PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL

FACULDADE DE BIOCIÊNCIAS

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E
MOLECULAR - PPGBCM

EFEITOS DA CAPSAICINA EM CÉLULAS ESTRELADAS HEPÁTICAS

Shanna Bitencourt

Orientador: Prof. Dr. Jarbas Rodrigues de Oliveira

Porto Alegre, 2012

PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL

FACULDADE DE BIOCIÊNCIAS

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E
MOLECULAR – PPGBCM

EFEITOS DA CAPSAICINA EM CÉLULAS ESTRELADAS HEPÁTICAS

Tese apresentada ao Programa de
Pós-Graduação em Biologia Celular e
Molecular da Pontifícia Universidade
Católica do Rio Grande do Sul como
requisito parcial para a obtenção do
grau de Doutora em Biologia Celular e
Molecular.

SHANNA BITENCOURT

ORIENTADOR: Prof. Dr. Jarbas R. de Oliveira

Porto Alegre, 2012

"Não tá morto quem peleia!"

Provérbio gaúcho

AGRADECIMENTOS

Aos meus colegas e amigos de Barcelona, pela amizade e parceria nesse ano incrível que foi 2010.

A minha querida Consuelo, pela amizade construída em Barcelona que permanece até hoje.

Ao Eduardo Pedrazza, muito obrigada por todas as vezes que abriste a porta do LID para acudir os desesperados da Biofísica!

Aos colegas de outros laboratórios que me ajudaram com essa pesquisa.

A todos aqueles que contribuíram de alguma forma para que mais essa etapa em minha vida fosse concluída. Muito obrigada de coração.

RESUMO

A fibrose hepática é a resposta cicatricial do fígado a lesões repetidas, caracterizada pelo rompimento da arquitetura hepática associada ao aumento da expressão dos componentes da matriz extracelular. As células estreladas hepáticas (HSCs) desempenham um papel-chave no processo de fibrogênese. No fígado normal, as HSCs encontram-se em sua forma quiescente de depósito de vitamina A. Durante a lesão hepática, essas células passam por uma ativação fenotípica, tornam-se miofibroblastos e adquirem propriedades fibrogênicas. A capsaicina, princípio ativo da pimenta vermelha, tem sido muito estudada nos últimos anos por possuir uma extensa gama de propriedades farmacológicas, que incluem efeitos analgésicos, anti-inflamatórios, antiproliferativos e anticarcinogênicos em uma variedade de tipos celulares. Por essa razão, o objetivo deste estudo foi investigar os efeitos *in vitro* da capsaicina na inativação, diferenciação e proliferação das HSCs, além de estudar os possíveis mecanismos envolvidos. Os resultados obtidos mostraram que a capsaicina é capaz de induzir o fenótipo quiescente nas HSCs via ativação de PPARγ e bloqueio da sinalização de TGF-β. O aumento nos níveis de citocinas antifibróticas (IFN-γ e IL-10) e a diminuição de mediadores pró-inflamatórios e pró-fibróticos (COX-2, MCP-1, colágeno tipo I) comprovaram que a capsaicina inibe a ativação e migração dessas células. Além disso, o mecanismo utilizado pela capsaicina para inibir a proliferação celular é via parada do ciclo celular. Essas descobertas mostram que a capsaicina tem potencial para ser um novo agente terapêutico no tratamento da fibrose hepática devido a suas ações antifibrogênicas e antiproliferativas.

Palavras-chave: fibrose hepática, capsaicina, célula estrelada hepática, proliferação, inflamação.

ABSTRACT

Liver fibrosis is the wound healing response to repeated injury of the liver. It is characterized by disruption of the liver architecture associated with increased expression of extracellular matrix components. Hepatic stellate cells (HSCs) play a key role in the process of fibrogenesis. In normal liver, HSCs are quiescent and its main function is to store vitamin A. During liver injury, these cells undergo activation, become myofibroblasts and acquire fibrogenic properties. Capsaicin, the active ingredient of red pepper has been extensively studied in recent years for possessing a wide range of pharmacological properties, including analgesic, anti-inflammatory, antiproliferative and anticarcinogenic in a variety of cell types. Therefore, the aim of this study was to investigate the *in vitro* effects of capsaicin on deactivation, differentiation and proliferation of HSCs, besides studying the possible mechanisms involved. The results showed that capsaicin is capable of inducing the quiescent phenotype in HSCs via PPARγ activation and blockage of TGF-β signaling. Increased levels of antifibrotic cytokines (IFN-γ and IL-10) and the reduction of pro-inflammatory and pro-fibrotic mediators (COX-2, MCP-1, type I collagen) showed that capsaicin inhibits the activation and migration of these cells. Furthermore, the mechanism used by capsaicin to inhibit cell proliferation is via cell cycle arrest. These findings demonstrate that capsaicin has the potential to be a novel therapeutic agent in the treatment of liver fibrosis due to its antifibrogenic and antiproliferative actions.

Key-words: hepatic fibrosis, capsaicin, hepatic stellate cell, proliferation, inflammation

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS

COX-2 – ciclo-oxigenase-2

ECM – matriz extracelular

HBV – vírus da hepatite B

HCV – vírus da hepatite C

HSC – célula estrelada hepática

IFN-α – interferon gama

IL1 – interleucina 1

IL6 – interleucina 6

IL10 – interleucina 10

IL12 – interleucina 12

MCP-1 – proteína quimiotática de monócitos 1

NF-κB – fator nuclear kappa B

PPARγ – receptor ativado por proliferador de peroxissomo gama

TGFβ – fator de transformação do crescimento beta

TNF-α – fator de necrose tumoral alfa

TRPV1 – receptor de potencial transitório vanilóide 1

SUMÁRIO

CAPÍTULO 1

1. INTRODUÇÃO
2. JUSTIFICATIVA
3. OBJETIVOS

1   INTRODUÇÃO

1.1   Fibrose Hepática

A fibrose hepática é uma resposta cicatricial reversível do fígado caracterizada pela produção e deposição excessiva de matriz extracelular (ECM), principalmente colágeno tipo I, após o dano hepático (1). É um processo dinâmico no qual as alterações ocorrem inicialmente pelo dano das células parenquimatosas e consecutiva inflamação (2). Se a lesão é aguda ou limitada, as alterações são transientes e a arquitetura hepática é restaurada. Entretanto, se o estímulo lesivo permanecer, o processo inflamatório se torna crônico e o acúmulo de ECM se mantém, levando a uma substituição progressiva do parênquima hepático por tecido cicatricial (Ilustração 1). As principais causas podem incluir infecção crônica por vírus da hepatite C (HCV) ou hepatite B (HBV), abuso de álcool, obesidade e esteato-hepatite não alcoólica (1, 3).

A cirrose é o estágio final da fibrose de fígado e é caracterizada morfologicamente pela presença de fibrose e formação de nódulos acompanhados de insuficiência hepática e hipertensão portal (4). A progressão para esse estágio final é lento, podendo levar 20-40 anos para se desenvolver em pacientes com lesão crônica de fígado. A cirrose representa, atualmente, uma das principais causas de morte nos países ocidentais (5).

1.2 Células Estreladas Hepáticas

Vários tipos celulares estão envolvidos na patogênese da fibrose hepática. Acredita-se que a células estreladas hepáticas (HSCs), localizadas no espaço perisinusoidal de Disse, sejam as principais produtoras de ECM no fígado (cerca de 80% de toda a produção de colágeno). Essas células correspondem a 15% do total de células do fígado e 30% das células não-parenquimatosas (6, 7). No fígado normal, as HSCs encontram-se em sua forma quiescente, não-proliferativa, armazenadora de vitamina A e produtora de metaloproteinases. Cerca de 50-80% do total de retinóides do corpo é estocado no fígado, dos quais 90% são armazenados nas células estreladas (8). Durante a lesão hepática, as HSCs perdem seu conteúdo lipídico e transdiferenciam-se em células com características de miofibroblastos e adquirem propriedades fibrogênicas (9). Essa transformação é o processo de ativação das HSCs e é considerada a chave da fisiopatologia da fibrogênese (6).

A ativação das HSCs pode ser dividida em duas fases: iniciação e perpetuação. A primeira fase está associada ao estímulo parácrino de células inflamatórias e hepatócitos lesados. As células de Kupffer podem estimular a proliferação, síntese de matriz, e perda dos retinóides pelas HSCs através da ação de citocinas como fator de transformação do crescimento β (TGF-β) e fator de necrose tumoral α (TNF-α) (10). A perpetuação resulta dos efeitos desses estímulos que mantêm o fenótipo ativado principalmente através do aumento da expressão de fatores de crescimento. Essa fase de ativação envolve no mínimo sete mudanças discretas no comportamento celular: proliferação, quimiotaxia, fibrogênese, contratilidade, degradação da matriz,

