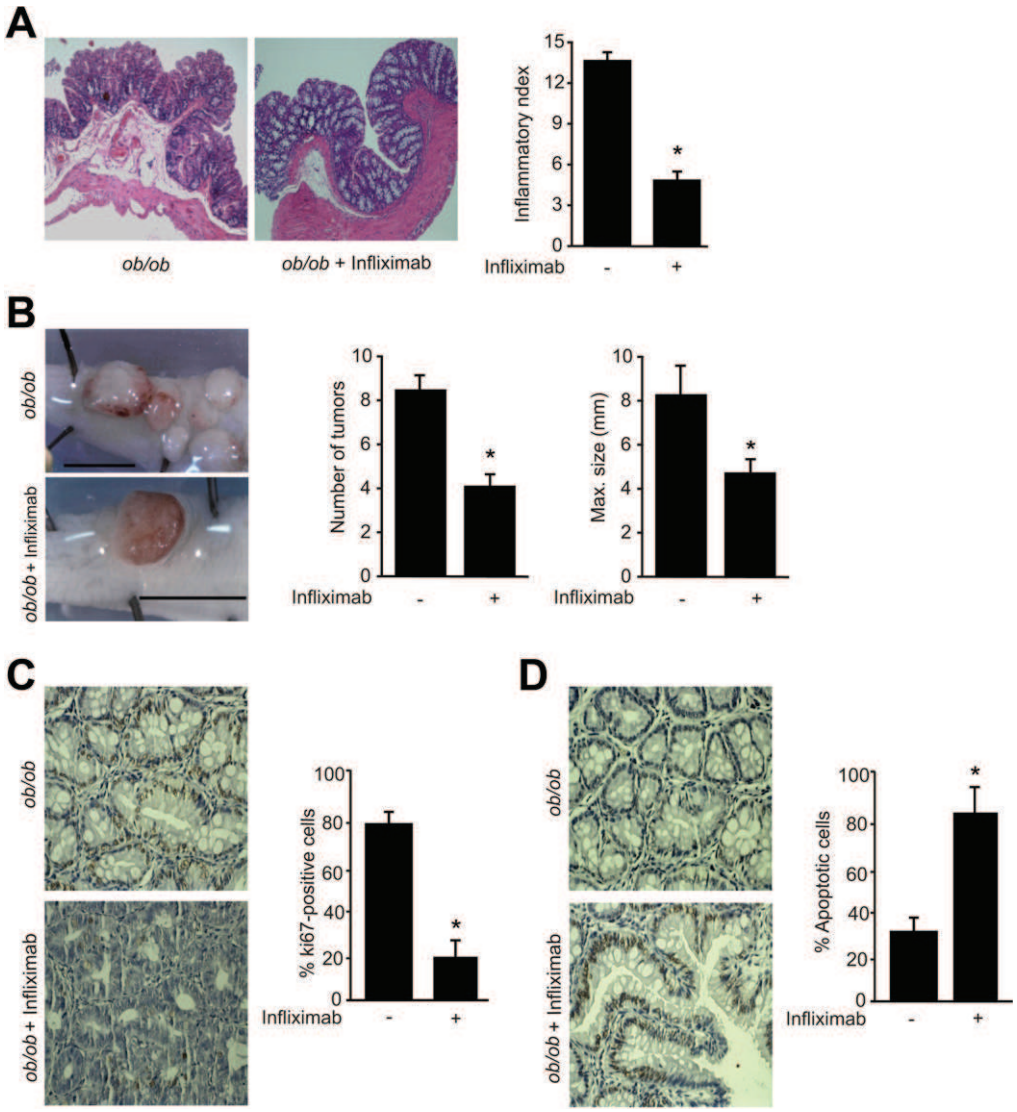

Figure 7. Absence of TNF-α protects from obesity effects on tumor formation. (*A*) H&E staining histologic section and inflammatory index and (*B*) macroscopic changes (*scale bars*, 5 mm), tumor incidence, and size of colons removed at the end of the protocol from *ob/ob* mice treated or not with infliximab. Representative microphotographs and quantification of (*C*) Ki67 and (*D*) apoptotic staining of colonic tissue sections, 4 fields per tumor section, mean ± SEM. *$P < .05$ vs control.

Discussion

The results of this study show that HFD induced and genetic obesity markedly increased the development of CRC. Our data indicate that obesity-induced colonic inflammation increases JNK and IKKβ expression and activity in the colon of AOM+DSS–treated mice. In addition, the HFD-fed and *ob/ob* mice also presented a significant increase in host insulin levels and an impairment in insulin activation of the IR/IRS-1/Akt pathway in the colon of these animals. Moreover, octreotide and pioglitazone treatments did not influence HT-29 xenograft growth in HFD-fed SCID mice. The administration of infliximab, a TNF-α inhibitor, reduced the magnitude of carcinogenesis in HFD-fed and in *ob/ob* mice, as well as inhibited the HT-29 xenograft growth in obese SCID mice, suggesting that TNF-α is a major modulator of the effects of obesity-induced CRC.

Epidemiologic studies have suggested that hyperinsulinemia is an important mechanism by which obesity can confer an increased risk of colon cancer, and insulin levels also may influence CRC recurrence.[6,24] However, we observed a blunted insulin-stimulated IR, IRS-1, and Akt phosphorylation in the colons of HFD-fed and *ob/ob* mice, providing a biochemical correlation for decreased colonic in vivo insulin sensitivity. Consistent with these results, octreotide and pioglitazone treatments were unable to decrease the tumor growth rate. It is interesting to note that octreotide, beyond reducing insulin secretion, also decreases the synthesis of several intestinal peptides, such as glucose-dependent insulinotropic polypeptide.[25] Glucose-dependent insulinotropic polypeptide is a hormone that can activate the PI3K/Akt/mTOR pathway in the gastrointestinal tract[26]; nevertheless, we did not observe decreased tumor growth or Akt phosphorylation with octreotide treatment.

Tumor-promoting inflammation is one of the enabling characteristics of carcinogenesis and reducing inflammation during tumor promotion is pivotal for chemoprevention.[27] TNF-α and its mediators, JNK and IKK, are critical in colon carcinogenesis induced by the treatment of AOM.[16–18] Our data show that obesity induced a great increase in the colonic inflammatory index, accompanied

by an increase in IKK and JNK phosphorylation in diet-induced and genetic obesity, as well as the treatment using the TNF-α blocker, infliximab, showed a consistent decrease in tumor growth in the HT-29 xenograft and in colitis-associated cancer development. Thus, the low-grade inflammatory state observed in obesity increased plasma TNF-α levels and created fertile soil in the colonic epithelium that increased DSS and AOM action.

TNF-α is an important component of the cancer network of inflammatory mediators, such as cytokines and vascular endothelial growth factor.[15] This network then acts by modulating the cancer microenvironment and allowing cancer growth.[28] It also has been shown that the PI3K/Akt pathway can be activated by TNF-α.[29,30] Moreover, the PI3K/Akt pathway is activated in patients with ulcerative colitis[31] and induces proinflammatory cytokine production.[32] Our findings showing a reduction in the Akt/mTOR pathway, which is known to be an important regulator of CRC growth,[33,34] after infliximab treatment of obese mice bearing colon cancer xenografts, suggests a major role of this pathway in obesity-mediated colon cancer growth.

Currently, infliximab is recommended for the treatment of complicated Crohn's disease and was approved for the treatment of ulcerative colitis that fails to respond to the standard therapies, such as corticosteroids and immunosuppressors.[35–37] In accordance with our study, neutralization of TNF-α is also an effective therapy in various animal models of T-cell–mediated colitis.[38,39] However, TNF-α neutralization in the DSS-induced colitis, an epithelial damage model, is still controversial.[40–42] These different results may be related to the environment that the animals were bred in or to the differences in the inflammatory levels obtained with the different protocols. It is interesting to note that despite the contradictory results related to colitis, the effect of TNF-α neutralization or inhibition of IKKβ was effective in reducing CRC in all studies.[16,18,42] The results presented herein extend these data, showing that infliximab also prevents obesity-mediated CRC. Altogether, these results suggest that the use of anti–TNF-α therapy could decrease both insulin resistance and colon cancer risk; although the possible contribution of anti–TNF-α therapy to improve insulin sensitivity in human beings still is controversial and deserves further investigation.[35,43–45] Along this line, chemoprevention of CRC in obese individuals with TNF-α neutralization should be evaluated.

In summary, our findings establish a role for TNF-α in obesity-mediated colon carcinogenesis and point to TNF-α blockers as attractive players in colon cancer chemoprevention for obese individuals.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2012.05.045.

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Reprint requests

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M.B.S.F. and G.Z.R. contributed equally to this work.

Conflicts of interest

The authors disclose no conflicts.

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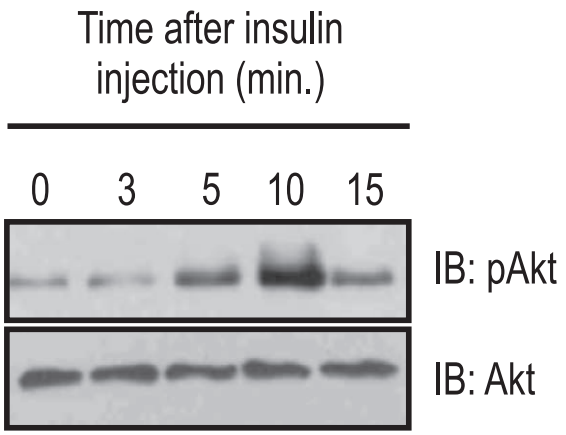
Supplementary Table 1. Components of Standard Chow and High-Fat Diet

Ingredients	Standard chow, *g*	High-fat diet, *g*
Casein	202	200
Sucrose	100	100
Cornstarch	397	115.5
Dextrinated starch	130.5	132
Lard	—	312
Soybean oil	70	40
Cellulose	50	50
Mineral mix AIN-93	35	35
Vitamin mix AIN-93	10	10
L-Cystine	3	3
Choline	2.5	2.5

AIN, American Institute of Nutrition.

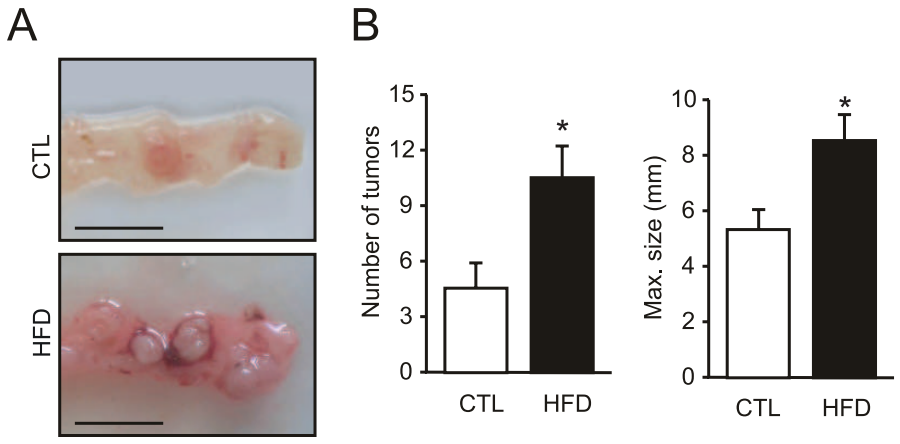


Supplementary Figure 1. Western blot of colonic time-course of insulin-induced Akt phosphorylation in lean mice.

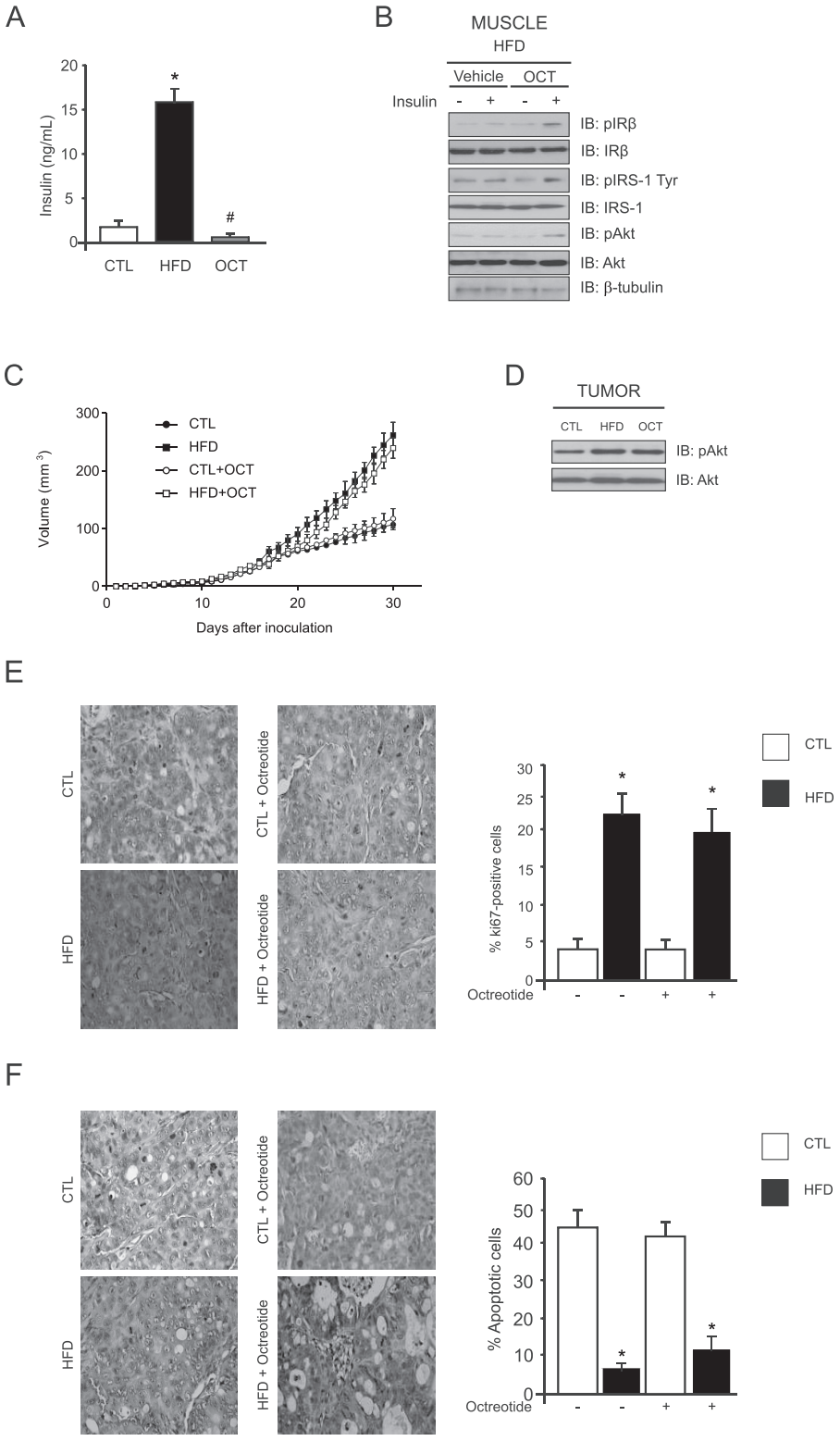
Supplementary Table 2. Characteristics of Chow-Fed and HFD-Fed C57BL6/J and SCID Mice and Chow-Fed C57BL6/J and ob/ob Mice

					SCID	
Characteristics	CTL	HFD	C57	ob/ob	CTL	HFD
Body weight, *g*	24.3 ± 2.8	33.2 ± 4.1[a]	22.3 ± 2.2	55.2 ± 3.5[a]	19.6 ± 1.6	28.5 ± 2.5[a]
Epididymal fat, *g*	4.0 ± 0.1	8.0 ± 0.5[a]	3.0 ± 0.1	11.0 ± 0.5[a]	0.05 ± 0.01	0.15 ± 0.02[a]
Insulin, *ng/mL*	3.8 ± 0.2	7.4 ± 0.5[a]	0.4 ± 0.1	12.46 ± 0.5[a]	0.53 ± 0.05	1.0 ± 0.15[a]
Leptin, *ng/mL*	2.1 ± 0.4	3.7 ± 0.5[a]	1.2 ± 0.4	ND	0.6 ± 0.01	1.5 ± 0.4[a]
TNF-α, *pg/mL*	53.2 ± 7.8	104 ± 9.6[a]	63.4 ± 0.6	169.6 ± 9.1[a]	2.57 ± 1.1	10.8 ± 5.4[a]
Kitt, *%/min*	5.1 ± 0.1	2.8 ± 0.4[a]	4.2 ± 0.1	3.3 ± 0.1[a]	6.0 ± 1.2	3.5 ± 1.1[a]

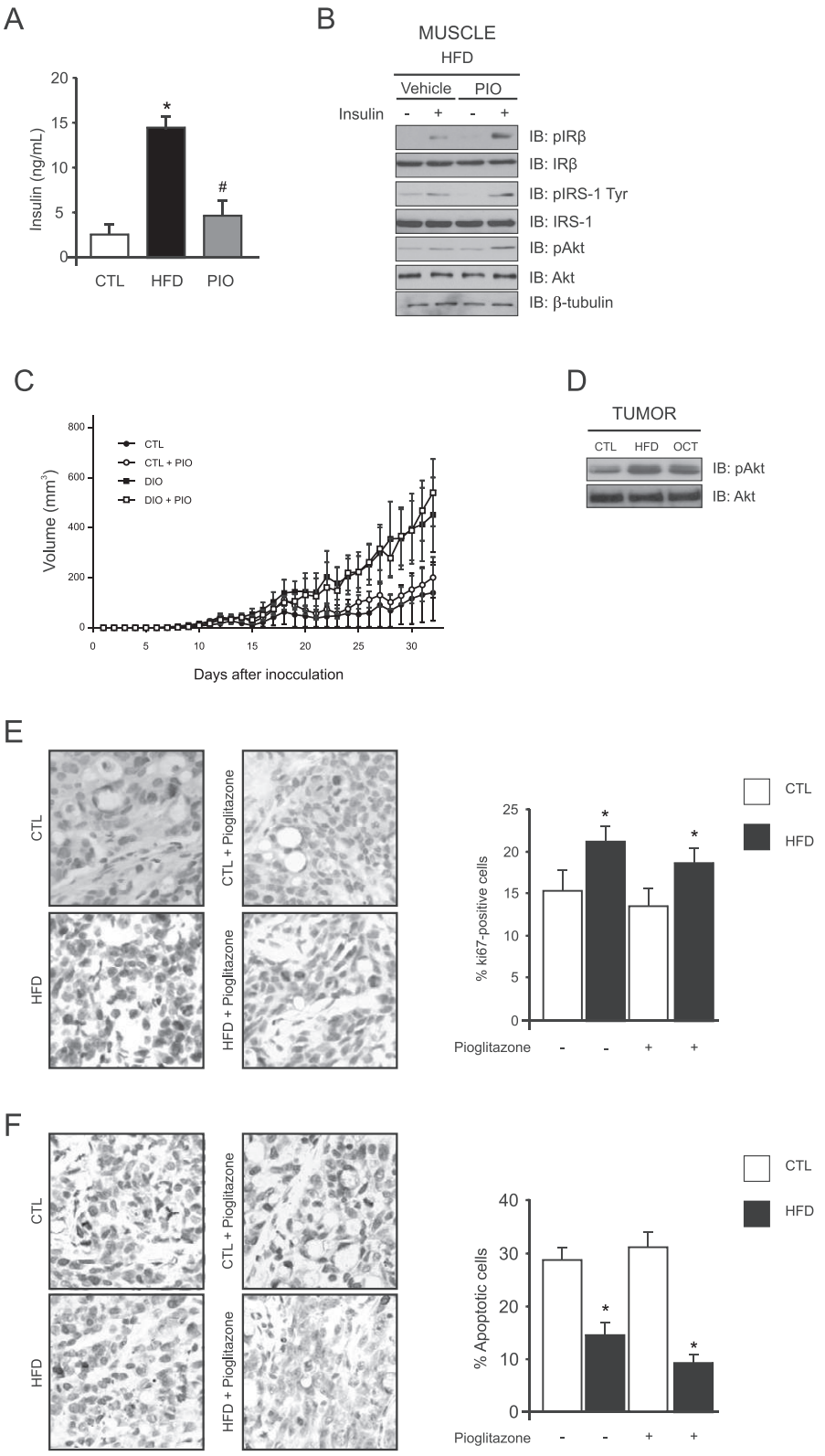
NOTE. n = 8 in each group. Values are presented as mean ± standard deviation.
CTL, control (chow-fed) mice; Kitt, rate constant for glucose disappearance in the insulin tolerance test.
[a]$P < .01$ vs respective control group.



Supplementary Figure 2. Diet-induced obesity promotes colon carcinogenesis in mice treated with AOM alone. (*A*) Macroscopic changes in colonic tissues. *Scale bars*, 5 mm. (*B*) Tumor incidence and size of colons removed at the end of the protocol from CTL and HFD-fed mice. Results are means ± SEM (n = 8). *$P < .05$ vs CTL.



Supplementary Figure 3. Octreotide reduces insulin levels with no effects on tumor growth, proliferation, and apoptosis. (*A*) Dosage of insulin from the serum of CTL, HFD, and HFD and octreotide-treated (OCT) mice. (*B*) Insulin-induced IRβ, IRS-1, Akt, and β-tubulin phosphorylation and expression in muscle lysates from HFD-fed mice, treated or not with OCT. (C) Tumor growth was measured for 30 days in SCID mice maintained on normal chow or HFD for 8 weeks before tumor inoculation with HT-29 cells (1×10^6 cells/mouse) and kept on normal chow or HFD for 30 days after inoculation and with the indicated treatment. (*D*) Western blots showing HT-29 xenografts lysates from CTL, HFD-fed mice, treated or not with OCT, immunoblotted with pAkt and Akt. (*E* and *F*) Representative microphotographs and quantification of (*E*) Ki67 and (*F*) apoptotic staining on HT-29 xenografts sections, 4 fields per tumor section, mean ± SEM. $^*P < .05$ vs control; $^{\#}P < .05$ vs HFD.



Supplementary Figure 4. Pioglitazone reduces insulin levels with no effects on tumor growth, proliferation, or apoptosis. (*A*) Dosage of insulin from the serum of CTL, HFD, and HFD and pioglitazone-treated (PIO) mice. (*B*) Insulin-induced IRβ, IRS-1, Akt, and β-tubulin phosphorylation and expression in muscle lysates from HFD-fed mice, treated or not with PIO. (*C*) Tumor growth was measured for 30 days in SCID mice maintained on normal chow or HFD for 8 weeks before tumor inoculation with HT-29 cells (1×10^6 cells/mouse) and kept on normal chow or HFD for 30 days after inoculation and with the indicated treatment. The results are means ± SD from 5–8 mice per group. (*D*) Western blots showing HT-29 xenograft lysates from CTL, HFD-fed mice, treated or not with PIO, immunoblotted with pAkt and Akt. (*E* and *F*) Representative microphotographs and quantification of (*E*) Ki67 and (*F*) apoptotic staining on HT-29 xenograft sections, 4 fields per tumor section, mean ± SEM. $^*P < .05$ vs control; $^\#P < .05$ vs HFD.

Supplementary Table 3. Characteristics of HFD-Fed Mice Treated With Vehicle or Infliximab

	HFD + vehicle	HFD + infliximab
TNF-α, *pg/mL*	133.4 ± 31.9	38.5 ± 23.4[a]
Leptin, *ng/mL*	1.95 ± 0.18	1.67 ± 0.43
Kitt, *%/min*	1.9 ± 0.6	5.1 ± 0.7[a]

NOTE. n = 4–5 in each group. Values are presented as mean ± standard deviation.
Kitt, rate constant for glucose disappearance in the insulin tolerance test.
[a]$P < .05$ vs vehicle-treated group.

CAPÍTULO II

ABSENCE OF DOUBLE –STRANDED RNA DEPENDENT PROTEIN KINASE PROTECTS FROM DEVELOPMENT OF COLITIS-ASSOCIATED CANCER

Running title: PKR protects against CAC

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Conflict of interest: The authors declare no conflict of interests.

ABSTRACT

The association between obesity and colon cancer development is a major conceptual advance in the understanding of colon cancer pathogenesis in the last decade and the role of obesity–related subclinical inflammation in carcinogenesis gained significance. It is believed that obesity acts as a tumor promoter, and it`s pro-tumorigenic effects depend mainly on low-grade inflammatory response caused by obesity-driven production of inflammatory and pro-tumorigenic cytokines (TNF and IL -6). A key feature of obesity-induced inflammation is the infiltration of macrophages in adipose tissue, leading to production of inflammatory cytokines and other mediators that also interfere with insulin signaling. Thus, the main objective of the study was to evaluate the role of PKR in the development of colon tumors in wild type and PKR knockout mice subjected to a high fat diet. In the present study, we manipulated the expression of PKR in myeloid cells to better define the role of PKR during tumorigenesis induced by colitis associated cancer and also their role in diet-induced inflammation and insulin resistance. We observe that PKR absence prevents colon tumor formation. We define the function of PKR in myeloid cells, where it mediates resistance to epithelial damage caused by DSS and prevents the formation of tumors. Moreover, the absence of PKR in myeloid cells also confers protection to insulin resistance induced by a high-fat diet, reducing the inflammation induced by obesity. These observations demonstrate that PKR can be a primary point during carcinogenesis associated with inflammation and may represent a promising target for therapeutic intervention.

INTRODUCTION

Colorectal cancer (CRC) remains a major health burden with more than one million cases worldwide and a mortality of approximately 33% in the developed world (Center et al., 2009; Walther et al., 2009). The association between obesity and the risk for CRC development is observed in both men and women (relative risk: 1.2–2.0) (Calle et al., 2003; Giovannucci, 2002). Among the major mediators of the inflammatory response is tumor necrosis factor (TNF)-α, whose over expression in adipose tissue is a common feature in human and animal models of obesity (Araújo et al., 2007; Osório-Costa et al., 2009). TNF−α, first identified as an antitumor agent, is now also recognized as a tumor-promoting cytokine that links inflammation and cancer (Balkwill, 2009). Furthermore, the increased TNF−α levels associated with obesity are a potent liver tumor promoter in mice (Park et al., 2010).

Chronic colon inflammation may release ROS and RNI (Hussain et al., 2003) that cause DNA damage and affect cell proliferation or survival and this way promote oncogenesis (Meira et al., 2008). Although, experimental evidences suggest that it acts mainly as a tumor promoter instead as an initiator (Coussens and Werb, 2002; Karin and Greten, 2005; Karin et al., 2006). Immune cells, frequently present in pre-neoplastic lesions produce a variety of cytokines and chemokines that increase local inflammatory response and increase pre-malignant cells survival and growth activating transcription factors such as NF-κB (Lin and Karin, 2007; Pikarsky et al., 2004). NF-κB-stimulated cytokines production by myeloid cells is essential for the colitis-associated cancer (CAC) growth, while NF-κB activation in epithelial intestinal cells promotes survival of newly formed

pre-malignant cells (Greten et al., 2004). These studies suggest that cytokines and growth factors produced after NF-κB activation in intestinal myeloid cells stimulate proliferation of pre-malignant intestinal epithelial cells in the first stages of CAC tumorigenesis. NF-κB inactivation in myeloid cells through IKKβ ablation inhibits production of inflammatory mediators such as IL-6 and TNF-α and prevents intestinal epithelias cells proliferation during CAC, decreasing the number of tumors formed and their size (Greten et al., 2004).

Metabolic diseases arise as groups including obesity, insulin resistance, type 2 diabetes, cardiovascular disease and cancer and are an important health problem worldwide, with limited treatment options. In the last decade, we have observed that the emergence of this group of diseases has strong inflammatory bases (Hotamisligil, 2006). During the course of obesity, a wide range of stress and inflammatory responses are evoked in metabolic tissues, leading to chronic local low grade inflammation which plays a central role in the inhibition of insulin receptor signaling and disturbances of systemic metabolic homeostasis. This atypical state involves immune and non-immune cells and pathways of immune engagement with nutrients and metabolites answer.

PKR can regulate or act together with major inflammatory signaling pathways that are involved in metabolic homeostasis, including the N-teminal kinase c-Jun (JNK) and IκB kinase (IKK). Studies in PKR-/- animals have shown that these mice present a lean phenotype and are protected against diet-induced obesity (Carvalho-Filho et al., 2012; Nakamura et al., 2010). In addition to being more lean and responsive to insulin, high-fat diet-induced PKR-/- mice exhibit reduced expression of proinflammatory cytokines such as IL-6 and TNFα, compared to wild type animals (Carvalho-Filho et al., 2012; Nakamura et

al., 2010). These data suggest that PKR may be involved in diet-induced inflammation. In both mice and humans, there is a consistent and marked increase in the phosphorylation of eIF2α and JNK activity in metabolic tissues of obese subjects (Carvalho-Filho et al., 2012; Gregor et al., 2009; Nakamura et al., 2010). In diet-induced obese mice, the absence of PKR protects against obesity and insulin resistance by preventing the activation of JNK and IKKβ, indicating that PKR is an important modulator of the insulin signaling under normal physiological conditions and in obesity (Carvalho-Filho et al., 2012; Nakamura et al., 2010).

In the present study, we manipulated the expression of PKR in myeloid cells to define the role of PKR during tumorigenesis induced by CAC and also their role in diet-induced inflammation and insulin resistance. We define the function of PKR in myeloid cells, where it mediates resistance to epithelial damage caused by DSS and prevents the formation of tumors. Moreover, the absence of PKR in myeloid cells also confers protection against insulin resistance induced by a high-fat diet, reducing the inflammation induced by obesity. These observations demonstrate that PKR can be a primary point during carcinogenesis associated with inflammation and may represent a promising target for therapeutic intervention.

METHODS

Antibodies, chemicals and buffers

All the reagents were from Sigma-Aldrich, unless otherwise specified. DSS was from MP Biochemicals (Solon, OH, USA). Anti-phospho-PKR, anti-PKR, anti-phospho-eIF2α, anti-eIF2α, anti-phospho-IRβ, anti-phospho-IRS-1 Tyr941, anti-IκBα, anti-phospho-JNK, anti-IL6 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-Akt and anti-β−actin antibodies were from Cell Signaling Biotechnology (Beverly, MA, USA).

Animals

The Ethics Committee of the State University of Campinas approved all experiments. Mice were provided from Central Breeding Center of the State University of Campinas and randomly divided into two groups, control and high-fat diet (HFD). The diet composition is described in the Supplemental Table 1.

Insulin tolerance test, serum insulin, leptin, IL-6 and TNF−α quantification

The mice were given an insulin tolerance test (ITT; 1.5 IU insulin/kg body weight) as described previously[20]. Plasma was separated by centrifugation (1,100xg) for 15 min at 4°C and stored at −80°C until the assay. Serum insulin and leptin were measured by using a mouse ELISA kit (Linco, St. Charles, MO, USA). Serum IL-6 and TNF−α were measured using a mouse ELISA kit (Thermo Scientific, Rockford, IL, USA).

Tumor induction and analysis

Four-week-old male mice (C57BL6/J Unib (WT) and Pkr-/- (PKR-/-)) were placed on standard chow or on a high-fat diet for one week and then injected intraperitoneally (i.p.) with 12.5 mg/kg AOM. After five days, 2.5% DSS (MW 36–50 kDa) was given in the drinking water for five days, followed by 14 days of regular water. This cycle was repeated twice and mice were sacrificed 30 days after the last cycle, at 20 weeks of age. Colons were removed, flushed with PBS, fixed in 4% paraformaldehyde and paraffin-embedded. Sections (5 μm) were cut and stained with hematoxylin and eosin (H&E). Tumor counts were performed in a blinded fashion. Tumor sizes were measured with calipers. Assessment of colitis disease scores was performed as previously described (Kojouharoff et al., 1997).

Cell culture

The mouse melanoma cancer cell line, B16-F10, was purchased from the Rio de Janeiro Cell Bank and cells were cultured in DMEM medium containing 10% fetal bovine serum with the addition of antibiotics and fungicides. Cells were maintained at 37°C in a humid atmosphere and 5% CO_2.