perda de lipídios e liberação de citocinas. Durante essa fase há uma liberação de estimulos pró-inflamatórios, pró-fibrogênicos e pró-mitogênicos que agem de forma autócrina e parácrina, além do remodelamento acelerado da ECM (11-13).
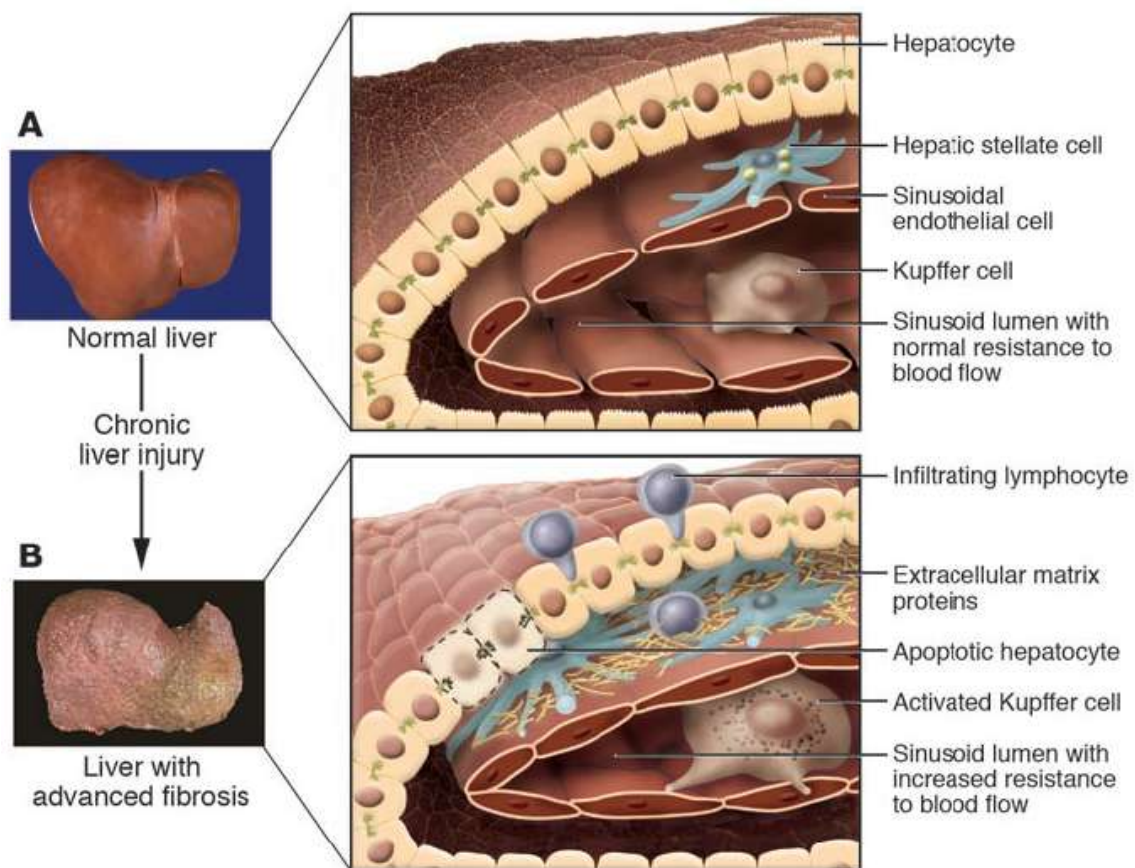

Ilustração 1. Arquitetura hepática normal (A) e associada à fibrose avançada (B). Após o dano crônico, linfócitos infiltram o parênquima hepático. Alguns hepatócitos entram em apoptose e as células de Kupffer se ativam, liberando mediadores inflamatórios. As HSCs entram em um processo de ativação e proliferam, além de secretar grandes quantidades de ECM (*Bataller & Brenner, 2005).*

1.2.1 PPAR-γ e as Células Estreladas Hepáticas

O receptor ativado por proliferador de peroxissomo (PPAR) é um fator nuclear transcricional ligante-dependente que possui um papel muito importante na adipogênese e metabolismo de ácidos graxos (14). Ele regula o armazenamento de ácidos graxos no tecido adiposo, além de estimular a maturação e diferenciação dos adipócitos (15). Ao mesmo tempo, sua ativação possui efeitos em diversos eventos fisiológicos e fisiopatológicos, incluindo a inibição da proliferação e indução de apoptose em uma variedade de células cancerosas. Estudos recentes mostraram que PPARγ possui efeitos protetores durante o reparo tecidual, além de modular a produção de certos mediadores inflamatórios (16).

O PPARγ é altamente expresso em HSCs quiescentes no fígado normal. A expressão e regulação desse fator nuclear são essenciais para a diferenciação das HSCs porque ele promove o armazenamento intracelular de lipídios enquanto suprime o gene do colágeno tipo I (17). Entretanto, os níveis de PPARγ e sua atividade são dramaticamente reduzidos durante a ativação das HSCs. Apesar do mecanismo molecular preciso por trás do potencial antifibrótico do PPARγ permaneça desconhecido, é bem possível que ele interaja com uma série de processos de sinalização que regulam a expressão de moléculas fibrogênicas pelas HSCs (16, 18). Em estudos anteriores foi comprovado que a expressão ectópica de PPARγ é suficiente para restaurar diversos marcadores do fenótipo quiescente e reduzir as características fibróticas das HSCs (19). A estimulação por seus agonistas inibe a proliferação das HSCs e a expressão de colágeno tipo I (20). Baseado nesses achados e

na eficácia dos ligantes de PPARγ demonstrados em modelos de fibrose hepática, esse fator nuclear pode ser considerado como alvo terapêutico (17).

1.2.2 Secreção de Fatores de Crescimento e Citocinas

As HSCs são uma fonte importantíssima na produção de citocinas e quimiocinas no fígado. Para quase todos os fatores de crescimento, as HSCs não apenas secretam citocinas, como também respondem a elas, enfatizando a importância da sinalização autócrina nessas células (21).

O TGF-β é um potente mediador pró-fibrogênico da resposta celular no fígado. Possui um papel importante no que diz respeito ao reparo tecidual, produção de ECM, regulação da proliferação e apoptose das HSCs (22). É constituído por três isoformas principais: β1, β2, β3. O TGF-β1 é a principal isoforma implicada na fibrose hepática; seus níveis estão marcadamente elevados em todas as etapas da fibrogênese (16). Sua liberação pelos hepatócitos lesados desencadeia a ativação das células de Kupffer e o recrutamento de outras células inflamatórias, como neutrófilos e linfócitos que consequentemente secretam TNF-α, interleucina 1 (IL-1), interleucina 6 (IL-6) e proteína quimiotática de monócitos 1 (MCP-1), acelerando a inflamação (6). Essas células também liberam fatores que podem influenciar a biologia das HSCs (23). A quimiotaxia é um fator essencial às HSCs, uma vez que as células migram para as áreas lesadas em resposta a fatores quimiotáticos (4). MCP-1 é uma das quimiocinas altamente expressas em áreas de remodelamento tecidual ativo durante a fibrogênese. Diferentes populações

celulares contribuem para a expressão de MCP-1, incluindo as próprias HSCs ativadas (24).

1.3 Reversão da Fibrose Hepática

Atualmente não há um tratamento padrão para a fibrose hepática (10). A terapia mais efetiva continua sendo a remoção do agente causador quando possível (25). Novas estratégias de tratamento têm sido criadas sobre uma base de evidências crescentes que documenta a reversibilidade da fibrose hepática e a compreensão detalhada da biologia das HSCs ativadas (26).

A redução da ativação das HSCs tornou-se a chave para o bloqueio da fibrogênese (12). Quando a agressão ao fígado é reduzida, juntamente com a diminuição da intensidade da inflamação e necrose hepatocelular, é observada uma redução no número das HSCs (27).

A diminuição da inflamação e da resposta imune, geralmente observadas em tratamentos antivirais, são medidas que podem evitar a ativação das HSCs. Muitos agentes possuem atividade anti-inflamatória, entre eles IFN-γ e IL-10, antagonistas de TGF-β, moduladores do fator nuclear kappa B (NF-κB) e agonistas de PPARγ. O desenvolvimento de antagonistas de citocinas pró-inflamatórias e de seus receptores podem trazer benefícios no tratamento da fibrose hepática(4).

Algumas terapias antifibróticas são direcionadas à indução da morte apoptótica das HSCs ativadas justamente por ser uma forma de controlar a fibrogênese sem causar a morte de outros tipos celulares (27). Muitos estudos mostram que o IFN-γ, além de sua atividade anti-inflamatória, também possui

ação sobre a proliferação das HSCs, induzindo a apoptose das células ativadas (25).

Devido às limitações de medidas preventivas e a falta de terapias estabelecidas para as doenças crônicas do fígado, a necessidade de desenvolver novos agentes antifibróticos torna-se fundamental (28). A terapia antifibrótica ideal, além de segura quando usada por períodos prolongados, deve ser específica para o fígado, não-hepatotóxica, seletiva para a cascata da fibrogênese, incluindo a inibição da deposição de matriz, síntese de colágeno e modulação da ativação das HSCs, podendo também estimular a apoptose dessas células, deve ser bem tolerada e se possível, de baixo custo (3).

A busca por produtos naturais que possuam efeitos antifibróticos vem crescendo cada vez mais devido a seus baixos efeitos adversos (29-32). Os nutracêuticos, alimentos ou parte deles que têm a capacidade de proporcionar benefício à saúde, são utilizados no tratamento de diversas doenças desde os tempos antigos. Atualmente, muitos estudos experimentais têm comprovado as evidências da utilização empírica (33).

1.4 Capsaicina

A capsaicina (8-methil-N-vanilil-trans-6-nonenamida) é o componente ativo das pimentas vermelhas, também conhecidas como pimentas chili. Ela faz parte de um grupo de compostos denominados capsaicinóides que são responsáveis pela pungência dos frutos das plantas do gênero *Capsicum*, membros da família *Solanaceae* (34). A capsaicina pura é um alcalóide cristalino, lipofílico, incolor, inodoro, solúvel em álcool e que apresenta a

fórmula molecular $C_{18}H_{27}NO_3$ (35). Sua estrutura pode ser visualizada na Ilustração 2. É altamente consumida mundialmente, especialmente como tempero, principalmente em países latinos e asiáticos (36). No Brasil, são produzidas inúmeras variedades de pimenta, com cultivos principalmente nos estados de São Paulo e Rio Grande do Sul. A comercialização desses frutos é responsável por movimentar em torno de oitenta milhões de reais/ano, sendo que só o comércio de sementes rende mais de três milhões (37).

A capsaicina é um agonista específico da família do receptor de potencial transitório vanilóide 1 (TRPV1) de canais iônicos, encontrados na membrana celular (38). A pungência e os efeitos nociceptivos da pimenta resultam dessa ligação (39). Porém, vários estudos demonstraram que a ação anti-inflamatória da capsaicina em células não-neuronais não é mediada pelos receptores vanilóides, indicando uma possível rota alternativa de sinalização (40, 41).

A capsaicina tem sido alvo de inúmeras pesquisas devido a sua extensa gama de propriedades farmacológicas, que inclui efeitos analgésicos, anti-inflamatórios, bem como antigenotóxicos, antimutagênicos e anticarcinogênicos (36, 42). Pesquisas recentes mostram que as propriedades quimiopreventivas da capsaicina podem estar envolvidas com a ação do tipo PPARγ-ligante (40).
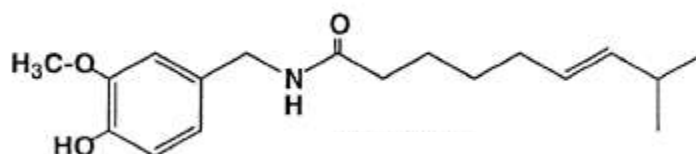
Ilustração 2. Estrutura química da capsaicina.

2   JUSTIFICATIVA

A fibrose hepática é uma complicação frequente de diversas doenças hepáticas crônicas. Seu estágio final, a cirrose, é considerado um dos maiores causadores de morte nos países ocidentais na atualidade. Sabendo-se que a fibrose pode ser reversível desde que o agente causador seja removido, a busca por terapêuticas antifibróticas eficazes torna-se relevante. Atualmente não há um tratamento padrão para a fibrose de fígado. Por essa razão, esse estudo tem como finalidade estudar a capsaicina como forma alternativa de tratamento, uma vez que inúmeros estudos já comprovaram seus efeitos hepatoprotetores. Dessa forma, torna-se pertinente a realização deste trabalho que busca um novo método de reverter o processo fibrótico de uma forma que seja acessível a todos.

3   OBJETIVOS

3.1   Objetivo geral

- Verificar os efeitos da capsaicina nas células estreladas hepáticas.

3.2   Objetivos específicos

- Verificar o efeito do uso da capsaicina na inativação e diferenciação das células GRX.
- Estudar a ação da capsaicina sobre a proliferação das células GRX.

CAPÍTULO 2

CAPSAICIN INDUCES DE-DIFFERENTIATION OF ACTIVATED HEPATIC STELLATE CELL

Capsaicin induces de-differentiation of activated hepatic stellate cell

Shanna Bitencourt, Fernanda C. de Mesquita, Eduardo Caberlon,
Gabriela V. da Silva, Bruno S. Basso, Gabriela A. Ferreira, and Jarbas R. de Oliveira

Abstract: Hepatic stellate cells (HSC) play a key role in liver fibrogenesis. Activation of PPARγ and inhibition of fibrogenic molecules are potential strategies to block HSC activation and differentiation. A number of natural products have been suggested to have antifibrotic effects for the de-activation and de-differentiation of HSCs. The purpose of this study was to investigate the in vitro effects of capsaicin on HSC de-activation and de-differentiation. The results demonstrated that capsaicin induced quiescent phenotype in GRX via PPARγ activation. Significant decrease in COX-2 and type I collagen mRNA expression was observed in the first 24 h of treatment. These events preceded the reduction of TGF-β1 and total collagen secretion. Thus, capsaicin promoted down-regulation of HSC activation by its antifibrotic and anti-inflammatory actions. These findings demonstrate that capsaicin may have potential as a novel therapeutic agent for the treatment of liver fibrosis.

Key words: hepatic stellate cell, capsaicin, fibrosis, peroxisome proliferator-activated receptor gamma, transforming growth factor-beta.

Résumé : Les cellules stellaires hépatiques (CSH) jouent un rôle clé dans la fibrogenèse du foie. L'activation du PPARγ et l'inhibition de molécules fibrogènes constituent des stratégies potentielles pour bloquer l'activation et la différenciation des CSH. Un certain nombre de produits naturels dont les effets antifibrotiques ont été suggérés ont été utilisés pour désactiver et dédifférencier les CSH. Le but de cette étude était d'examiner les effets in vitro de la capsaïcine sur la désactivation et la dédifférenciation des CSH. Les résultats ont démontré que la capsaïcine induisait la quiescence des cellules GRX par l'intermédiaire de l'activation du PPARγ. Une diminution significative de l'expression de l'ARNm de COX-2 et du collagène de type I était observée durant les 24 premières heures de traitement. Ces événements précédaient la réduction de TGF-β1 et la sécrétion de collagène total. Ainsi, la capsaïcine favorise la régulation à la baisse de l'activation des CSH par ses actions antifibrotiques et anti-inflammatoires. Ces données démontrent que la capsaïcine peut avoir du potentiel en tant que nouvel agent thérapeutique pour traiter la fibrose du foie.

Mots-clés : cellules stellaires hépatiques, capsaïcine, fibrose, PPARγ, TGF-β.

[Traduit par la Rédaction]

Introduction

Hepatic stellate cells (HSCs) are a major regulator in liver homeostasis and play a central role in the development and maintenance of liver fibrosis. In normal liver, HSCs store vitamin A in cytoplasmic lipid droplets, show a low proliferate rate, and express markers that are characteristic of adipocytes (e.g., PPARγ) (Friedman 2008b). In pathological conditions, HSCs activate, lose retinoids, proliferate, and enhance collagen synthesis (Pinheiro-Margis et al. 1992). GRX, the oldest immortal murine HSC line, derived from inflammatory fibrogranulomatous lesions in liver, displays myofibroblast-like cell morphology and secretes collagen type I and III (Borojevic et al. 1985). These cells have pro-inflammatory and pro-fibrogenic properties, but as has been previously described, the GRX induction to quiescent phenotype by several agents that modify lipid metabolism decrease collagen production and store lipid droplets (Borojevic et al. 1990; Cardoso et al. 2003; Margis and Borojevic 1989; Martucci et al. 2004).

Antifibrotic agents targeting HSC activation have been proposed as a therapeutic target against liver fibrosis. Previous reports have shown that the stimulation of PPARγ activity by its ligands and agonists down-regulates HSC activation and counterbalance proliferative and fibrogenic responses (Li et al. 2008). Flavonoids, catechins, and alkaloids are some of a number of natural products that have been sug-

S. Bitencourt, F.C. de Mesquita, E. Caberlon, G.V. da Silva, B.S. Basso, G.A. Ferreira, and J.R. de Oliveira. Laboratório de Pesquisa em Biofísica Celular e Inflamação, Pontifícia Universidade Católica do Rio Grande do Sul, PUCRS, Avenida Ipiranga 6681, prédio 12, bloco C, sala 221, CEP 90619-900, Porto Alegre, Rio Grande do Sul, Brazil.

Corresponding author: Jarbas R. de Oliveira (e-mail: jarbas@pucrs.br).

gested to have antifibrotic effects used for de-activation and de-differentiation of HSCs (Bragança de Moraes et al. 2012; Fu et al. 2008; Kawada et al. 1998; Shin et al. 2011; Singal et al. 2011; Souza et al. 2008).

Capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide) is a pungent component of a great variety of capsicum fruits, including chili pepper (Chen et al. 2011). The effects of capsaicin have been widely studied, and antioxidative, antiproliferative, and anticarcinogenic effects have been documented (Gupta et al. 2010; Materska and Perucka 2005; Oyagbemi et al. 2010). Prior studies have shown that capsaicin is a naturally occurring PPARγ ligand, and its actions include inhibition of pro-inflammatory molecules and apoptosis of a great range of cells (Kim et al. 2004; Park et al. 2004).

The purpose of this investigation was to study the role of capsaicin with respect to de-differentiation of activated HSCs and further examine its potential action for modulating profibrogenic molecules. Capsaicin may have potential as a novel therapeutic agent for the treatment of liver fibrosis.

Materials and methods

Cell culture

All the experiments were performed using the murine GRX hepatic stellate cell line, which retains key features of activated HSCs (Borojevic et al. 1985). Cells were obtained from the Rio de Janeiro Cell Bank (Federal University, Rio de Janeiro, Brazil). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum (FBS) (Invitrogen, USA), 2 g/L HEPES buffer, 3.7 g/L NaHCO$_3$, and 1% penicillin and streptomycin (Invitrogen). Cells were seeded in tissue culture plates and incubated at 37 °C in a humidified atmosphere of 5% CO$_2$.

Capsaicin treatment

GRX cells were incubated with capsaicin (*Capsicum sp.*, >95%, Sigma Chemical Co., USA): 10 μmol/L, 50 μmol/L, or 100 μmol/L. Analyses were performed 24 h and (or) 10 days after treatment. All experiments were performed thrice and in triplicate.

Detection of lipid droplets by Oil Red-O staining

To visualize cell morphology and lipid accumulation, on day 10, cells were stained with Oil Red-O (ORO) (Sigma Chemical Co., USA) (Ramírez-Zacarías et al. 1992). Briefly, after fixing cells with 10% formaldehyde, ORO (0.35 g, 60% isopropanol) was added for 15 min. Intracellular lipid droplets were examined using an inverted light microscope.