Tumor graft models

Four-week-old male C57BL6/J Unib mice (n=10 per group) were inoculated subcutaneously in the dorsal region with $1x10^6$ B16-F10 cells. Tumor volume (V) was calculated daily by measuring length (L) and width (W) of the tumor with calipers and using the formula V= {W x L x [(W+L)/2]} x 0.52.

Treatment with PKR inhibitor (Merck) began 7 days after cells inoculation. PKR inhibitor (20mg/kg) was given subcutaneously every two days.

Tissue extracts

Mice were anesthetized with sodium amobarbital (15 mg/kg body weight, i.p.). Tumors and colons were removed, minced coarsely, and homogenized in extraction buffer (1% Triton-X 100, 100 mM Tris, pH 7.4, containing 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PMSF, and 0.1 mg of aprotinin/ml). The extracts were centrifuged at 11,000 rpm and 4°C, and the supernatants of these tissues used.

Bone marrow transplantation

5-week-old male mice were lethally irradiated (1050rad) and transplanted with 1 x 10^6 bone marrow cells from WT or PKR-/- mice.

Peritoneal macrophage isolation

Mice were killed by cervical dislocation, abdominal wall was exposed, and sterile PBS injected into cavity. After a 10 seconds gentle shake, peritoneal lavage fluid containing cells was withdrawn and plated on cell culture dishes in DMEM media, supplemented with fetal bovine serum 10% at 37°C for one hour. Then, adherent macrophages (>90% of the cells) were treated with 10ng/mL of LPS for three hours. Afterward, cells were collected and mRNA was analyzed.

Protein analysis by immunobloting

Whole tissue extracts were homogenized in extraction buffer, treated with Laemmli sample buffer containing 100 mM DTT, and heated in a boiling water bath. For total extracts, similar-sized aliquots (50 μg protein) were subjected to SDS-PAGE. Proteins were resolved in 8-15% SDS gels and blotted onto nitrocellulose membranes (Bio-Rad). Band intensities were quantified by optical densitometry of developed autoradiographs using UN-SCAN-IT Gel (Silk Scientific, Inc. Orem, UT, USA).

Statistical analysis

Data are presented as means ± SEM of at least three independent experiments. Statistical analysis was performed by employing the ANOVA test with the *Bonferroni post test*. Significance was established at the $p<0.05$ level.

RESULTS

Absence of PKR protects against tumor formation

It has been demonstrated that mice lacking PKR have lower levels of inflammation, with reduced JNK activity and lower level of inflammatory cytokines such as TNF-α and IL-6 under a high-fat diet (Nakamura et al., 2010). As TNF-α is involved in development of colon cancer in mice (Flores et al., 2012), we sought to examine the effect of complete absence of PKR on CAC development . We therefore injected wild-type (WT) and PKR-/- mice with the procarcinogen azoxymethane (AOM) followed by three rounds of dextran sodium sulfate (DSS) exposure to promote colitis. The PKR deficiency decreased tumor frequency and numbers in mice (Figure 1A and B), although there was no statistically difference in tumor size (Figure 1C).

Pharmacological inhibition of PKR protects against tumor formation

We also inhibited PKR activity with a pharmacological inhibitor and observed that inhibition of PKR was able to reduce tumor growth of a subcutaneous tumor graft (Figure 2A, B and C). Furthermore, we submitted PKR inhibitor-treated WT mice to the AOM +DSS tumor induction protocol and observed that when PKR is inhibited, tumor incidence

and number are decreased (Figure 2 D and E), while there is no statistically difference in tumor size (Figure 2 F). Taken together, these data indicate that PKR is important for tumor development but not for tumor growth in CAC.

PKR activity in the colon of obese mice

Studies demonstrated that obesity activates the inflammatory pathway in the colon (Flores et al., 2012) and that PKR is also active in liver and white adipose tissue of obese mice (Carvalho-Filho et al., 2012; Nakamura et al., 2010). We next sought to evaluate PKR activity in the colon of lean and obese WT and PKR-/- mice. As indicated in Figure 3A, high-fat diet increased phosphorylation and activity of PKR in the colon. We also examined the effect of AOM and DSS treatment on PKR activity in the colon of mice. DSS is known to cause inflammation of the colon and we observe that in WT mice DSS treatment increased PKR activity (Figure 3B).

PKR absence protects against obesity-induced colon tumor formation

We next examined the effect of obesity on WT and PKR-/- mice colon tumor formation. Consistent with previous findings (Flores et al., 2012), WT obese mice exhibited increased frequency and the number of colon tumors (Figure 4), although no difference was

observed in tumor size. As expected, lack of PKR protected against tumor formation in lean mice, as well as in high-fat diet treated mice (Figures 4A and B). As observed, high-fat diet was not able to increase tumor formation in PKR-/- mice. We also analyzed circulating cytokines levels of WT and PKR-/- mice in standard or high-fat diet. As observed in Figures 4D and E, high-fat diet was only able to increase the level of TNF-α and IL-6 in WT mice.

PKR is required for LPS-mediated induction of proinflammatory cytokines in macrophages

It was previously reported that PKR induces an inflammatory response in macrophages (Carvalho-Filho et al., 2012). Therefore, we tested whether PKR directly regulates expression of proinflammatory cytokines in macrophages exposed to LPS. Incubation of WT peritoneal macrophages with LPS caused an increase in mRNA levels of IL1β, IL-6 and TNF-α but not in iNOS (Figures 5A-D). Real-time PCR analysis showed that LPS-induction of the inflammatory cytokines mRNA was attenuated in PKR-/- macrophages (Figure 5A-D). Furthermore, anti-inflammatory IL-10 mRNA expression was reduced in both WT and PKR-/- macrophages after LPS induction (Figure 5 E). Together, these results support the hypothesis that PKR is an important component of the inflammatory response triggered by exposure of macrophages to LPS, one of the molecules associated with obesity.

Deletion of PKR in myeloid cells decreases obesity-induced colon tumor formation

To address the role of PKR in myeloid cells, we injected PKR-/- bone marrow in WT mice and WT bone marrow in PKR-/- mice. The effect of the deletion of PKR was analyzed. We detected reduced expression of proinflammatory cytokines TNF-α and IL-6 on animals with PKR-/- bone marrow under a high-fat diet treatment than in WT bone marrow mice with a high-fat diet (Figures 6 A-D).

Deletion of PKR in myeloid cells also reduced tumor incidence in the CAC model (Figures 6E and F) of high-fat diet fed animals. Mice also showed a reduction in tumor counts (Figures 6G and H) and even a protection in tumor size (Figures 6I and J). Mice with PKR-/- bone marrow present similar number and size of tumors, independently of the diet, while mice with WT bone marrow are much more susceptible to the inflammatory effects of the high-fat diet in promoting tumor growth. These data support the notion that PKR in myeloid cells can promote tumor growth and development through production of tumor-promoting factors.

Resistance to diet-induced obesity is due to PKR deficiency in myeloid cells

Previous studies have shown that PKR-/- mice are resistant to diet-induced obesity (Carvalho-Filho et al., 2012; Nakamura et al., 2010). To determine if myeloid PKR

deficiency prevents obesity, WT and PKR-/- mice, as well as the bone marrow transplant groups, were fed normal chow or high-fat diet for eight weeks starting three weeks posttransplantation. As expected, no differences in body mass were observed in chow-fed mice and PKR-/- mice on high-fat diet were protected from obesity (Figures 7A and B). Importantly, mice reconstituted with PKR-/- bone marrow (PKR-/- → WT) also were protected from high-fat diet weight gain.

PKR deficiency in myeloid cells improves insulin sensitivity

As expected, PKR-/- mice maintained in high-fat diet had improved glucose and insulin tolerance compared to similarly fed WT mice. PKR-/- → WT on high-fat diet also exhibited improved glucose and insulin tolerance compared to similarly fed WT → WT mice (Figures 7C-F). To test whether PKR deficiency in the hematopoietic compartment improves insulin signaling, we measured proteins from the insulin signaling pathway in muscle of chow and high-fat diet fed mice with WT or PKR-/- bone marrow (Figures 7G and H). Together, these data demonstrate that depletion of PKR in myeloid cells leads to improved insulin sensitivity in both high-fat diet and chow fed mice.

We also assessed whether the inflammatory pathway also showed further improvement in signaling. As shown in Figure 7I, high-fat diet in WT bone marrow increased IL-6 and JNK phosphorylation and decrease IκBα expression.

DISCUSSION

A connection between inflammation and cancer has been suspected for a long time. Overweight and obesity greatly increases colon cancer risk, especially in men (Calle and Kaaks, 2004; Calle et al., 2003). Despite the magnitude of the effect and the very large number (900,000) of individuals on which the epidemiological study that provided this insight was based, the mechanism by which obesity increases risk of death from colon cancer remained unknown. Furthermore, the tumor-promoting effect of obesity in colon cancer depends to a large extent on the low-grade inflammatory response it induces, which involves elevated production of TNF-α and IL-6, both of which are tumor-promoting cytokines (Lin and Karin, 2007). Notably, IL-6 signaling through ERK and STAT3 can reduce epithelial cell apoptosis while stimulating cell proliferation, as recently found in CAC (Grivennikov et al., 2009). Furthermore, expression of IL-6 is elevated in cirrhosis and hepatocelularcarcinoma (HCC) (Tilg et al., 1992; Trikha et al., 2003). Elevated serum IL-6 also correlates with a higher risk of progression from chronic viral hepatitis to HCC (Nakagawa et al., 2009; Wong et al., 2009). Furthermore, IL-6 -/- male mice no more responded to the tumor-promoting effect of obesity (Park et al., 2010). Although TNF-α and IL-6 may not be the only mediators responsible for obesity-induced tumor promotion, it is well-established that their production is elevated in obese mice due to the low-grade inflammatory response caused by lipid accumulation (Arkan et al., 2005; Hotamisligil, 2006; Shoelson et al., 2007; Solinas et al., 2007). Regardless of its source, it is established that IL-6 can propagate inflammation (Kamimura et al., 2003).

Although cell-autonomous events such as proliferation and death evasion control tumor development, the tumor microenvironment also makes a major contribution and influences the physiology of malignant cells (Radisky and Bissell, 2004). Nearly all tumors contain inflammatory and immune cells, such as dendritic cells, macrophages, and lymphocytes, which produce cytokines and other factors that promote tumor growth and survival (Balkwill et al., 2005; Coussens and Werb, 2002; Lin and Karin, 2007). The most obvious tumor-promoting role of immune cells is manifested in inflammation-associated cancers, where tumors arise and grow at sites of chronic inflammation. In previous work, inhibition of NF-κB activation in myeloid cells, exerted through the cell-type-specific ablation of IKKβ, inhibits the proliferation of premalignant IECs in a mouse model of CAC (Greten et al., 2004). Myeloid cells in the lamina propria of mice subjected to CAC induction produce cytokines that stimulate the proliferation of adjacent premalignant IECs, which harbor activating b-catenin mutations (Greten et al., 2004).

During early CAC induction, when it acts as a tumor promoter, IL-6 is produced mainly by myeloid cells (Grivennikov et al., 2009). Correspondingly, mice lacking IL-6 in bone marrow-derived cells exhibited fewer tumors, with a substantial reduction in overall CAC tumor load (Grivennikov et al., 2009). Similarly, we also demonstrate that mice lacking PKR in bone marrow derived cells also showed fewer and smaller tumors.

Besides its importance during early tumor promotion, IL-6 signaling also affects tumor growth during late stages of CAC (Becker et al., 2004). Interestingly, IL-6 signaling during that stage increases TNF-α production, and interference with TNF-α signaling curtails tumor growth and reduces IL-6 production. Thus, the beneficial effect of TNF-α

inhibition may be partially due to reduced IL-6 expression. While TNF-α can directly induce IL-6 production by NF-κB-, NF-IL-6-, and AP-1-dependent mechanisms (Legrand-Poels et al., 2000), it also can facilitate the recruitment and survival of proinflammatory immune cells capable of IL-6 production (Kollias et al., 1999). Conversely, IL-6 can directly induce TNF-α transcription and sustains chronic inflammation, in particular by ensuring the continuous presence of TNF-α-producing cells (Atreya et al., 2000). Such cross-regulation is not unique to IL-6 and TNF-a since, for example, IL-1 can also induce IL-6 production (Legrand-Poels et al., 2000) and have an indirect impact on immune cell recruitment (Dinarello, 1994). Indeed, IL-1α released by necrotic hepatocytes induces IL-6 synthesis (Sakurai et al., 2008), whereas IL-1β is involved in CAC development (Garlanda et al., 2007; Xiao et al., 2007) and is also a well-established IL-6 inducer in immune cells and even in IECs (Parikh et al., 1997). We previously established a role for TNF-a in obesity-mediated colon carcinogenesis and that the use of TNF-a blockers should be evaluated in colon cancer chemoprevention for obese individuals (Flores et al., 2012).

It has become apparent that the interaction between transformed cells and their environment, including innate immune cells, fibroblasts and endothelial cells can be instrumental in both tumor promotion and progression of many epithelial tumors, especially in the context of chronic inflammation (Balkwill and Mantovani, 2001; Coussens and Werb, 2002). The deletion of PKR in myeloid cells results in a marked decrease in inflammation-induced proliferation and tumor size. Interestingly, the deletion of PKR in myeloid cells had a more pronounced effect on tumor number than tumor size. This

strongly suggests that PKR in myeloid cells is most likely involved in tumor development than in tumor growth.

The role of macrophages in tumor development in contrast to tumor progression has not been clearly established, although these cells are important components of the tumor microenvironment (Balkwill and Mantovani, 2001; Coussens and Werb, 2002). Elimination of macrophages using a CSF-1null-mutation illustrated that macrophage recruitment is important for progression of mammary gland tumors, since invasive growth and metastasis were significantly attenuated (Lin et al., 2001). In addition to identifying a key molecular mechanism connecting inflammation and cancer, our results suggest that specific pharmacological inhibition of PKR may be very effective in prevention of CAC. Nevertheless, given the important role of PKR in promoting innate immune responses there is a certain risk associated with long-term use of such drugs, a problem that needs to be addressed before realizing the full benefit of PKR inhibitors in chemoprevention of CAC or other cancers associated with chronic inflammation.

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FIGURE LEGENDS

Figure 1: Analysis of tumor formation in WT and PKR-/- mice after AOM and 3 doses of DSS: (A) tumor incidence, (B) number of tumors and (C) tumor size in WT mice and PKR-/-. Data shown are mean ± SEM. * $p < 0.05$ vs. WT (n = 5 experiments).

Figure 2: Effect of pharmacological inhibition of PKR. (A) Growth curve of LLC tumors in WT mice treated with pharmacological inhibitor of PKR (PKRi) or vehicle. (B) Expression and phosphorylation of PKR and (C) expression and phosphorylation of eIF2α protein in LLC tumor tissue of WT mice treated with pharmacological inhibitor of PKR (PKRi) or vehicle. (D) tumor incidence, (E) number of tumors and (F) tumor size of AOM and 3 doses of DSSin WT mice treated with pharmacological inhibitor of PKR (PKRi) or vehicle. Data shown are mean ± SEM. * $p < 0.05$ vs. vehicle (n = 8-10 animals per group).

Figure 3: Expression and phosphorylation of PKR and eIF2α proteins in colonic mucosa of WT and PKR-/- mice. (A) Phosphorylation and expression of PKR and eIF2α proteins in the colon of WT and PKR-/- mice subjected to the control diet (CTL) or high-fat diet (HFD) for 8 weeks without treatment with AOM and DSS. (B) expression and phosphorylation of PKR and eIF2α proteins in the colon of WT and PKR-/- mice subjected to the control diet (CTL) or high-fat diet (HFD) for 8 weeks and treated with AOM and DSS.

Figure 4: Analysis of tumor formation in WT and PKR-/- mice after treatment with AOM and DSS and control (CTL) or high-fat diet (HFD): (A) Incidence of tumors, (B) number of tumors and (C) size of tumors in WT and PKR-/- mice treated with AOM and DSS in animals subjected to control diet (CTL) or high-fat diet (HFD) for 8 weeks. Data shown are mean ± SEM. (n = 4-7 experiments).