Quantification of lipid accumulation

The estimation of cell accumulation of lipid droplets was based on the protocol described by Bouraoui et al. (2008). The procedure is based on ORO staining of intracellular lipid droplets and Coomassie brilliant blue staining (Sigma) of cellular proteins. Briefly, cells were fixed with perchloric acid and incubated with ORO dissolved in propylene glycol (2 mg/mL) for 2 h. The ORO within the lipid droplets was extracted using isopropanol. The absorbance was read at 492 nm using an ELISA plate reader. Next, the wells were washed for protein determination. Cells were incubated with

Coomassie brilliant blue staining for 1 h. After washing, the cells were incubated with propylene glycol for 3 h at 60 °C. The absorbance was read at 620 nm. The specific lipid content was calculated as the ratio of absorbance value obtained for ORO and Coomassie brilliant blue staining.

TGF-β quantification

TGF-β1 concentration was measured in cell supernatant using a commercially available ELISA kit (R and D Systems, USA). The kit contained a specific monoclonal antibody immobilized on a 96-well microtiter plate that bound TGF-β1 in the aliquot and a second enzyme-conjugated specific polyclonal antibody. Following several washings to remove unbound substances and antibodies, a substrate solution was added to the wells. Color development was stopped by sulfuric acid, and optical density was determined at 540 nm with the correction wavelength set at 570 nm in an ELISA plate reader. Results were calculated on a standard curve concentration and multiplied for the dilution factor. TGF-β1 levels were expressed as picograms per milliliter.

Measurement of collagen content

Collagen content in GRX cells was measured using picrosirius red. Briefly, picro-sirius red was added to cell supernatant to form a collagen–dye complex. After centrifugation, unbound dye was removed, and the collagen–dye complex was dissolved in NaOH. The absorbance was measured at 540 nm in an ELISA plate reader. Each sample was normalized to the relative amount of total protein measured by the Bradford method (Bradford 1976). Results were expressed as percentage of control.

RNA extraction and RT-PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, USA). RNA was reverse transcribed into cDNA, using Superscript III First-Strand Synthesis SuperMix (Invitrogen, USA) according to the manufacturer instructions. Table 1 shows the primers sets used. Polymerase chain reaction products were electrophoresed using 1.5% agarose gel, containing ethidium bromide (5 μg/mL). The gel was visualized using UV light and photographed. Densitometric quantitation of band intensity was performed using ImageJ software (NIH, USA). All the results correspond to percentage of control using β-actin as internal control gene.

Cell viability

Viable cells were counted by examining cell number with the MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) assay (Invitrogen, USA), measuring mitochondrial respiratory function (Mosmann 1983). GRX cells were seeded into 24-well plates at a density of 5×10^4 cells/well and treated with capsaicin (as described above) for 10 days. Cells were incubated in medium containing 10% MTT for 3 h and dissolved with isopropanol. Absorbance at 540 nm and a reference at 620 nm were measured using an ELISA reader. For determination of cell number, cells were counted using a hemocytometer. Trypan blue analyses were performed to determine cellular viability.

Statistics

Data are reported as mean ± SD. Each experiment was

PROOF/ÉPREUVE

Table 1. Sequence of primers used for RT-PCR.

Primers	Forward primer (5′–3′)	Reverse primer (5′–3′)	Reference
PPAR-γ	TGGAATTAGATGACAGTGACTTGG	CTCTGTGACGATCTGCCTGAG	(Guimarães et al. 2007)
Type I collagen	AGAACATCACCTATCACTGCAAGA	GTGGTTTTGTATTCGATGACTGTCT	(Brun et al. 2005)
COX-2	CTGGTGCCTGGTCTGATGATG	GGCAATGCGGTTCTGATACTG	(Inoue et al. 2003)
β-actin	TATGCCAACACAGTGCTGTCTGG	TACTCCTGCTTGCTGATCCACAT	(Guimarães et al. 2007)

performed at least three independent times and in triplicate. Statistical tests were performed using SPSS software (version 13.0, SPSS Inc., USA). Results were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. The level of significance was set at $P < 0.05$.

Results

Capsaicin induces de-differentiation of GRX cells

Capsaicin led the phenotype conversion of myofibroblast-like (activated) phenotype into fat storing (quiescent) phenotype. Typical changes in cell morphology during de-differentiation were observed. Cells lost their elongated and parallel strand appearance and acquired a larger and polygonal shape. Intracellular lipid droplets were mostly visible in GRX cells treated with all capsaicin concentrations in a dose-dependent manner. The induction of lipid droplets was not synchronous, and groups of cells fully induced into the quiescent phenotype were present with cells that had not yet started to accumulate lipid. Capsaicin increased considerably these vacuoles of rich intracellular lipids around the nucleus from the fifth day on. After 10 days most cells exhibited the fat storing phenotype, as detected by phase contrast microscopy after staining with ORO (Fig. 1D). In contrast, control cells preserved their myofibroblast-like morphology, devoid of large lipid droplets (Fig. 1A). These findings were confirmed by colorimetric quantification of intracellular lipids concentration (Fig. 1E). The amount of ORO detected at day 10 was up to 1.3-fold higher compared with that of control cells staining ($P < 0.05$). Thus, it demonstrated that lipid accumulation is proportional to the concentration of capsaicin.

To confirm cell de-differentiation, the mRNA expression of PPARγ was measured after 24 h and 10 days of treatment. The expression of PPARγ mRNA was significantly increased after 10 days of culture compared with that at 24 h ($P < 0.01$) (Fig. 2).

Capsaicin promotes antifibrotic effects in GRX

To measure the antifibrotic effects of capsaicin, the amount of secreted TGF-β1 present in media from cultured GRX was quantified by ELISA. At 24 h no difference was detected. Thus, as the cells began to differentiate and change in morphology, a significant decrease of TGF-β1 levels was detected. After 10 days, GRX cells treated with 50 μmol/L and 100 μmol/L of capsaicin exhibited a strong reduction of TGF-β1 secretion when compared to the control group ($P < 0.001$) (Fig. 3).

As the activated HSC is the predominant hepatic cell type in the liver responsible for the increased synthesis and deposition of type I collagen during fibrosis, we decided to quantify the amount of total collagen secreted by GRX cells and to measure the mRNA expression of type I collagen. The rate of total collagen secretion decreased after 24 h ($P < 0.05$) and 10 days ($P < 0.01$) (Fig. 4), as the mRNA expression of type I collagen was down-regulated at 24 h, remaining low until day 10 ($P < 0.01$) (Fig. 5).

COX-2 mRNA expression was also used as a marker of activation of the hepatic stellate cell line to confirm the effect of capsaicin. Also as expected, the mRNA expression of COX-2 (Fig. 6) followed the same pattern as type I collagen. These findings indicate that capsaicin has anti-inflammatory effect on GRX cells, and that it is able to decrease the fibrotic responses of GRX.

Effects on cell proliferation

The anti-proliferative activity on GRX cells was determined by cell viability using the MTT assay and direct cell count. As shown on Fig. 7A, GRX cell proliferation was dose-dependently inhibited by capsaicin. At a concentration of 100 μmol/L, capsaicin inhibited cell viability up to 60% when compared with untreated control in a dose-dependent manner ($P < 0.001$). To confirm the results of MTT assay, cells were counted using a hemocytometer. Capsaicin significantly reduced the cell number at all evaluated ~~evaluated~~ concentrations (Fig. 7B). The decrease was up to 4 times compared with control cells ($P < 0.001$). In addition, capsaicin did not increase lactate dehydrogenase (LDH) release in the culture medium (data not shown), indicating that the anti-proliferative effects of capsaicin were not related to the non-specific cytotoxic effects.

Discussion

The morphological feature of HSC is the appearance of microscopic cytoplasmic lipid droplets (Shirakami et al. 2012). In the present study, we demonstrated that capsaicin is capable of inducing de-differentiation of myofibroblast to lipocyte phenotype in GRX, a murine hepatic stellate cell line. The culture of GRX in the presence of capsaicin resulted in an increased lipid accumulation, as evidenced by ORO staining and confirmed by lipid quantitation. Therefore, capsaicin acts as a potent inducer of the quiescent phenotype in a time and concentration-dependent manner.

PPARγ is a key adipogenic transcription factor that plays an important role in adipogenesis and fatty acid metabolism. This nuclear transcription factor is highly expressed in quiescent HSC, and its activity affects cell differentiation pathway through a variety of mechanisms (Guimarães et al. 2007; Tsukamoto et al. 2006). Furthermore, PPARγ activation is necessary for inhibiting cell proliferation, inducing apoptosis, suppressing ECM gene expression, and restoring lipid storage capacity (Wang et al. 2011; Zhang et al. 2012). As some authors suggest, capsaicin may have a PPARγ ligand-like action

PROOF/ÉPREUVE

Fig. 1. Oil Red-O (ORO) staining and lipid quantitation of GRX cells at day 10. (A) Control cells; (B), (C), (D) cells treated with capsaicin at 10 μmol/L, 50 μmol/L, and 100 μmol/L, respectively. Bar length = 20 μm. (E) Specific lipid content expressed spectrophotometrically as the ratio of absorbance value obtained for ORO and Coomassie brilliant blue staining. Results are expressed as mean ± SD. *$P < 0.05$: control vs. treated cells.
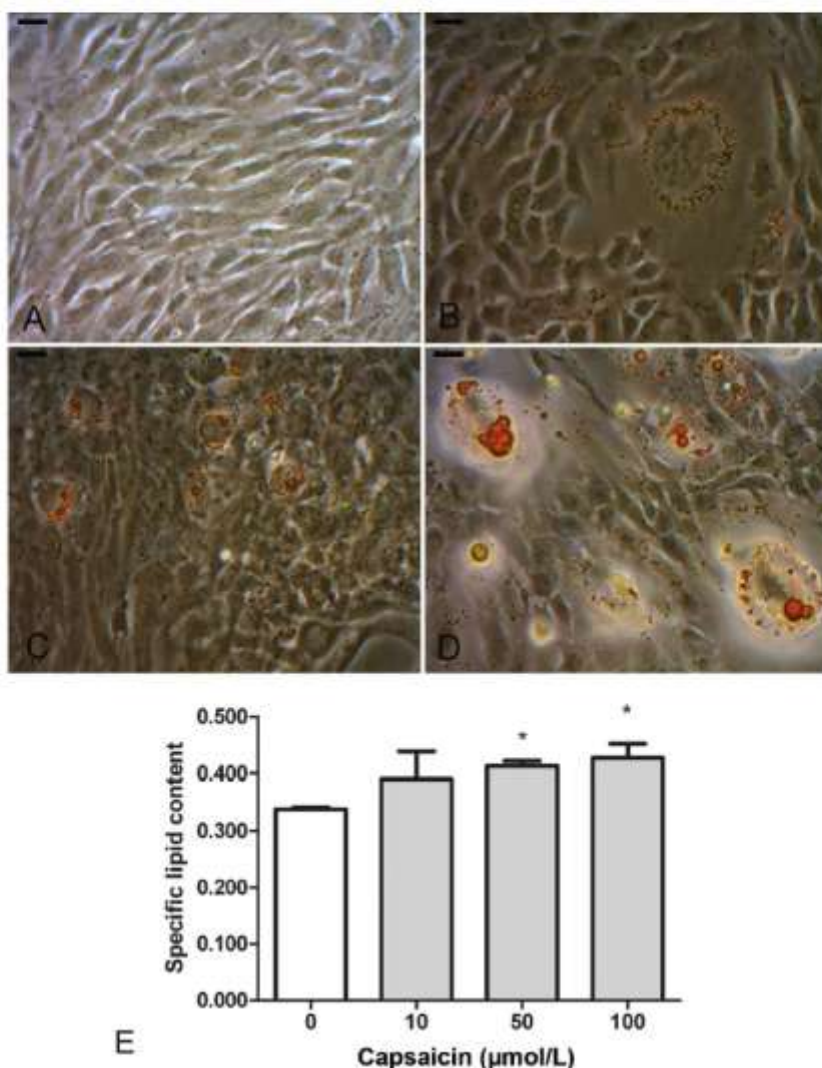
(Kim et al. 2004; Park et al. 2004). For that reason, we decided to confirm cell de-differentiation by analyzing PPARγ activity. We thought that capsaicin-induced de-differentiation may be associated with the PPARγ pathway. We evaluated PPARγ mRNA expression in GRX cells treated with capsaicin for 24 h and 10 days and found the induction of the lipocyte phenotype was associated with up-regulation of PPARγ mRNA expression after the 10 day treatment. These results suggest that PPARγ participates as a transcriptional factor of adipogenesis during GRX de-differentiation induced by capsaicin.

TGF-β is the most important profibrogenic mediator of cellular responses in the liver. It not only activates HSC but also stimulates ECM synthesis, and regulates cell proliferation and differentiation (Bataller and Brenner 2005). TGF-β1 is the major isoform implicated in hepatic fibrosis. During fibrogenesis, tissue and blood levels of active TGF-β1 are elevated. Thereby, its concentrations are correlated with the severity of liver fibrosis (Friedman 2008a; Gressner et al. 2002). Fibrotic HSC can itself secrete a substantial amount of TGF-β1 that can maintain self-activation (Bataller and Brenner 2005; Kisseleva and Brenner 2008). As TGF-β is the central regulator cytokine of tissue fibrosis, we next analyzed TGF-β1 concentrations in GRX cells supernatant. A significant decrease of TGF-β1 protein levels was detected after 10 days of treatment with capsaicin. The results indicate a possible antifibrotic effect of capsaicin in HSC via reduced generation of active TGF-β1. Prior researches have shown that an interference in TGF-β1 synthesis blocks the up-regulation of ECM expression and production of collagen strik-

27

Fig. 2. Effects of capsaicin (CPS) on PPAR-γ mRNA expression of GRX cells treated for 24 h and 10 days. β-actin was an internal control for equal loading. Data are expressed as mean ± SD ($n = 3$). Results are presented as percentage of control. * $P < 0.01$ compared with control.
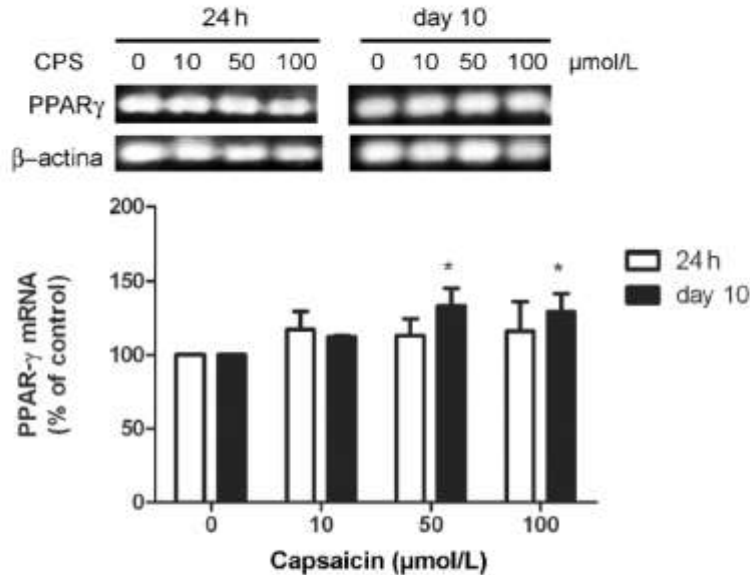

Fig. 3. ELISA assay of TGF-β1 in cell supernatant of 24 h and 10-day treatments. Data represent the mean ± SD ($n = 3$). TGF-β1 levels were expressed as picograms per milliliter. * $P < 0.001$ compared with control.
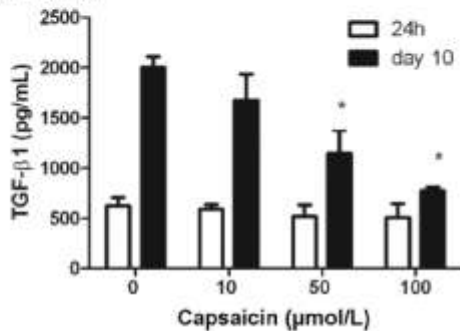

Fig. 4. Total collagen content in cell supernatant of 24 h and 10-day treatments. Data represent the mean ± SD ($n = 3$). Results were expressed as percentage of control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with control.

Fig. 5. Effects of capsaicin (CPS) on type I collagen mRNA expression of GRX cells treated for 24 h and 10 days. Data represent the mean ± SD ($n = 3$). β-actin was an internal control for equal loading. Results are presented as percentage of control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with control.

ingly decreasing fibrosis (Ye and Dan 2010). Accumulating evidence indicates that activation of PPARγ disrupts the TGF-β signaling pathway and inhibits its probrotic effect (Zhang et al. 2012). Besides, other studies have shown that the anti-inflammatory action of capsaicin is due to its suppression of pro-inflammatory cytokines, such as NF-κB and TNF-α, via PPARγ activation (Park et al. 2004). Thus, the decrease of TGF-β levels after 10 days of treatment corrobo-
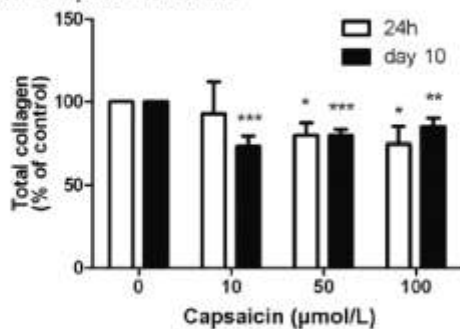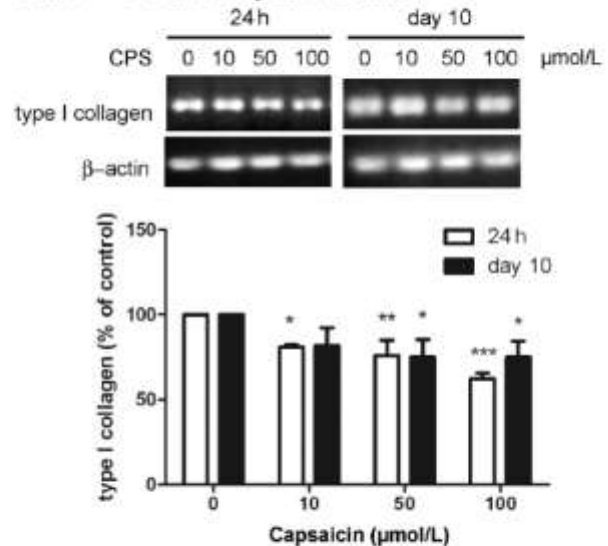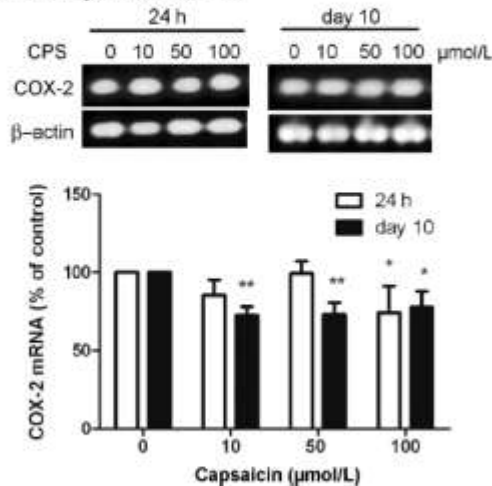
PROOF/ÉPREUVE