Figure 5: Analysis of mRNA expression in peritoneal macrophages of WT and PKR-/- mice stimulated with LPS (A) IL-1β, (B) IL-6, (C) iNOS, (D) TNF-α and (E) IL-10. Data shown are mean ± SEM. * $p < 0.05$ vs WT without LPS and # $p < 0.05$ vc PKR-/- without LPS

Figure 6: PKR-/- myeloid cells protect against obesity induced colon tumor formation. (A-D) serum dosage of TNF-a and IL-6, (E and F) tumor incidence, (G and H) tumor number and (I and J) of the indicated groups. WT with WT bone marrow, WT → WT, PKR-/- with PKR-/- bone marrow PKR-/- → PKR-/-, WT with PKR-/- bone marrow, PKR-/- → WT and PKR-/- with WT bone marrow, WT → PKR-/-) after treatment with AOM and DSS and control diet (CTL) or high-fat diet (HFD): Data shown are mean ± SEM. * $p < 0.05$ vs WT→WT CTL, # $p < 0.05$ vs. PKR-/-→WT CTL and $ $p < 0.05$ vs. WT→WT HFD.

Figure 7: Characterization of the insulin sensitivity of the bone marrow transplantation animals (A) Weight of transplanted animals underwent control diet (CTL) or high fat diet

(HFD) for 8 weeks. (B) Glucose Tolerance Test (GTT) of transplanted animals underwent control diet (CTL) or high-fat diet (HFD) for 8 weeks. (C) insulin tolerance test (Kitt) of transplanted animals in the control diet (CTL) or high-fat diet (HFD) for 8 weeks. (D) protein expression and phosphorylation of the insulin signaling pathway in the colon of transplanted animals subjected to control diet (CTL) or high-fat diet (HFD) for 8 weeks. (E) Protein expression and phosphorylation of the insulin signaling pathway in the colon of transplanted animals subjected to control diet (CTL) or high-fat diet (HFD) for 8 weeks and stimulated with insulin at the time of extraction. (F) Phosphorylation and protein expression of inflammatory pathway in colon transplanted animals underwent control diet (CTL) or high-fat diet (HFD) for 8 weeks. WT with WT bone marrow, WT → WT, PKR-/- with PKR-/- bone marrow PKR-/- → PKR-/-, WT with PKR-/- bone marrow, PKR-/- → WT and PKR-/- with WT bone marrow, WT → PKR-/-) after treatment with AOM and DSS. Data shown are mean ± SEM. * $p < 0.05$ vs. WT → WT CTL # $p < 0.05$ vs PKR -/- WT → CTL $ $p < 0.05$ vs. WT → WT HFD , † $p < 0.05$ vs. WT → PKR -/- CTL ‡ $p < 0.05$ vs PKR -/- → PKR -/- CTL .

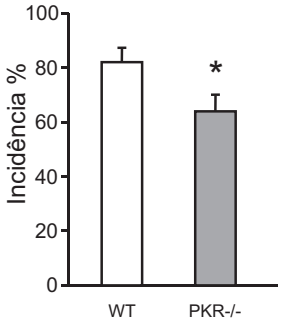
SUPLEMENTAL MATERIAL

Supplemental Table1: Components of Standard Chow and High-fat Diet

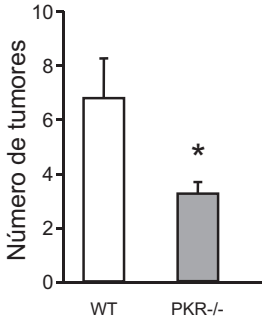
Ingredients	Standard Chow (g)	High Fat Diet (g)
Casein	202	200
Sucrose	100	100
Cornstarch	397	115.5
Dextrinated starch	130.5	132
Lard	-	312
Soybean oil	70	40
Cellulose	50	50
Mineral mix American Institute of Nutrition (AIN)-93	35	35
Vitamin mix AIN-93	10	10
L-Cystine	3	3
Choline	2.5	2.5

Figure 1

A.


Incidência %
100
80
60
40
20
0
*
WT
PKR-/-

B.


Número de tumores
10
8
6
4
2
0
*
WT
PKR-/-

C.

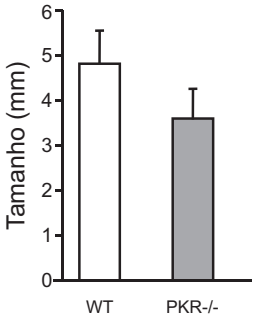
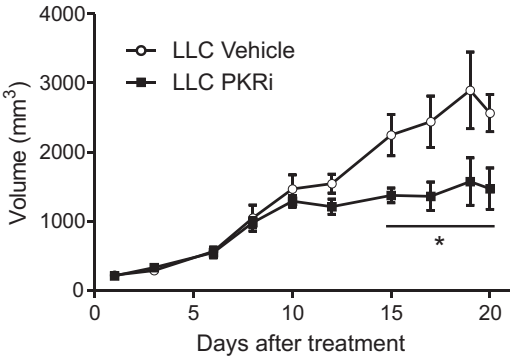
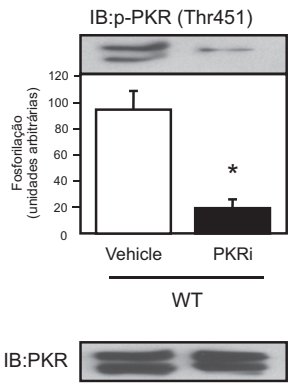

Tamanho (mm)
6
5
4
3
2
1
0
WT
PKR-/-

Figure 2

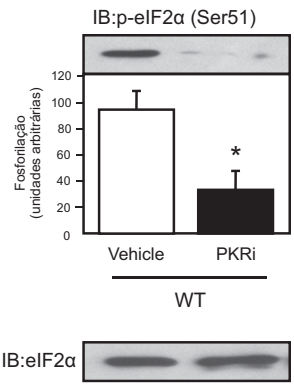
A.



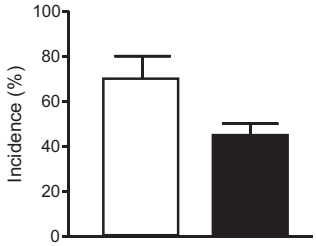
B.



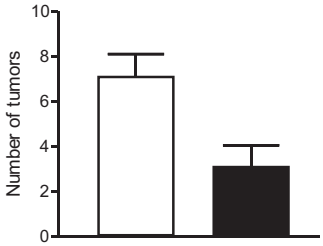
C.



D.



E.



F.

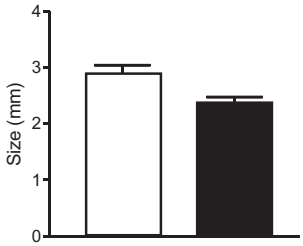


Figure 3

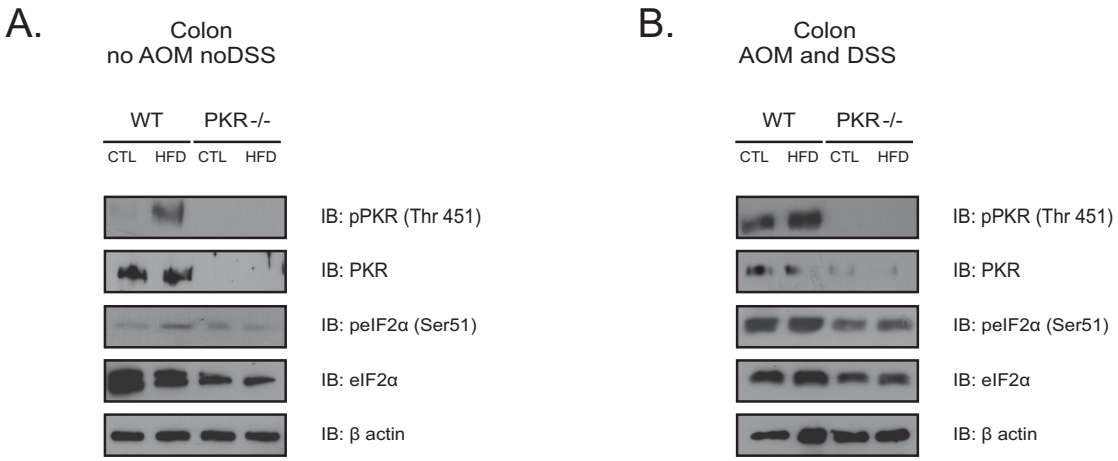

A.
Colon
no AOM noDSS
WT
PKR-/-
CTL
HFD
CTL
HFD
IB: pPKR (Thr 451)
IB: PKR
IB: peIF2α (Ser51)
IB: eIF2α
IB: β actin
B.
Colon
AOM and DSS
WT
PKR-/-
CTL
HFD
CTL
HFD
IB: pPKR (Thr 451)
IB: PKR
IB: peIF2α (Ser51)
IB: eIF2α
IB: β actin

Figure 4

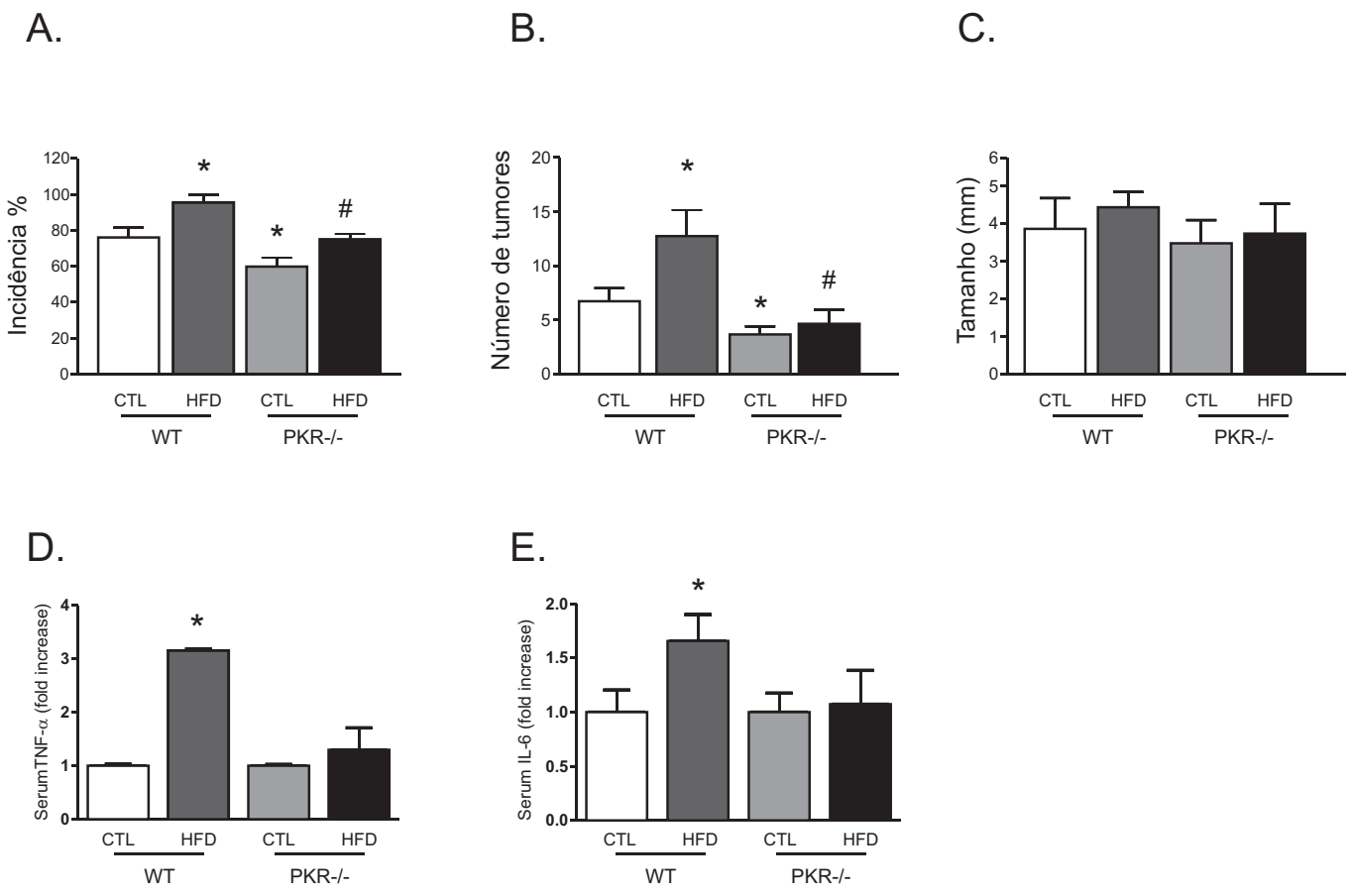

A.
Incidência %
120
100
80
60
40
20
0
*
*
#
CTL
HFD
CTL
HFD
WT
PKR-/-
B.
Número de tumores
20
15
10
5
0
*
*
#
CTL
HFD
CTL
HFD
WT
PKR-/-
C.
Tamanho (mm)
6
5
4
3
2
1
0
CTL
HFD
CTL
HFD
WT
PKR-/-
D.
SerumTNF-α (fold increase)
4
3
2
1
0
*
CTL
HFD
CTL
HFD
WT
PKR-/-
E.
Serum IL-6 (fold increase)
2.0
1.5
1.0
0.5
0.0
*
CTL
HFD
CTL
HFD
WT
PKR-/-