6

Fig. 6. Effects of capsaicin (CPS) on COX-2 mRNA expression of GRX cells treated for 24 h and 10 days. Data represent the mean $\pm$ SD ($n = 3$). β-actin was an internal control for equal loading. Results are presented as percentage of control. * $P < 0.05$, ** $P < 0.01$ as compared with control.
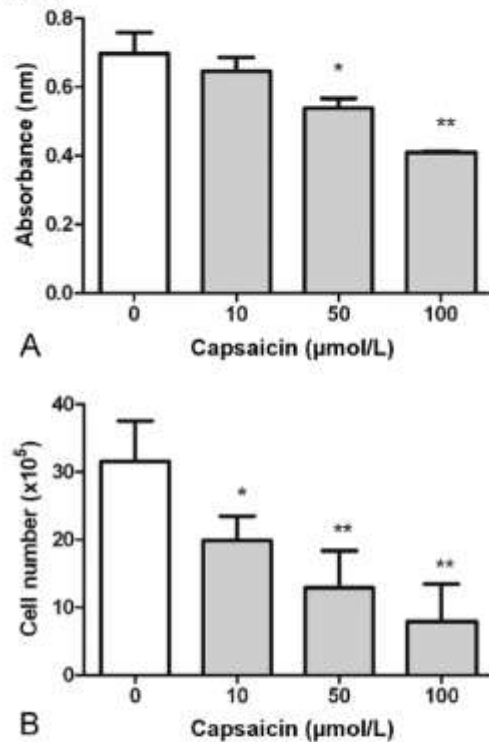
Fig. 7. Effect of capsaicin on viability of GRX cells. Cells were treated for 10 days with different concentrations of capsaicin. (A) Cell viability assessed by MTT assay. Data represent the mean $\pm$ SD ($n = 3$). Results were expressed as absorbance reading. * $P < 0.05$, ** $P < 0.001$ compared with control. (B) Cell viability assessed by direct cell count. Data represent the mean $\pm$ SD ($n = 4$). Results were expressed as cell number. * $P < 0.05$, ** $P < 0.001$ compared with control.

rates with PPARγ data. Accordingly, we cautiously suggest that capsaicin mediates the blockage of the TGF-β signaling pathway via PPARγ activation.

GRX cells, as many activated HSCs, secrete high levels of collagen type I and III (Borojevic et al. 1985; Herrmann et al. 2007). Up-regulation of collagen synthesis during activation is one of the most remarkable responses of HSC to chronic hepatic damage. Type I collagen expression can be regulated both transcriptionally and post-transcriptionally by a number of stimuli and pathway, the most potent stimulus is TGF-β. Therefore, type I collagen is a critical parameter reflecting the metabolism of collagen in the process of liver fibrosis (Gressner et al. 2002). Our results in this report demonstrated that capsaicin not only significantly diminished the amount of total collagen in culture media but also down-regulated type I collagen mRNA expression within the first 24 h of incubation and remaining decreased for 10 days. Interestingly, the short-term effect of capsaicin in type I collagen mRNA expression is not due to PPARγ activation or by a decrease in TGF-β.

Many factors have been identified to contribute to HSC activation, including COX-2, an important enzyme in starting inflammatory reaction. HSCs and Kupffer cells are the major source of COX-2 up-regulation in liver fibrosis (Nanji et al. 1997). The role of COX-2 in HSC activation is controversial (Zhao et al. 2011). Several investigators have reported that COX-2 participates in hepatic fibrosis mediating liver damage and inflammation, and the blockage of its expression is associated with antifibrotic activity (Efsen et al. 2001; Jeong et al. 2010; Zhao et al. 2012). Other data suggest COX-2 may also have anti-inflammatory properties (Gilroy et al. 1999; Paik et al. 2009). In addition, recent work revealed relevant interactions between the PPAR and COX pathways (Eibl 2008). Several PPARγ ligands can suppress COX-2 expression, or they can interfere with downstream targets.

These data suggest that PPARγ signaling and the COX-2 pathway are intertwined (Hazra et al. 2008).

As previously reported, capsaicin can inhibit NF-κB activation, leading to the reduction of pro-inflammatory mediators such as COX-2, iNOS, and PGE2 (Kim et al. 2003). Therefore, we hypothesized that the anti-inflammatory action of capsaicin might modulate COX-2 mRNA expression. As expected, our results showed that the mRNA expression of COX-2 is already diminished in the first 24 h with capsaicin (100 µmol/L). Our findings corroborate with previous data confirm that inhibitors of COX-2 reduce activation of HSC and decrease production and secretion of collagen by HSC (Paik et al. 2009). Thus, we suggest that the anti-inflammatory action of capsaicin against activated HSCs may be the first trigger to GRX de-activation and differentiation.

In conclusion, capsaicin led the phenotype conversion of GRX cells by its antifibrotic and anti-inflammatory actions. The mainly cellular and molecular mechanisms involved in the anti-inflammatory effect of capsaicin is the reduction of the pro-inflammatory mediator COX-2, which seems to be the first trigger to the induction of de-differentiation in GRX. Moreover, capsaicin exerts an antifibrotic action related by up-regulation of PPARγ mRNA expression, and,

PROOF/ÉPREUVE

consequently, the modulation of profibrogenic genes. Increasing knowledge of the antifibrotic properties of capsaicin can be important for the development of new antifibrotic and anti-inflammatory agents in chronic liver diseases.

Acknowledgements

References

Bataller, R., and Brenner, D.A. 2005. Liver fibrosis. J. Clin. Invest. 115(2): 209–218. PMID:15690074.

Borojevic, R., Monteiro, A.N., Vinhas, S.A., Domont, G.B., Mourao, P.A., Emonard, H., et al. 1985. Establishment of a continuous cell line from fibrotic schistosomal granulomas in mice livers. In Vitro Cell. Dev. Biol. 21(7): 382–390. doi:10.1007/BF02623469. PMID:4030623.

Borojevic, R., Guaragna, R.M., Margis, R., and Dutra, H.S. 1990. In vitro induction of the fat-storing phenotype in a liver connective tissue cell line-GRX. In Vitro Cell. Dev. Biol. 26(4): 361–368. doi:10.1007/BF02623827. PMID:2188940.

Bouraoui, L., Gutierrez, J., and Navarro, I. 2008. Regulation of proliferation and differentiation of adipocyte precursor cells in rainbow trout (Oncorhynchus mykiss). J. Endocrinol. 198(3): 459–469. doi:10.1677/JOE-08-0264. PMID:18579724.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72(1–2): 248–254. doi:10.1016/0003-2697(76)90527-3. PMID:942051.

Brun, P., Castagliuolo, I., Pinzani, M., Palu, G., and Martines, D. 2005. Exposure to bacterial cell wall products triggers an inflammatory phenotype in hepatic stellate cells. Am. J. Physiol. Gastrointest. Liver Physiol. 289(3): G571–G578. doi:10.1152/ajpgi.00537.2004. PMID:15860640.

Cardoso, C.C., Paviani, E.R., Cruz, L.A., Guma, F.C., Borojevic, R., and Guaragna, R.M. 2003. Effect of pentoxifylline on arachidonic acid metabolism, neutral lipid synthesis and accumulation during induction of the lipocyte phenotype by retinol in murine hepatic stellate cell. Mol. Cell. Biochem. 254(1–2): 37–46. doi:10.1023/A:1027356412399. PMID:14674680.

Chen, X.W., Serag, E.S., Sneed, K.B., and Zhou, S.F. 2011. Herbal bioactivation, molecular targets and the toxicity relevance. Chem. Biol. Interact. 192(3): 161–176. doi:10.1016/j.cbi.2011.03.016. PMID:21459083.

Bragança de Moraes, C.M., Melo, D.A.S., Santos, R.C.V., Bitencourt, S., Mesquita, F.C., Santos de Oliveira, F., et al. 2012. Antiproliferative effect of catechin in GRX cells. Biochem. Cell Biol. 90(4): 575–584. doi:10.1139/o2012-010. PMID:22574829.

Efsen, E., Bonacchi, A., Pastacaldi, S., Valente, A.J., Wenzel, U.O., Tosti-Guerra, C., et al. 2001. Agonist-specific regulation of monocyte chemoattractant protein-1 expression by cyclooxygenase metabolites in hepatic stellate cells. Hepatology, 33(3): 713–721. doi:10.1053/jhep.2001.22761. PMID:11230753.

Eibl, G. 2008. The Role of PPAR-gamma and Its Interaction with COX-2 in Pancreatic Cancer. PPAR Res. 2008: 326915. doi:10.1155/2008/326915. PMID:18615182.

Friedman, S.L. 2008a. Hepatic fibrosis–overview. Toxicology, 254(3): 120–129. doi:10.1016/j.tox.2008.06.013. PMID:18662740.

Friedman, S.L. 2008b. Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. Physiol. Rev. 88(1): 125–172. doi:10.1152/physrev.00013.2007. PMID:18195085.

Fu, Y., Zheng, S., Lu, S.C., and Chen, A. 2008. Epigallocatechin-3-gallate inhibits growth of activated hepatic stellate cells by enhancing the capacity of glutathione synthesis. Mol. Pharmacol. 73(5): 1465–1473. doi:10.1124/mol.107.040634. PMID:18230716.

Gilroy, D.W., Colville-Nash, P.R., Willis, D., Chivers, J., Paul-Clark, M.J., and Willoughby, D.A. 1999. Inducible cyclooxygenase may have anti-inflammatory properties. Nat. Med. 5(6): 698–701. doi:10.1038/9550. PMID:10371510.

Gressner, A.M., Weiskirchen, R., Breitkopf, K., and Dooley, S. 2002. Roles of TGF-beta in hepatic fibrosis. Front. Biosci. 7(1–3): d793–d807. doi:10.2741/gressner. PMID:11897555.

Guimarães, E.L.M., Franceschi, M.F.S., Andrade, C.M.B., Guaragna, R.M., Borojevic, R., Margis, R., et al. 2007. Hepatic stellate cell line modulates lipogenic transcription factors. Liver Int. 27(9): 1255–1264. doi:10.1111/j.1478-3231.2007.01578.x. PMID:17919238.

Gupta, S.C., Kim, J.H., Prasad, S., and Aggarwal, B.B. 2010. Regulation of survival, proliferation, invasion, angiogenesis, and metastasis of tumor cells through modulation of inflammatory pathways by nutraceuticals. Cancer Metastasis Rev. 29(3): 405–434. doi:10.1007/s10555-010-9235-2. PMID:20737283.

Hazra, S., Peebles, K.A., Sharma, S., Mao, J.T., and Dubinett, S.M. 2008. The Role of PPARgamma in the Cyclooxygenase Pathway in Lung Cancer. PPAR Res. 2008: 790568. doi:10.1155/2008/790568. PMID:18769553.

Herrmann, J., Gressner, A.M., and Weiskirchen, R. 2007. Immortal hepatic stellate cell lines: useful tools to study hepatic stellate cell biology and function? J. Cell. Mol. Med. 11(4): 704–722. doi:10.1111/j.1582-4934.2007.00060.x. PMID:17760834.

Inoue, H., Kondo, A., and Togari, A. 2003. Activation of the peripheral sympathetic nervous system increased the expression of cyclooxygenase-2 (COX-2) mRNA in mouse calvaria. Neurosci. Lett. 338(1): 37–40. doi:10.1016/S0304-3940(02)01352-6. PMID:12565135.

Jeong, S.W., Jang, J.Y., Lee, S.H., Kim, S.G., Cheon, Y.K., Kim, Y.S., et al. 2010. Increased expression of cyclooxygenase-2 is associated with the progression to cirrhosis. Korean J. Intern. Med. 25(4): 364–371. doi:10.3904/kjim.2010.25.4.364. PMID:21179273.

Kawada, N., Seki, S., Inoue, M., and Kuroki, T. 1998. Effect of antioxidants, resveratrol, quercetin, and N-acetylcysteine, on the functions of cultured rat hepatic stellate cells and Kupffer cells. Hepatology, 27(5): 1265–1274. doi:10.1002/hep.510270512. PMID:9581680.

Kim, C.S., Kawada, T., Kim, B.S., Han, I.S., Choe, S.Y., Kurata, T., and Yu, R. 2003. Capsaicin exhibits anti-inflammatory property by inhibiting IkB-a degradation in LPS-stimulated peritoneal macrophages. Cell. Signal. 15(3): 299–306. doi:10.1016/S0898-6568(02)00086-4. PMID:12531428.

Kim, C.-S., Park, W.-H., Park, J.-Y., Kang, J.-H., Kim, M.-O., Kawada, T., et al. 2004. Capsaicin, a spicy component of hot pepper, induces apoptosis by activation of the peroxisome proliferator-activated receptor gamma in HT-29 human colon cancer cells. J. Med. Food, 7(3): 267–273. doi:10.1089/jmf.2004.7.267. PMID:15383218.

Kisseleva, T., and Brenner, D.A. 2008. Fibrogenesis of parenchymal organs. Proc. Am. Thorac. Soc. 5(3): 338–342. doi:10.1513/pats.200711-168DR. PMID:18403330.

Li, J.T., Liao, Z.X., Ping, J., Xu, D., and Wang, H. 2008. Molecular mechanism of hepatic stellate cell activation and antifibrotic therapeutic strategies. J. Gastroenterol. 43(6): 419–428. doi:10.1007/s00535-008-2180-y. PMID:18600385.

Margis, R., and Borojevic, R. 1989. Retinoid-mediated induction of the fat-storing phenotype in a liver connective tissue cell line

PROOF/ÉPREUVE

30

(GRX). Biochim. Biophys. Acta, **1011**(1): 1–5. doi:10.1016/0167-4889(89)90069-4. PMID:2923869.

Martucci, R.B., Ziulkoski, A.L., Fortuna, V.A., Guaragna, R.M., Gumg, F.C., Trugo, L.C., and Borojevic, R. 2004. Beta-carotene storage, conversion to retinoic acid, and induction of the lipocyte phenotype in hepatic stellate cells. J. Cell. Biochem. **92**(2): 414–423. doi:10.1002/jcb.20073. PMID:15108365.

Materska, M., and Perucka, I. 2005. Antioxidant activity of the main phenolic compounds isolated from hot pepper fruit (*Capsicum annuum* L). J. Agric. Food Chem. **53**(5): 1750–1756. doi:10.1021/jf035331k. PMID:15740069.

Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods, **65**(1–2): 55–63. doi:10.1016/0022-1759(83)90303-4. PMID:6606682.

Nanji, A.A., Miao, L., Thomas, P., Rahemtulla, A., Khwaja, S., Zhao, S., et al. 1997. Enhanced cyclooxygenase-2 gene expression in alcoholic liver disease in the rat. Gastroenterology, **112**(3): 943–951. doi:10.1053/gast.1997.v112.pm9041257. PMID:9041257.

Oyagbemi, A.A., Saba, A.B., and Azeez, O.I. 2010. Capsaicin: a novel chemopreventive molecule and its underlying molecular mechanisms of action. Indian J. Cancer, **47**(1): 53–58. doi:10.4103/0019-509X.58860. PMID:20071791.

Paik, Y.H., Kim, J.K., Lee, J.I., Kang, S.H., Kim, D.Y., An, S.H., et al. 2009. Celecoxib induces hepatic stellate cell apoptosis through inhibition of Akt activation and suppresses hepatic fibrosis in rats. Gut, **58**(11): 1517–1527. doi:10.1136/gut.2008.157420. PMID:19201774.

Park, J.Y., Kawada, T., Han, I.S., Kim, B.S., Goto, T., Takahashi, N., et al. 2004. Capsaicin inhibits the production of tumor necrosis factor alpha by LPS-stimulated murine macrophages, RAW 264.7: a PPARgamma ligand-like action as a novel mechanism. FEBS Lett. **572**(1–3): 266–270. doi:10.1016/j.febslet.2004.06.084. PMID:15304360.

Pinheiro-Margis, M., Margis, R., and Borojevic, R. 1992. Collagen synthesis in an established liver connective tissue cell line (GRX) during induction of the fat-storing phenotype. Exp. Mol. Pathol. **56**(2): 108–118. doi:10.1016/0014-4800(92)90028-A. PMID:1587337.

Ramírez-Zacarías, J.L., Castro-Muñozledo, F., and Kuri-Harcuch, W. 1992. Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with oil red O. Histochemistry, **97**(6): 493–497. doi:10.1007/BF00316069. PMID:1385366.

Shin, E., Lee, C., Sung, S.H., Kim, Y.C., Hwang, B.Y., and Lee, M.

K. 2011. Antifibrotic activity of coumarins from Cnidium monnieri fruits in HSC-T6 hepatic stellate cells. J. Nat. Med. **65**(2): 370–374. doi:10.1007/s11418-010-0485-7. PMID:21082271.

Shirakami, Y., Lee, S.A., Clugston, R.D., and Blaner, W.S. 2012. Hepatic metabolism of retinoids and disease associations. Biochim. Biophys. Acta, **1821**(1): 124–136. doi:10.1016/j.bbalip.2011.06.023. PMID:21763780.

Singal, A.K., Jampana, S.C., and Weinman, S.A. 2011. Antioxidants as therapeutic agents for liver disease. Liver Int. **31**(10): 1432–1448. doi:10.1111/j.1478-3231.2011.02604.x. PMID:22093324.

Souza, I.C., Martins, L.A., Coelho, B.P., Grivicich, I., Guaragna, R. M., Gottfried, C., et al. 2008. Resveratrol inhibits cell growth by inducing cell cycle arrest in activated hepatic stellate cells. Mol. Cell. Biochem. **315**(1–2): 1–7. doi:10.1007/s11010-008-9781-x. PMID:18454344.

Tsukamoto, H., She, H., Hazra, S., Cheng, J., and Miyahara, T. 2006. Anti-adipogenic regulation underlies hepatic stellate cell transdifferentiation. J. Gastroenterol. Hepatol. **21**(Suppl. 3): S102–S105. doi:10.1111/j.1440-1746.2006.04573.x. PMID:16958658.

Wang, Z., Xu, J.P., Zheng, Y.C., Chen, W., Sun, Y.W., Wu, Z.Y., and Luo, M. 2011. Peroxisome proliferator-activated receptor gamma inhibits hepatic fibrosis in rats. Hepatobiliary Pancreat. Dis. Int. **10**(1): 64–71. doi:10.1016/S1499-3872(11)60009-X. PMID:21269937.