Figure 5

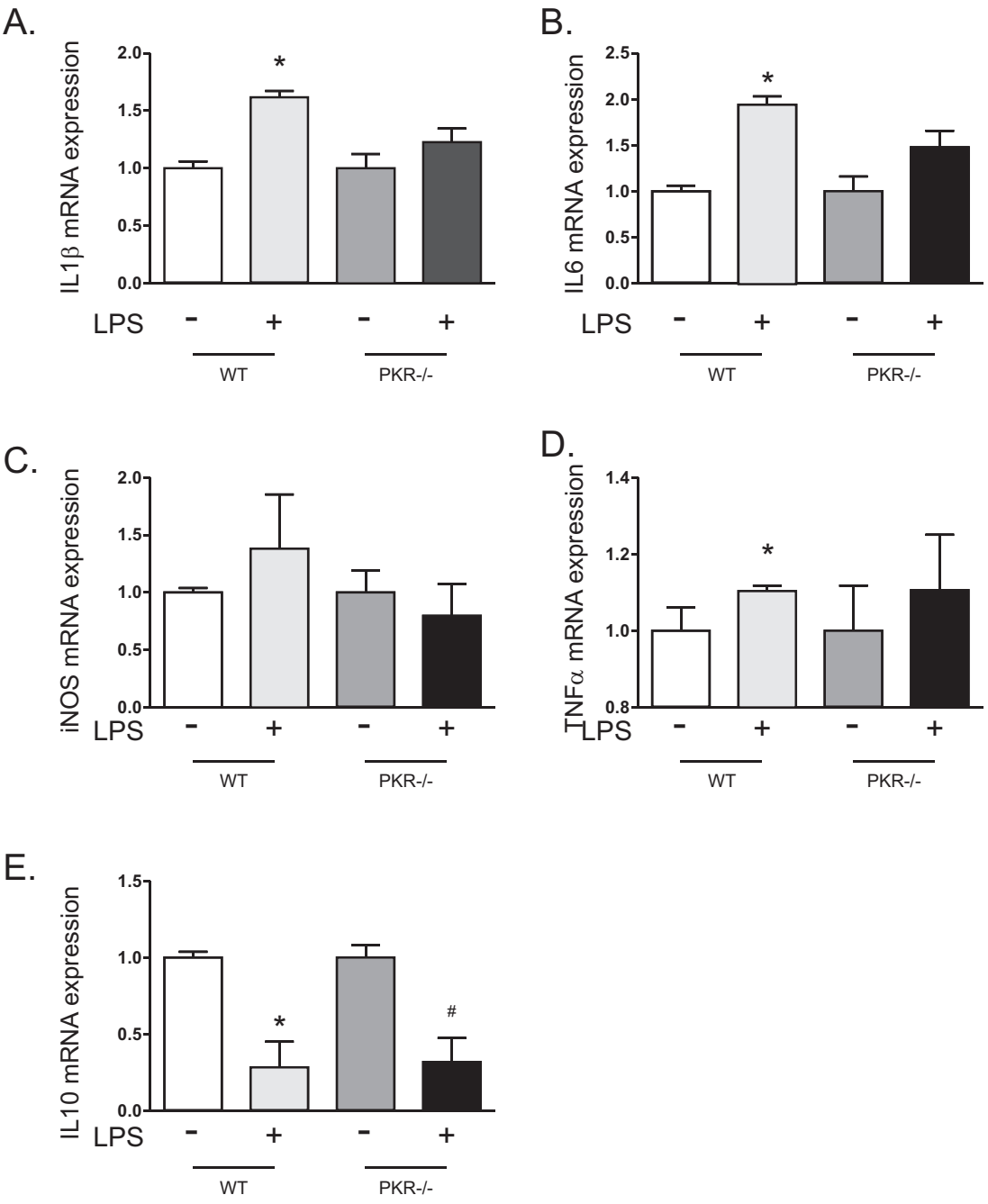
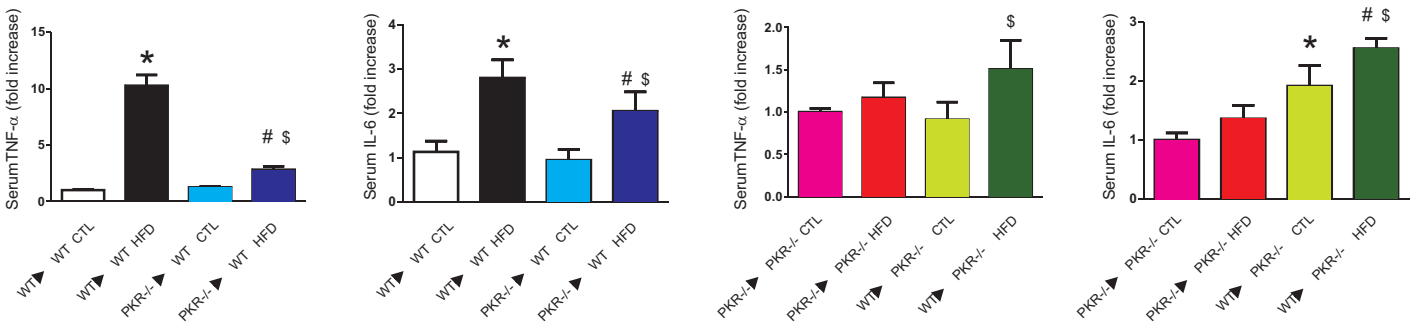
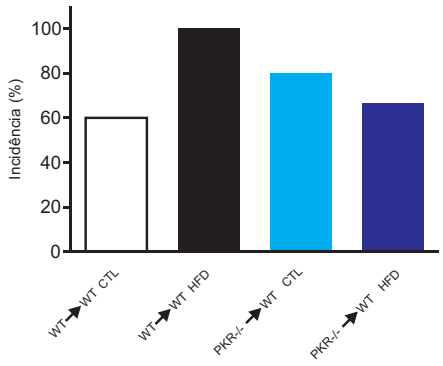

A.
IL1β mRNA expression
2.0
1.5
1.0
0.5
0.0
*
LPS
-
+
-
+
WT
PKR-/-
B.
IL6 mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
*
LPS
-
+
-
+
WT
PKR-/-
C.
iNOS mRNA expression
2.0
1.5
1.0
0.5
0.0
LPS
-
+
-
+
WT
PKR-/-
D.
TNFα mRNA expression
1.4
1.2
1.0
0.8
*
LPS
-
+
-
+
WT
PKR-/-
E.
IL10 mRNA expression
1.5
1.0
0.5
0.0
*
#
LPS
-
+
-
+
WT
PKR-/-

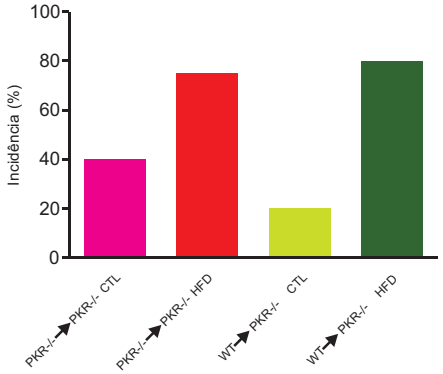
Figure 6



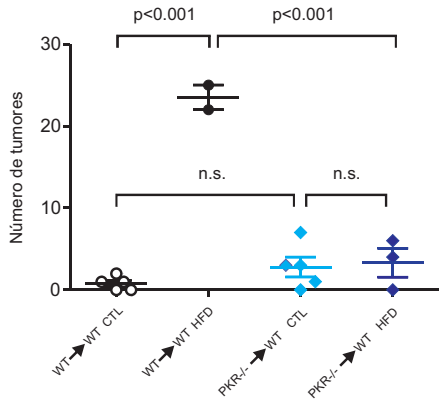
A.



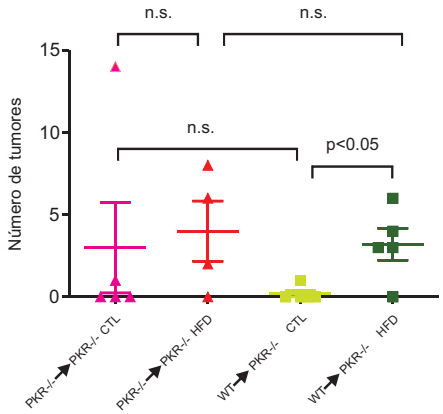
B.



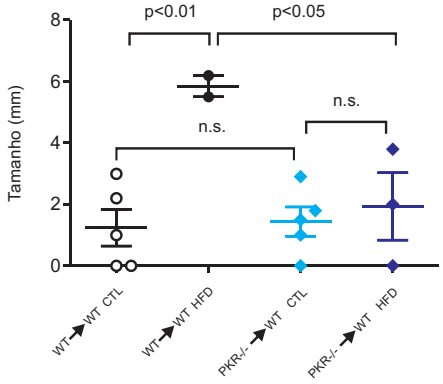
C.



D.



E.



F.

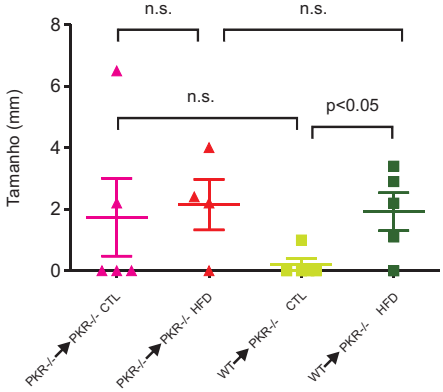
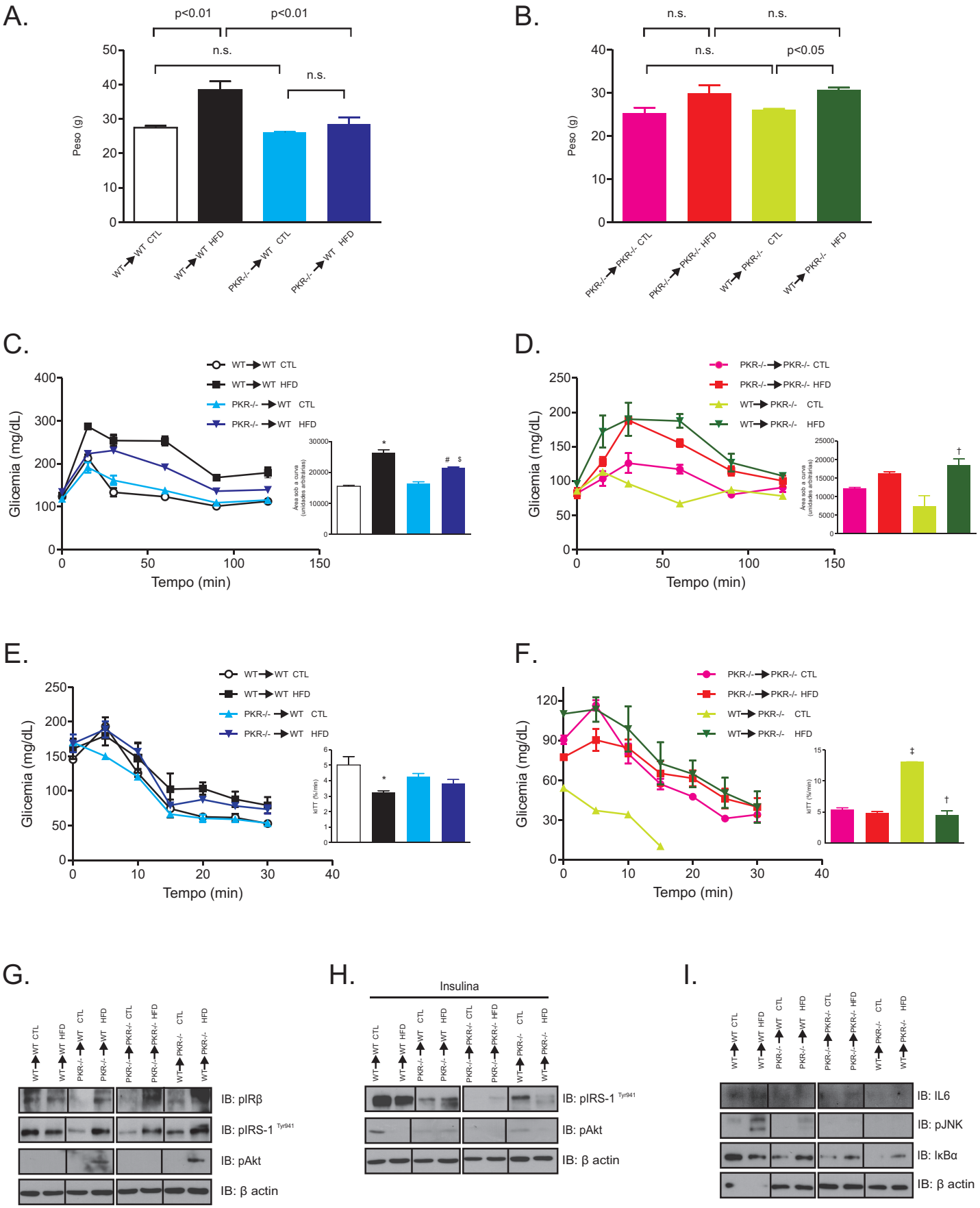


Figure 7


A.
p<0.01
p<0.01
n.s.
n.s.
Peso (g)
WT→WT CTL
WT→WT HFD
PKR-/-→WT CTL
PKR-/-→WT HFD
B.
n.s.
n.s.
n.s.
p<0.05
Peso (g)
PKR-/-→PKR-/- CTL
PKR-/-→PKR-/- HFD
WT→PKR-/- CTL
WT→PKR-/- HFD
C.
WT→WT CTL
WT→WT HFD
PKR-/-→WT CTL
PKR-/-→WT HFD
Glicemia (mg/dL)
Tempo (min)
Área sob a curva (unidades arbitrárias)
D.
PKR-/-→PKR-/- CTL
PKR-/-→PKR-/- HFD
WT→PKR-/- CTL
WT→PKR-/- HFD
Glicemia (mg/dL)
Tempo (min)
Área sob a curva (unidades arbitrárias)
E.
WT→WT CTL
WT→WT HFD
PKR-/-→WT CTL
PKR-/-→WT HFD
Glicemia (mg/dL)
Tempo (min)
kITT (%/min)
F.
PKR-/-→PKR-/- CTL
PKR-/-→PKR-/- HFD
WT→PKR-/- CTL
WT→PKR-/- HFD
Glicemia (mg/dL)
Tempo (min)
kITT (%/min)
G.
WT WT CTL
WT WT HFD
PKR-/- WT CTL
PKR-/- WT HFD
PKR-/- PKR-/- CTL
PKR-/- PKR-/- HFD
WT PKR-/- CTL
WT PKR-/- HFD
IB: pIRβ
IB: pIRS-1 Tyr941
IB: pAkt
IB: β actin
H.
Insulina
WT WT CTL
WT WT HFD
PKR-/- WT CTL
PKR-/- WT HFD
PKR-/- PKR-/- CTL
PKR-/- PKR-/- HFD
WT PKR-/- CTL
WT PKR-/- HFD
IB: pIRS-1 Tyr941
IB: pAkt
IB: β actin
I.
WT WT CTL
WT WT HFD
PKR-/- WT CTL
PKR-/- WT HFD
PKR-/- PKR-/- CTL
PKR-/- PKR-/- HFD
WT PKR-/- CTL
WT PKR-/- HFD
IB: IL6
IB: pJNK
IB: IκBα
IB: β actin

DISCUSSÃO

DISCUSSÃO

A relação entre inflamação e câncer é suspeita há muito tempo. O sobrepeso e a obesidade aumentam o risco de câncer de cólon, especialmente em homens (Calle e Kaaks, 2004;. Calle et al, 2003). Apesar da magnitude do efeito e do grande número (900.000) de indivíduos em que se baseou o estudo epidemiológico que proporcionou este conhecimento, o mecanismo pelo qual a obesidade aumenta o risco de morte por câncer de cólon permaneceu desconhecido. Além disso, o efeito da obesidade como indutor de tumor de cólon depende, em grande parte, da resposta inflamatória de baixo grau por ela induzida, que envolve a produção elevada de TNF-α e IL-6, ambas citocinas atuam como promotoras de tumores (Lin e Karin, 2007). Notavelmente, a sinalização de IL-6 por meio de ERK e STATt3 pode diminuir a apoptose de células epiteliais, enquanto estimula a proliferação celular, como recentemente encontrado no câncer associado à colite (Grivennikov et al., 2009). Além disso, a expressão de IL-6 é elevada na cirrose hepática e em carcinoma hepatocelular (HCC) (Tilg et al, 1992;. Trikha et al, 2003). Elevadas concentrações séricas de IL-6 também se correlaciona com um maior risco de progressão da hepatite viral crônica para HCC (Nakagawa et al, 2009; Wong et al, 2009). Além disso, camundongos machos sem IL-6 não mais responderam ao efeito de promoção tumoral da obesidade (Park et al, 2010). Apesar de TNF-α e IL-6 não serem os únicos mediadores responsáveis pela promoção de tumores induzidos pela obesidade, é bem estabelecido que a sua produção é elevada em animais obesos devido à resposta inflamatória de baixo grau causada pelo acúmulo de lipídios (Arkan et al, 2005; Hotamisligil, 2006; Shoelson et al, 2007; Solinas et al, 2007). Independentemente de sua origem, é reconhecido que a IL-6 pode propagar a inflamação (Kamimura et al., 2003).

Os resultados deste estudo mostram que obesidade, induzida ou genética, aumenta significativamente o desenvolvimento do câncer colo-retal. Os nossos dados indicam que a inflamação do cólon induzida pela obesidade aumenta a expressão e atividade de JNK e IKKβ no cólon de camundongos tratados com AOM+DSS. Além disso, os camundongos obesos também apresentaram um aumento significativo nos níveis de insulina e uma deficiência na ativação da via da insulina no cólon destes animais.

Estudos epidemiológicos sugerem que a hiperinsulinemia é um importante mecanismo pelo qual a obesidade pode conferir um risco aumentado de câncer de cólon, e os níveis de insulina também podem influenciar o reaparecimento do câncer colo-retal (Sandhu MS, et al., 2002; Ma J et al., 2004). No entanto, observamos uma diminuição da sensibilidade à insulina no cólon. Consistente com estes resultados, os tratamentos de octreotideo e pioglitazona melhoraram a sensibilidade à insulina mas não foram capazes de diminuir a taxa de crescimento do tumor. É interessante notar que o octreotideo, além de reduzir a secreção da insulina, também diminui a síntese de vários peptídeos intestinais, tais como polipeptídeo insulinotrópico dependente da glicose (GIP) (Ranganath LR et al., 1999). GIP é um hormônio que pode ativar a via PI3K/Akt/mTOR no trato gastrointestinal (Baggio LL, et al., 2007); no entanto, não observamos diminuição do crescimento tumoral ou fosforilação de Akt no o tratamento com octreotideo. Os nossos dados mostram que a obesidade é acompanhada por um grande aumento do índice inflamatório do cólon, e também por um aumento na fosforilação de JNK e IKK, bem como o tratamento com o bloqueador de TNF-α, infliximabe, mostrou uma diminuição consistente no crescimento de tumores em xenoenxertos de HT-29, e no desenvolvimento de câncer associado à colite. Assim, o estado inflamatório de baixo grau observado na obesidade aumentou os níveis

plasmáticos de TNF-α e criou o "solo fértil" no epitélio do cólon que aumenta a ação de DSS e AOM.

Embora os eventos normais de células, como a proliferação e evasão de morte controlem o desenvolvimento tumoral, o microambiente tumoral também faz uma contribuição importante e influencia a fisiologia das células malignas (Radisky e Bissell, 2004). Quase todos os tumores contem células inflamatórias e imunes, tais como as células dendríticas, macrófagos e linfócitos, que produzem citocinas e outros fatores que promovem o crescimento tumoral e a sobrevivência (Balkwill et al, 2005;. Coussens e Werb, 2002; Lin e Karin, 2007). O papel de promotor tumoral mais óbvio de células imunes é manifestada em câncer associado à inflamação, em que os tumores surgem e crescem em locais de inflamação crônica. Em trabalhos anteriores, a inibição da ativação do NF-κB em células mielóides inibiu a proliferação de células epiteliais intestinais pré-malignas em um modelo de câncer associado à colite (Greten et al., 2004).

Durante a indução inicial de câncer associado à colite, a IL-6 é produzida principalmente por células mielóides (Grivennikov et al., 2009). Correspondentemente, camundongos sem IL-6 em células derivadas de medula óssea exibiram menos tumores, com uma redução substancial na carga total de tumores (número e tamanho) (Grivennikov et al., 2009). Da mesma forma, nós também demonstramos que camundongos sem PKR nas células derivadas da medula óssea também apresentaram menos tumores e de tamanhos menores .

Tornou-se aparente que a interação entre as células transformadas e o seu ambiente, incluindo as células imunes inatas, fibroblastos e células endoteliais pode ser um instrumento tanto para a promoção quanto para a progressão tumoral de muitos tumores

epiteliais, especialmente no contexto de inflamação crônica (Balkwill e Mantovani, 2001 ; Coussens e Werb, 2002). A supressão de PKR em células mielóides resulta em uma diminuição acentuada na proliferação induzida por inflamação e tamanho do tumor. Curiosamente, a supressão de PKR em células mielóides teve um efeito mais pronunciado no número de tumores do que o tamanho do tumor. Isto sugere fortemente que a PKR em células mielóides é provavelmente mais envolvida no desenvolvimento do tumor do que no crescimento do tumor.

O papel dos macrófagos no desenvolvimento tumoral, em contraste com seu papel na progressão tumoral, não tem sido claramente estabelecida, embora essas células são componentes importantes do microambiente tumoral (Balkwill e Mantovani, 2001; Coussens e Werb, 2002). A eliminação de CSF-1 em macrófagos ilustra que o recrutamento de macrófagos é importante para a progressão de tumores de glândulas mamárias, uma vez que o crescimento invasivo e metástases foram significativamente atenuados (Lin et al., 2001).

Além de identificar um mecanismo molecular chave que conecta inflamação e câncer, aumento de TNF-α e carcinogênese no cólon mediada pela obesidade, os nossos resultados sugerem que o bloqueio de TNF-α possa atuar como participante atraente na quimioprevenção do câncer de cólon de indivíduos obesos. Além disso, a inibição farmacológica específica de PKR pode ser muito eficaz na prevenção do câncer associado à colite. No entanto, dado o importante papel da PKR na promoção de respostas imunes inatas, há um certo risco associado ao uso prolongado de tais drogas e mais estudos são necessários.

CONCLUSÕES

CONCLUSÕES

Conclusão Geral

No presente trabalho é apresentado que em situações de obesidade, onde encontramos um estado de baixo grau de inflamação, mas com elevação significativa dos níveis de citocinas inflamatórias circulantes, o TNF-α contribui para a formação e crescimento de tumores de cólon em camundongos obesos. Além disso, nossos dados indicam que os bloqueadores de TNF-α poderiam ser usados como agentes quimiopreventores em câncer de cólon em indivíduos obesos.

Além disso, também mostramos que a ausência da proteína PKR protege contra a inflamação associada à obesidade e por isso também protege contra a formação de tumores de cólon em camundongos obesos. Além de identificar um mecanismo molecular chave que conecta inflamação e câncer, os nossos resultados sugerem que a inibição farmacológica específica de PKR pode ser muito eficaz na prevenção de câncer associado à colite.

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APÊNDICES

APÊNDICE I

INSULIN RECEPTOR SUBSTRATE 1 MEDIATES IRINOTECAN-INDUCED PI3K/AKT/mTOR PATHWAY ACTIVATION IN COLON CANCER

Running title: Function of IRS-1 in irinotecan treatment

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Conflict of interest

The authors declare no conflict of interests.

Keywords: mTOR; IRS-1; Irinotecan

ABSTRACT

Aim: Resistance of tumors to treatment is a common clinical problem in human colorectal cancer (CRC). Irinotecan, an inhibitor of topoisomerase I, potently activates mTOR signaling, a mechanism that is involved in CRC refractoriness to chemotherapy. Herein, we investigated the mechanisms involved in irinotecan-induced mTOR activation.

Methods: Human CRC xenografted into severe combined immunodeficient (SCID) mice and the CRC cell lines (HT-29 and CACO-2) were treated with irinotecan or inhibitors of PI3K signaling or a combination of both drugs. We analyzed the effects of irinotecan in intracellular signaling using immunoblot, immunohistochemistry, cell viability and apoptosis assays and protein tyrosine phosphatase (PTP) 1B activity assay.

Results: SN38, the active metabolite of irinotecan, induces multiples changes in IRS-1 serine phosphorylation and decreased interaction of SOCS3 with IRS-1, leading to IGFR and EGFR activation. IRS-1 knockdown as well as PI3K inhibition abrogated the SN38 activation of IGFR, EGFR and PI3K/Akt/mTOR pathway, and sensitized colorectal cancer cell lines to SN38 *in vitro*. Accordingly, treatment with IRS-1 antisense oligonucleotide or rapamycin potentiates the anti-tumor effects of irinotecan HT-29 xenograft tumor.

Conclusions: Irinotecan leads to IGF1-R, EGFR and PI3K/AKT/mTOR activation through modulation of IRS-1 function in colon cancer, supporting the idea that halting the tyrosine kinase receptors pathway is an interesting approach to optimize irinotecan based chemotherapy as well as highlights IRS-1 phosphorylation status and SOCS3 as potential prognosis biomarker in CRC patients treated with irinotecan.

INTRODUCTION

Irinotecan, is an antitumor agent that causes cellular damage via inhibition of topoisomerase I(1). Recently, combination of this drug with tyrosine kinase inhibitors was approved for clinical use based on the reduced likelihood of irinotecan resistance(2-4). Interestingly, not only blocking tyrosine kinases, but also inhibiting PI3K/Akt/mTOR pathway enhance apoptosis induced by SN38, the active form of irinotecan, in human hepatoma cells(5). Furthermore, rapamycin increased irinotecan activity in colon cancer cells through a cooperative modulation of mTOR/HIF-1α axis(6). However, the mechanism by which irinotecan modulates the PI3K/Akt/mTOR pathway remains to be identified.

Type I insulin-like growth factor receptor (IGF1R) and epidermal growth factor receptor (EGFR) signaling pathway are important growth-regulatory pathways in colon cancer(7, 8). Ligand binding induces these receptor activation and phosphorylation of tyrosine residues on substrates and adaptor proteins such as insulin receptor substrate 1 (IRS-1)(7, 9). Tyrosine phosphorylated IRS-1 can recruit other signaling messengers, including PI3K/Akt/mTOR pathway(7, 10), which inhibits apoptosis and regulates proliferation and growth(11).

Several lines of evidence implicate IRS-1 as a critical target for the negative regulation of this pathway. First, serine phosphorylation of IRS-1 by distinct kinases, such as JNK(12), IKK(13), ERK(14), PKCζ(15), PKC θ(16), Akt(17), GSK-3(18), IRAK(19), mTOR(20) decreases tyrosine kinase induced IRS-1 phosphorylation. Second, protein tyrosine phosphatases (PTPs), such as PTP1B,

dephosphorylate and inhibit IRS-1 recruitment of PI3K (21, 22). Finally, association of SOCS3 with IRS-1 results in decreased phosphorylation of Akt (23-25).

In the current study, we evaluated if irinotecan affects IRS-1 function. We demonstrated that irinotecan by changing IRS-1 serine phosphorylation status and decreasing SOCS3 expression, promotes activation of EGFR and IGFR in colon cancer. Collectively, these findings provide a molecular basis for combinatorial use of irinotecan and tyrosine kinase inhibitors.

MATERIALS AND METHODS

Antibodies and chemicals. Rapamycin was from Sigma-Aldrich, SN38 was from Tocris (Bristol, UK). Irinotecan was from Sanofi Aventis. Anti-phospho[Ser473] Akt, anti-Akt, anti-phospho[Thr669] pEGFR, anti-EGFR, Anti-phospho[Tyr895] IRS-1, anti-phospho[Ser307] IRS-1, anti-phospho[Ser636/639] IRS-1, anti-phospho[Ser1101] IRS-1, anti-IRS-1, anti-phospho[Ser2448] mTOR, anti-mTOR, anti-phospho[Thr389] p70S6K, and anti-p70S6K were from Cell Signaling Technology (Beverly, MA). Anti-PTP1B was purchased from Abcam (Cambridge, MA). Tyrosine phosphopeptide (TSTEPQ-pY-QPGENL), Malachite Green solution A, and Malachite Green were from Millipore (Billerica, MA). Routine reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Cell culture and siRNA transfections. HT-29 and Caco-2 colon cancer cell lines were maintained in McCoys´s and DMEM, respectively, supplemented with 10% FBS and penicillin/streptomycin. siRNAs corresponding to human IRS-1 were transfected into cells by using LipofectAMINE^{tm}2000 (Invitrogen) according to the manufacturer´s instructions.

Cell viability. Cells were seeded at a density of 20,000 cells/well and allowed to adhere overnight and treated with the indicated drugs as described in the figures legends. Cell viability 48h after treatment was determined by the addition of 0.3mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide. After 4h

of incubation, the formazan was solubilized in ethanol and the absorbance determined by spectrophotometer at 570nm.

Western Blot analysis. Cells were lysed in a freshly prepared ice-cold buffer(26) and Western blot was performed. Briefly, total extracts were prepared and 50μg total proteins were separated by SDS-PAGE and blotted onto nitrocellulose membrane. After incubation with antibodies, immune complexes were detected using the SuperSignal West Pico Chemiluminescent kit (Pierce Biotechnology).

PTP1B phosphatase assay. HT-29 and Caco-2 cell lines were sonicated in lysis buffer (100mM Imidazol-HCl, 200mM EDTA, 200mM EGTA, 10mg/ml aprotinin, 10mg/ml leupeptin, 300mM Benzamidine, 100mM PMSF, 10mg/ml pepstatin). The cell lysate was immunoprecipitated with 5μg anti-PTP1B and protein A-Sepharose and washed three times with TBS. The measurement of PTP1B activity was carried out in specific buffer (25mM Hepes, pH 7.2, 2.5mM EDTA, 50mM NaCl, 5mM DTT) using 250μM synthetic tyrosine phosphopeptide (TSTEPQ-pY-QPGENL) as the substrate for 15 minutes, at 30ºC. The release of phosphate group was measure using Malachite green detection solution at OD 660nm.

Xenografts studies. Six-week-old male SCID mice were provided by the University of Campinas – Central Breeding Center and inoculated subcutaneously with 1×10^6 HT-29 cells. Once tumors became palpable, tumor volume was calculated as previously(26). Treatments were initiated when tumors reached 50–

100mm^3. Mice were treated with 50mg/kg irinotecan per week, 25μg rapamycin per day, 0.4ng/kg IRS-1 antisense oligonucleotide per day, alone or in combination. All studies were conducted in compliance with Ethics Committee of the University of Campinas guidelines.

Immunohistochemistry and TUNEL assay, Ki-67 and TUNEL staining were performed as described previously(26).

Statistical analysis. All experiments were repeated two to four times after experimental conditions were optimized. Statistical analysis was performed by using an one-way ANOVA test with *Bonferroni post hoc* test, and $P<0.05$ was considered to be statistically significant.

RESULTS

SN38 promotes IGF1R, EGFR, and IRS-1 activation.

SN38 increased tyrosine phosphorylation of IGF1R, EGFR and IRS-1 in a dose-dependent manner after 4 hours of SN38 treatment in HT-29 human colon cancer cells (Figure 1A). Accordingly, p70S6K phosphorylation increased in a time- (Figure 1B) and dose-dependent (data not shown) manner after SN38 treatment. In contrast, IRS-1 phosphorylation at Ser-636/639 and Ser-1101 residues decreased in HT-29 cell line after SN38 administration (Figure 1B). IRS-1 Ser-312 phosphorylation, however, increased after exposure to the drug. Consistent with increased IRS-1 Ser-312 phosphorylation, SN38 increased phosphorylation of JNK, IKK and p38 (Figure 1C).

In order to evaluate if the increased IRS-1 tyrosine phosphorylation after SN38 treatment was caused by decreased expression of PTP1B (Figure 1B), we performed a PTP1B phosphatase assay. Nonetheless, SN38 treatment did not change PTP1B activity, as shown in figure 1D. Thus, we tested if SN38 treatment could influence the expression SOCS3. Interestingly, SN38 treatment decreased the association of SOCS3 with IRS-1 (Figure 1E), which might lead to the observed increase in IRS-1 tyrosine phosphorylation.

IRS-1 knockdown sensitizes colorectal cancer cell line HT-29 and Caco-2 to SN38 in vitro.

Since SN-38 induced activation of IRS-1, we decided to knockdown the IRS-1 expression (Figure 2A). HT-29 and Caco-2 treatment with both IRS-1 siRNA

and SN38 for 72 hours decreased cell viability by approximately 10% (Figure 2B) and 8% (Figure 2C), respectively, when compared to SN38 group alone.

The SN38 administration increased the IGF1R phosphorylation levels, and treatment of the cells with the IRS-1 siRNA abrogated this effect in both HT-29 and Caco-2 cell lines (Figures 2D and 2E, respectively). The IRS-1, Akt, and mTOR phosphorylation increased after SN38 treatment, whereas the Akt and mTOR activation was unresponsive to SN38 administration in both cell lines lacking IRS-1. Thus, SN38 promotes Akt/mTOR activation through IRS-1 phosphorylation, and inhibition of this protein abrogates this effect.

PI3K and mTOR inhibitors sensitize colorectal cancer cell lines to SN38 in vitro.

As shown in Figure 3A, the PI3K inhibitor decreased the HT-29 viability by approximately 55%, whereas the SN38 treatment decreased the viability by approximately 27% compared to untreated group. The combination of both drugs dramatically decreased the viability of the HT-29 by 72%, a 16% decrease in cell viability when compared to SN38 treatment alone. Treatment of HT-29 cell line with 10nM rapamycin reduced cell viability by about 11%. When this dose of rapamycin was combined with SN38, viability was reduced by 44%, a 17% reduction in cell viability *versus* SN38 alone.

In the Caco-2 cell lines, the viability after LY294002 treatment was also reduced by approximately 55% of control group, whereas the SN38 treatment decreased the viability of the cell by 40% compared to untreated group (Figure 3B).

The combination of both drugs further decreased the viability to 67% of the untreated group. Surprisingly, in the Caco-2 cell line the combination of SN38 with rapamycin did not showed any additive effect on cell viability compared to the SN38 treatment alone (Figure 3B).

As previously observed, SN38 treatment induced IRS-1 Tyr-895, Akt Ser-473, mTOR Ser-2448, and p70S6K Thr-389 phosphorylation when compared to control group (Figure 3C and D). LY294002 reduced SN38-induced Akt and mTOR activation (Figures 3C and 3D, respectively), thereby pointing out the involvement of PI3K in SN38-dependent AKT activation. Pretreatment of HT-29 and Caco-2 with rapamycin blunted the mTOR and p70S6K phosphorylation induced by SN38 in both cell lines, but it also increased the IRS-1 and Akt phosphorylation. mTOR activity dampens PI3K signaling by promoting p70S6K-mediated inhibitory phosphorylation of IRS-1, addition of the mTOR inhibitor impairs this mechanism through the inhibition of the p70S6K activity, increasing the Akt phosphorylation observed after SN38 administration. This feedback mechanism may be involved in the Caco-2 resistance to the concomitant treatment with SN38 and rapamycin (Figure 3B).

Interestingly, administration of SN-38 to HT-29 cells increased the phosphorylation of p53, although no further increase after administration of LY294002 or rapamycin was observed (Figure 3C). In the Caco-2 cell line, however, p53 was not inducible by the DNA damaging agent SN38, suggesting the absence of a functional protein (Figure 3D).

Irinotecan and Rapamycin synergize to inhibit tumor growth in mice bearing colon cancer xenografts

Tumor growth was reduced in mice treated with irinotecan (50mg/kg) or rapamycin (25μg), while combined treatment completely arrested tumor growth (Figure 4A). Figure 4B shows that irinotecan and rapamycin alone increased the number of TUNEL-positive cells compared to sections from control-treated tumors. However, the combination did not further increase the number of TUNEL-positive cells. On the other hand, the concomitant treatment with irinotecan and rapamycin dramatically decreased the number of Ki67-positive cells compared to either treatment alone (Figure 4B).

As shown in Figure 4C, irinotecan significantly increase the phosphorylation of IGF1R, IRS-1, Akt, mTOR and p70S6K protein compared to the control xenografts. As expected, tumors from animals treated with rapamycin showed lowered mTOR and p70S6K phosphorylation compared to control group. Coherently, in the animals receiving irinotecan and rapamycin, the induction of mTOR and p70S6K phosphorylation by irinotecan was blunted.

Irinotecan and IRS-1 knockdown synergize to inhibit tumor growth in mice bearing colon cancer xenografts

Tumors were roughly half the size in mice treated with a combination of IRS-1 ASO and irinotecan versus mice treated with either agent alone and about a third versus mice in control group (Figure 5A). As shown in Figure 5B, irinotecan alone significantly increase the phosphorylation of IGF1R, IRS-1, Akt, mTOR and

p70S6K protein compared to the control tumors. As expected, tumors from animals treated with IRS-1 ASO showed lowered IRS-1 content compared to control group. We also observed a decrease phosphorylation of Akt, mTOR and p70S6K in tumors from IRS-1 ASO treated mice. Interestingly, in the animals receiving irinotecan and IRS-1 ASO, the induction of mTOR and p70S6K phosphorylation by irinotecan was blunted significantly. These data demonstrate the ability of IRS-1 ASO to blunt irinotecan-dependent mTOR and p70S6K activation *in vivo*.

DISCUSSION

The present study shows that irinotecan acts by increasing the activation of IGF1R, EGFR and IRS-1 in HT-29 and Caco-2 colon cancer cell lines through changes in the IRS-1 serine phosphorylation status and inhibition of SOCS3, further activating the Akt/mTOR pathway. The administration of IRS-1 siRNA or the PI3K inhibitor decreases this activation, and sensitizes colon cancer cell lines to irinotecan. Moreover, in contrast to single-agent therapy, xenografted tumors treated with a combination of irinotecan and IRS-1 ASO showed potent inhibition of IRS-1, Akt, and mTOR, which was accompanied by a reduction in tumor volume, a similar effect was observed with the combination of irinotecan and rapamycin.

Previous studies suggest that SOCS3 act as a negative modulator of carcinogenesis. For instance, exogenous administration of SOCS3 inhibits proliferation and invasion of A549 lung cancer cells(27) and loss of SOCS3 expression is associated with poor clinical outcome in breast cancer(28). In parallel, it is observed that increased expression of SOCS3 inhibits IRS1 tyrosine phosphorylation(29). Our results show that irinotecan in spite of not changing PTP1B activity caused a dramatic decrease of SOCS3, and this was associated with an increase in tyrosine phosphorylation of IRS1 and membrane tyrosine kinases, an effect that consistently increased the PI3K/Akt/mTOR pathway. Thus, we propose that the loss of SOCS3 expression represents a critical event, permissive for the increased survival of HT-29 and Caco-2 cell lines.

In accordance with the hypothesis that IRS-1 is central to irinotecan-induced activation of PI3K/Akt/mTOR pathway, SN38 treatment decreased phosphorylation

of two serines residues required for inhibition of IRS-1. On the other hand, SN38 administration increased kinase activity of JNK, IKK and p38 and phosphorylation of IRS-1 on Ser-312. Interestingly, the inhibition of NF-B, a target of IKK, sensitizes chemo resistant tumors to the apoptotic potential of irinotecan resulting in tumor regression(30, 31). In agreement with the observed increased activity of PI3K/Akt/mTOR pathway, Copps et al. showed that IRS-1 Ser-307 phosphorylation, the mice analogous to human Ser-312, plays a permissive role in insulin-stimulated IRS-1 tyrosine phosphorylation and signal transduction(32). In aggregate, our findings suggest the existence of a short negative feedback loop, wherein, IRS-1 serine phosphorylation status modulates its additional activation through changes in the tyrosine kinase receptor activity(33). Another mechanism that may co-occur to sustain the increased activity of PI3K/Akt/mTOR pathway is a gain of interaction between irinotecan-induced IRS-1 serine phosphorylation and PI3K(34).

Intriguingly, despite the additive effects of irinotecan and IRS-1 ASO combination we just observed a synergistic effect of mTOR inhibition and irinotecan in HT-29 cells. Considering that the proliferation of Caco-2 cells was inhibited with both IRS-1 ASO and PI3K inhibitors and that it was recently reported a gain of interaction between IRS-1 and mutated PI3K, our finding suggest that IRS-1/PI3K interaction is critical to Caco-2 cells replication(34, 35) Since Caco-2 cell lines did not showed increased p53 phosphorylation after SN38 treatment, it is also possible that the synergistic effect between irinotecan and rapamycin be dependent on activation of p53. In accordance, Beuvink and colleagues(36)

showed that the antitumor activity of rapamycin and cisplatin treatment in cultured tumor cells depends on an intact p53 response.

Collectively, we suggest that the irinotecan-induced modification of IRS-1 function leads potentiation of the activity of IGFR and EGFR as well as their downstream effectors contribute to the amplified responses observed in the combinatorial use of tyrosine kinase inhibitors and irinotecan. Thus, the irinotecan-induced changes in IRS-1 function may be associated with the clinically significant activity of cetuximab or panitumumab, monoclonal antibodies that specifically block the EGFR, combined with irinotecan, even in patients with irinotecan-refractory colorectal cancer(2, 37, 38). In this line, we can also speculate that irinotecan-induced changes in IRS-1 function could be involved in the increased overall survival observed in the association of vascular endothelial growth factor (VEGF) blockade and irinotecan in the treatment of human colon cancer (39, 40). Therefore, the role of the commonly used chemotherapy in the activation of intracellular signaling pathways should be focused to the discovery of biomarkers and so IRS-1 serine phosphorylation status and its association with SOCS3 should be explored as biomarkers in colorectal cancer patients treated with the association of irinotecan and inhibitors of tyrosine kinases.

Taken together, our findings identify IGF1R, EGFR and PI3K/Akt/mTOR pathway activation as a consequence of irinotecan-induced changes in IRS-1 activity and underscore, the potential of a combined therapeutic approach with irinotecan and inhibitors of tyrosine kinase receptors and of PI3K/Akt/mTOR pathway for the treatment of colon cancer.

CONFLICT OF INTERESTS STATEMENT

The authors have declared no competing interests.

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FIGURE LEGENDS

Figure 1. SN38 increases the activity of the IRS-1/Akt/mTOR pathway. (A) HT-29 cell were treated with increasing concentrations of SN38 for 4 hours and the phosphorylation levels of IGF-I, EGFR, and IRS-1 analyzed by Western blot; (B) HT-29 cells were treated with 100 nM SN38 for 30 minutes, 1, 2, 4 and 6 hours and the phosphorylation levels of IGF-IR, IRS-1, and p70S6K analyzed by Western blot. (C) HT-29 cell were treated with increasing concentrations of SN38 for 4 hours and the phosphorylation levels of JNK, IKK, and p38 analyzed by Western blot. (D) PTP1B-specific phosphatase activity. (E) HT-29 cell were treated with increasing concentrations of SN38 for 4 hours and the association between IRS1 and SOCS3 analyzed by Western blot; Representative blots of three independent experiments.

Figure 2. IRS-1 knockdown reverses the p70S6K activation caused by SN38 treatment in human colon cancer cell lines. (A) IRS-1 was efficiently knockdown by siRNA treatment; (B) HT-29 and (C) CACO-2 cell lines were transfected with IRS-1 siRNA and 24 hours post-transfection treated with SN38 for 48 hours. Cell viability was measure using the MTT assay. Data are mean values ± SD from experiment performed in triplicate and repeated and least three times. *$p<0.01$; (D) HT-29 and (E) CACO-2 cell lines were transfected with IRS-1 siRNA and after 24 hours treated with SN38 for 4 hours. IGF1R, IRS-1, Akt, mTOR, and p70S6K phosphorylation levels were detected by Western blot. Representative blots of three independent experiments.

Figure 3. The PI3K inhibitor, LY294002, reverses the p70S6K activation caused by SN38 treatment in human colon cancer cell lines. (A) HT-29 and (B) CACO-2 cell lines were treated with SN38, LY294002, and rapamycin for 48 hours. Cell viability was measure using the MTT assay. Data are mean values ± SD from experiment performed in triplicate and repeated and least three times. *$p<0.01$; (C) HT-29 and (D) CACO-2 cell pre-treated with LY294002 or rapamycin for 2 hours and then treated with SN38 for 4 hours. IRS-1, Akt, mTOR, and p70S6K phosphorylation levels were detected by Western blot. Representative blots of three independent experiments.

Figure 4. Irinotecan and rapamycin synergize in vivo to reduce HT-29 tumor growth. 1.0×10^6 HT-29 cells were injected subcutaneously into the flank of SCID mice. Once the tumor reached 50-100 mm^3, treatments were initiated as indicated in Methods, n=8-12 mice per group mean ± SEM; (A) Tumor growth was measured daily after treatment with Irinotecan (50 mg/kg) or Rapamycin (25 µg) alone or in combination; (B) Representative microphotographs of TUNEL-positive and of Ki-67-positive staining cells per field, 4 field per tumor section, mean ± SEM; (C) The HT-29 tumor lysates were detected by Western

blot with the indicated antibodies. Representative blots of three independent experiments. *, P ≤ 0.05 vs. control; #, P ≤ 0.05 vs. irinotecan; †, P ≤ 0.05 vs. rapamycin.

Figure 5. Irinotecan and IRS-1 knockdown synergize in vivo to reduce HT-29 tumor growth. 1.0 x 10^6 HT-29 cells were injected subcutaneously into the flank of SCID mice. Once the tumor reached 50-100 mm^3, treatments were initiated as indicated in Methods, n=6-10 mice per group mean ± SEM; (A) Tumor growth was measured daily after treatment with Irinotecan (50 mg/kg) or IRS-1 antisense oligonucleotide (ASO IRS-1) alone or in combination; (B) The HT-29 tumor lysates were detected by Western blot with the indicated antibodies. Representative blots of three independent experiments.

Figure 1

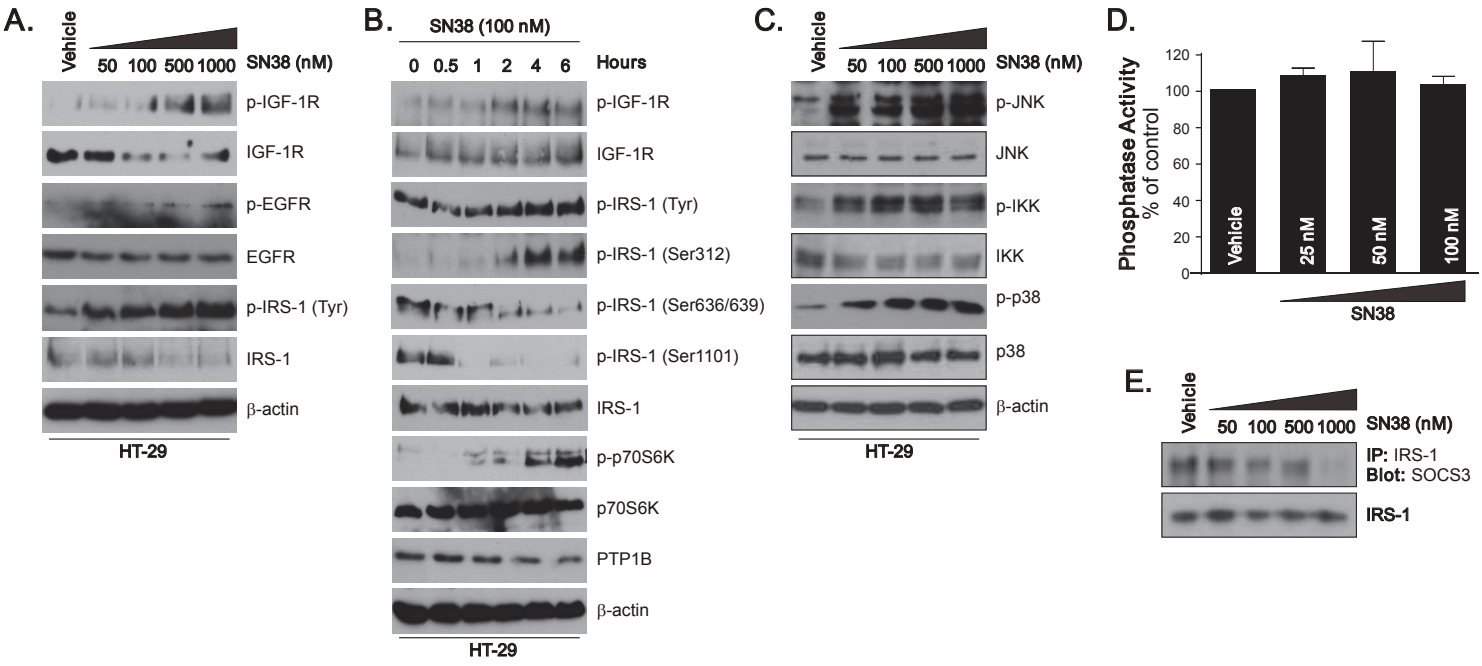

A.
Vehicle
50 100 500 1000
SN38 (nM)
p-IGF-1R
IGF-1R
p-EGFR
EGFR
p-IRS-1 (Tyr)
IRS-1
β-actin
HT-29
B.
SN38 (100 nM)
0 0.5 1 2 4 6
Hours
p-IGF-1R
IGF-1R
p-IRS-1 (Tyr)
p-IRS-1 (Ser312)
p-IRS-1 (Ser636/639)
p-IRS-1 (Ser1101)
IRS-1
p-p70S6K
p70S6K
PTP1B
β-actin
HT-29
C.
Vehicle
50 100 500 1000
SN38 (nM)
p-JNK
JNK
p-IKK
IKK
p-p38
p38
β-actin
HT-29
D.
Phosphatase Activity
% of control
120
100
80
60
40
20
0
Vehicle
25 nM
50 nM
100 nM
SN38
E.
Vehicle
50 100 500 1000
SN38 (nM)
IP: IRS-1
Blot: SOCS3
IRS-1

Figure 2

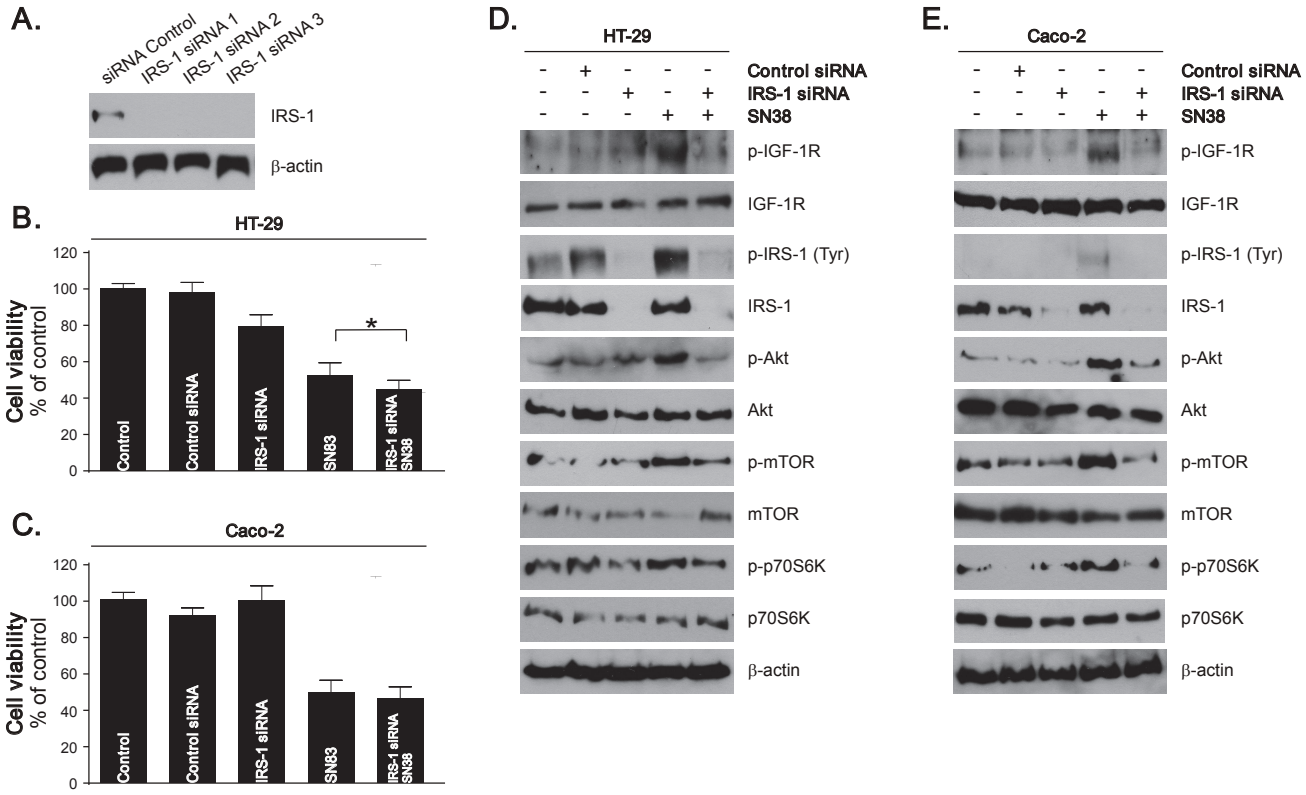

A.
siRNA Control
IRS-1 siRNA 1
IRS-1 siRNA 2
IRS-1 siRNA 3
IRS-1
β-actin
B.
HT-29
Cell viability
% of control
120
100
80
60
40
20
0
Control
Control siRNA
IRS-1 siRNA
SN83
IRS-1 siRNA SN38
*
C.
Caco-2
Cell viability
% of control
120
100
80
60
40
20
0
Control
Control siRNA
IRS-1 siRNA
SN83
IRS-1 siRNA SN38
D.
HT-29
Control siRNA
IRS-1 siRNA
SN38
p-IGF-1R
IGF-1R
p-IRS-1 (Tyr)
IRS-1
p-Akt
Akt
p-mTOR
mTOR
p-p70S6K
p70S6K
β-actin
E.
Caco-2
Control siRNA
IRS-1 siRNA
SN38
p-IGF-1R
IGF-1R
p-IRS-1 (Tyr)
IRS-1
p-Akt
Akt
p-mTOR
mTOR
p-p70S6K
p70S6K
β-actin

Figure 3

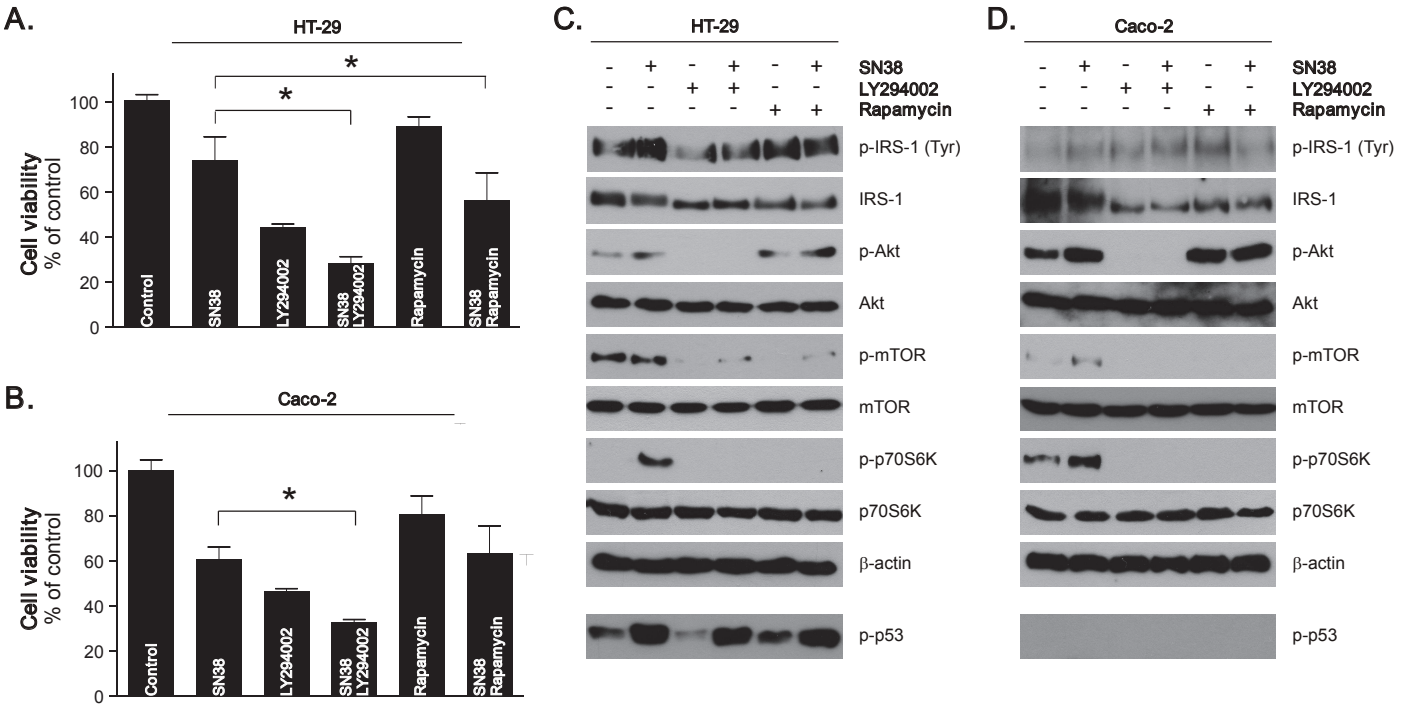
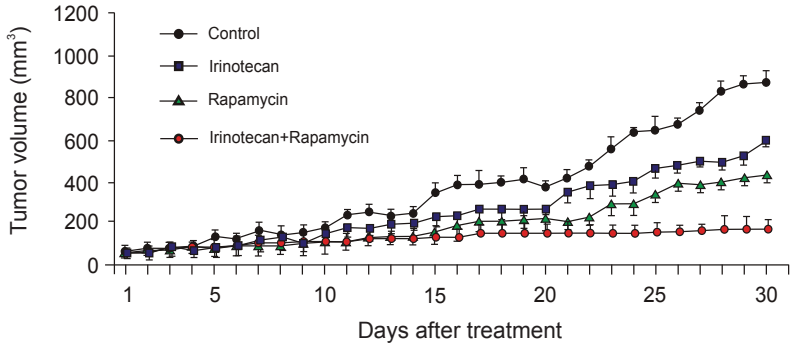
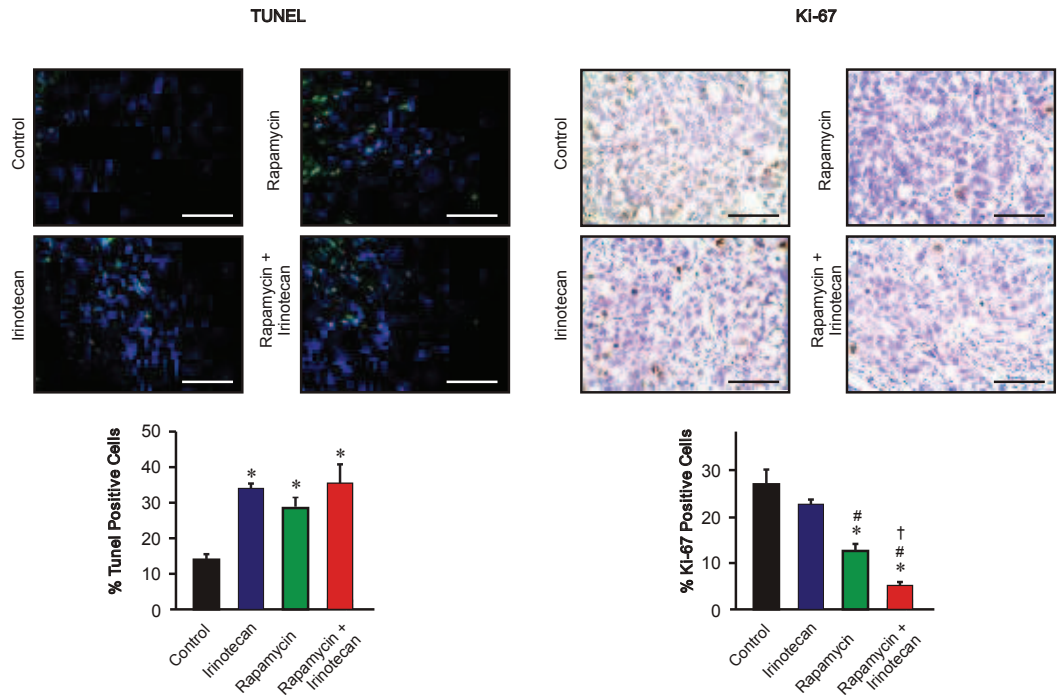

A.
HT-29
Cell viability
% of control
100
80
60
40
20
0
Control
SN38
LY294002
SN38 LY294002
Rapamycin
SN38 Rapamycin
*
*
B.
Caco-2
Cell viability
% of control
100
80
60
40
20
0
Control
SN38
LY294002
SN38 LY294002
Rapamycin
SN38 Rapamycin
*
C.
HT-29
SN38
LY294002
Rapamycin
p-IRS-1 (Tyr)
IRS-1
p-Akt
Akt
p-mTOR
mTOR
p-p70S6K
p70S6K
β-actin
p-p53
D.
Caco-2
SN38
LY294002
Rapamycin
p-IRS-1 (Tyr)
IRS-1
p-Akt
Akt
p-mTOR
mTOR
p-p70S6K
p70S6K
β-actin
p-p53

Figure 4.

A.


Tumor volume (mm3)
1200
1000
800
600
400
200
0
Control
Irinotecan
Rapamycin
Irinotecan+Rapamycin
1
5
10
15
20
25
30
Days after treatment

B.


TUNEL
KI-67
Control
Rapamycin
Irinotecan
Rapamycin + Irinotecan
% Tunel Positive Cells
% KI-67 Positive Cells
Control
Irinotecan
Rapamycin
Rapamycin + Irinotecan

C.

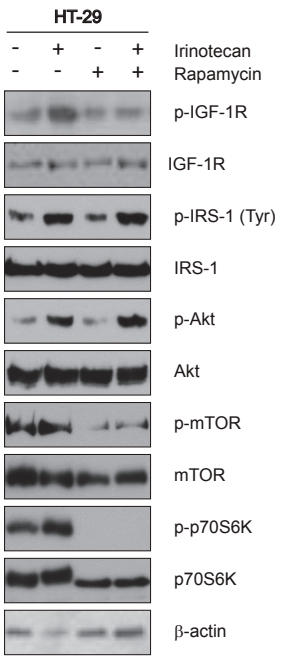
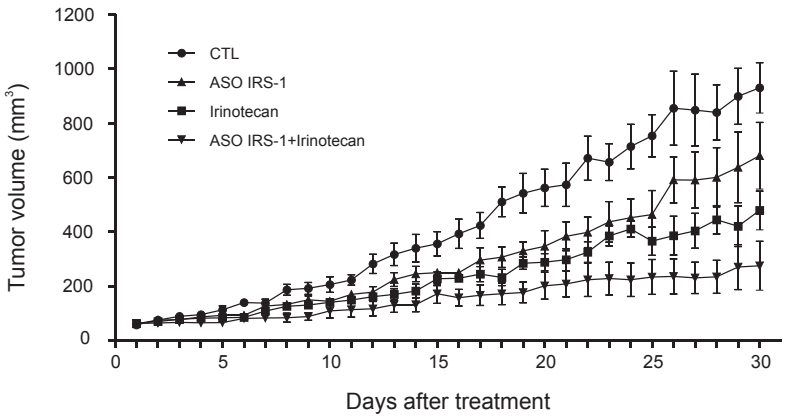
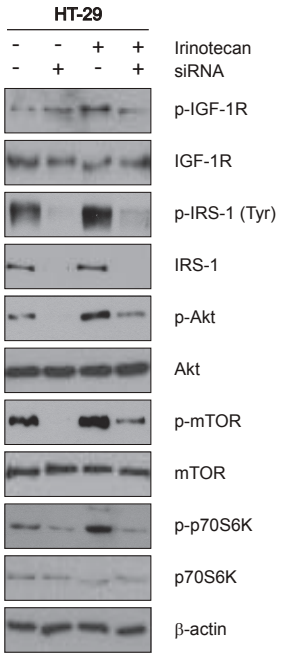

HT-29
Irinotecan
Rapamycin
p-IGF-1R
IGF-1R
p-IRS-1 (Tyr)
IRS-1
p-Akt
Akt
p-mTOR
mTOR
p-p70S6K
p70S6K
β-actin

Figure 5.

A.


Tumor volume (mm3)
1200
1000
800
600
400
200
0
0
5
10
15
20
25
30
Days after treatment
CTL
ASO IRS-1
Irinotecan
ASO IRS-1+Irinotecan

B.


HT-29
- - + + Irinotecan
- + - + siRNA
p-IGF-1R
IGF-1R
p-IRS-1 (Tyr)
IRS-1
p-Akt
Akt
p-mTOR
mTOR
p-p70S6K
p70S6K
β-actin

APÊNDICE II

Lista de Publicações no período

Reis, L.O., Ferrari, K., Zamuner, M., **ROCHA, G.Z.,** Billis, A., Fávaro, W.J. "Urothelial carcinogen resistance driven by stronger Toll-like receptor 2 (TLR2) and Uroplakin III (UP III) defense mechanisms: a new model". World J Urol. 2014 May 29.

Oliveira, A.G., Araujo, T.G., Carvalho, B.M., Guadagnini, D., **ROCHA, G.Z..**, Bagarolli, R.A., Carvalheira, J.B., Saad, M.J.."Acute exercise induces a phenotypic switch in adipose tissue macrophage polarization in diet-induced obese rats".Obesity (Silver Spring). 2013 21(12):2545-56.

Pimentel, G.D.; Ropelle, E.R.; **ROCHA, G.Z.**; Carvalheira, J.B. "The role of neuronal AMPK as a mediator of nutritional regulation of food intake and energy homeostasis". Metabolism, 2013 v. 62 (2): 171-8.

Flores, M.B.S.*; **ROCHA, G.Z.*;** Damas-Souza, D.M.; Osório-Costa, F.; Dias, M.M.; Ropelle, E.R.; Camargo, J.A.; de Carvalho, R.B.; Carvalho, H.F.; Saad, M.J.A.; Carvalheira, J.B.C. "Obesity-Induced Increase in Tumor Necrosis Factor-α Leads to Development of Colon Cancer in Mice". Gastroenterology, 2012, v.143 (3): 741-753. *Equal contribution

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