Ye, Y., and Dan, Z. 2010. All-trans retinoic acid diminishes collagen production in a hepatic stellate cell line via suppression of active protein-1 and c-Jun N-terminal kinase signal. J. Huazhong Univ. Sci. Technolog. Med. Sci. **30**(6): 726–733. doi:10.1007/s11596-010-0648-5. PMID:21181362.

Zhang, F., Lu, Y., and Zheng, S. 2012. Peroxisome proliferator-activated receptor-gamma cross-regulation of signaling events implicated in liver fibrogenesis. Cell. Signal. **24**(3): 596–605. doi:10.1016/j.cellsig.2011.11.008. PMID:22108088.

Zhao, L., Gandhi, C.R., and Gao, Z.H. 2011. Involvement of cytosolic phospholipase A2 alpha signalling pathway in spontaneous and transforming growth factor-beta-induced activation of rat hepatic stellate cells. Liver Int. **31**(10): 1565–1573. doi:10.1111/j.1478-3231.2011.02632.x. PMID:22093332.

Zhao, Y., Wang, Y., Wang, Q., Liu, Z., Liu, Q., and Deng, X. 2012. Hepatic stellate cells produce vascular endothelial growth factor via phospho-p44/42 mitogen-activated protein kinase/cyclooxygenase-2 pathway. Mol. Cell. Biochem. **359**(1–2): 217–223. doi:10.1007/s11010-011-1016-x. PMID:21863308.

PROOF/ÉPREUVE

CAPÍTULO 3

EFFECTS OF CAPSAICIN ON PROLIFERATION AND CYTOKINE RELEASE OF ACTIVATED HEPATIC STELLATE CELLS

[Artigo submetido –

Biochemical Pharmacology]

EFFECTS OF CAPSAICIN ON PROLIFERATION AND CYTOKINE RELEASE OF ACTIVATED HEPATIC STELLATE CELLS

Shanna Bitencourt[1], Fernanda Mesquita[1], Bruno Basso[1], Isabella Gadegast[1], Lucas Rizzo[2], Moises Bauer[2], Ramon Bartrons[3], Francesc Ventura[3], Jose Luis Rosa[3], Jarbas Oliveira[1].

1 Laboratório de Pesquisa em Biofísica Celular e Inflamação, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre-RS, Brazil.

2 Laboratório de Imunologia do Envelhecimento, Instituto de Pesquisas Biomédicas, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre-RS, Brazil.

3 Departament de Ciències Fisiològiques II, Campus de Bellvitge, Universitat de Barcelona, L'Hospitalet de Llobregat, Barcelona, Spain.

* To whom correspondence should be addressed at Laboratório de Pesquisa em Biofísica Celular e Inflamação, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Avenida Ipiranga 6681, prédio 12, bloco C, sala 221, CEP 90619-900, Porto Alegre, Rio Grande do Sul, Brazil. Phone: +55 51 33534147. Fax: +55 51 33203568 E-mail: jarbas@pucrs.br

ABSTRACT

Hepatic stellate cells (HSCs) undergo a process of activation characterized by increased proliferation, motility and synthesis of extracellular matrix components. Activated HSCs are central to the pathogenesis of liver fibrosis. This process is driven to a large extent by autocrine and paracrine secretion of cytokines. Capsaicin, the active component of chili pepper, has been reported to have antiproliferative and anti-inflammatory effects on a variety of cell lines. In this study, we aimed to investigate whether capsaicin induces HSC growth arrest besides its modulating effect on pro and anti-inflammatory cytokines release. Apoptosis and cell cycle arrest were investigated as well as inflammatory cytokines (IL-12, IFN-γ, IL-10 and MCP-1) determined by flow cytometer after 24-hour and 10-day exposure to capsaicin. Our results showed that capsaicin reduces cell proliferation within 24 h. No programmed cell death is induced in the same period; however, cell cycle is arrested at stage G0/G1. Inflammatory cytokines IL-12, IFN-γ and IL-10 have their concentrations increased after 10-day treatment, with no change in the short period of 24 h. MCP-1 has a significant increase in the first 24 h, though its concentrations decrease up to 9-fold in day 10. In conclusion, the present study showed that capsaicin inhibits HSC proliferation via cell cycle arrest at G0/G1 phase. In addition, it down-regulates HSC activation by increasing secretion of IFN-γ and IL-10 besides impairing chemotaxis signaling. Thus, these results suggest that capsaicin can suppress HSC activation.

Keywords: hepatic stellate cell, capsaicin, growth arrest, inflammatory cytokine

1. INTRODUCTION

Increased inflammation and cytokine signaling are critical components in progression of liver disease. Following liver injury, several cell types can secrete inflammatory cytokines, specially hepatic stellate cells (HSCs) [1]. The recruitment of HSCs to sites of injury is thought to be an early step in their function in repair and matrix remodeling. These cells undergo a process of activation headed for a phenotype characterized by increased proliferation, motility, contractility and synthesis of extracellular matrix (ECM) components. Stimulation of HSC is regulated by several soluble factors, including cytokines, chemokines and growth factors [2]. HSC activation is the major contributor to development of hepatic fibrosis and thus an important therapeutic target [3].

Previously published works demonstrate the efficacy of natural products for the treatment of hepatic fibrosis. The use of nutraceuticals that can inhibit the proliferation of activated HSCs have a great potential in reversing fibrosis [4]. Capsaicin, the major ingredient of hot peppers, is known to have anti-inflammatory and antiproliferative properties. A number of studies have shown that capsaicin results in growth arrest or apoptosis induction in several cell lines [5]. Our previous study demonstrated that capsaicin has a peroxisome proliferator-activated receptor (PPAR)-ligand action and antifibrotic effect in HSCs, including the inhibition of the proinflammatory mediator transforming growth factor β (TGF-β) [6].

In order to clarify the mechanism underlying the antiproliferative and antifibrotic effects of capsaicin, we investigated whether capsaicin induces

programmed cell death or alters cell cycle besides its modulating effect on inflammatory cytokines release, which play key roles in liver fibrogenesis.

2. METHODS

2.1. CELL CULTURE AND TREATMENT

All the experiments were performed with GRX cell, a murine hepatic stellate cell line. Cells were obtained from the Rio de Janeiro Cell Bank (PABCAM, Federal University, Rio de Janeiro, Brazil). GRXs were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA), 2 g/L HEPES buffer, 3.7g/L $NaHCO_3$ and 1% penicillin and streptomycin (Invitrogen) in a humidified atmosphere of 5% CO_2 at 37ºC. Cells were incubated with 100 μM capsaicin (*Capsicum sp.* > 95%, Sigma-Aldrich, St. Louis, MO) for 24 h and/or 10 days. All experiments were repeated at least three times.

2.2. VIABLE CELL COUNTING

GRX cells were seeded into 24-well plates at a density of 5×10^5 cells/well and treated with capsaicin for 24 h. For determination of cell number, cells were counted using a hemocytometer. Trypan blue analyses were performed to determine cellular viability.

2.3. QUANTIFICATION OF APOPTOSIS

Apoptosis was assessed using the FITC Annexin V Apoptosis Detection Kit I (BD Bioscience, San Jose, CA). Briefly, after treating with capsaicin for 24 h, cells were washed twice with PBS and ressuspended in binding buffer before

addition of annexin V-FITC and propidium iodide (PI). Cells were vortexed and incubated for 15 min in the dark at room temperature. A total of 10 000 events were acquired for each assayed sample. All data were acquired with a FACSCanto II flow cytometer (BD Biosciences). Data were analyzed using the FlowJo 7.2.5 software (Tree Star Inc., Ashland, OR). Results are displayed as scatter dots allowing discrimination between viable cells, apoptotic cells with an intact membrane and cells undergoing secondary necrosis.

2.4. ANALYSIS OF MITOCHONDRIAL MEMBRANE POTENTIAL ($\Delta\Psi$m)

Breakdown of $\Delta\Psi$m was determined by FACS analysis using the MitoScreen Kit (BD Biosciences). JC-1 (5,5,6,6-tetra-chloro-1,1,3,3-tetraethylbenzimidazol-carbocyanine iodide) dye, which is selectively incorporated into mitochondria, is a sensitive and reliable method to detect changes of the mitochondrial membrane potential ($\Delta\Psi$m) [7, 8]. After incubation for 24 h with capsaicin, cells were stained with 0.5 ml JC-1 solution for 15 min at 37°C. Stained GRX were washed twice in JC-1 MitoScreen wash buffer. A total of 10 000 events were acquired for each assayed sample. All data were obtained immediately after staining on a FACSCanto II flow cytometer with CellQuest PRO v4.0.2 software (BD Biosciences). Results are displayed as scatter dots allowing discrimination between polarized and depolarized cells.

2.5. CELL CYCLE ANALYSIS

To determine the effect of capsaicin on the cell cycle, defined as G0/G1, S and G2/M phase, GRX cells were first synchronized by serum starvation and then

exposed to capsaicin for 24 h. Cell cycle phase analysis was performed using the FITC BrdU Flow Kit (BD Biosciences). Briefly, after treatment and BrdU labeling, cells were harvested by trypsinization and adjusted to 1 x 10^6 cells/mL. Samples were fixed with BD Cytofix/Cytoperm Buffer (BD Biosciences). Following fixation, cells were treated with DNase for 20 min to expose BrdU epitopes. After, DNA was stained for cell cycle analysis using 7-AAD dye. The DNA profiles were determined by FACSCanto II flow cytometer (BD Biosciences) and analyzed using the FlowJo 7.2.5 software (Tree Star Inc.).

2.6. QUANTIFICATION OF CYTOKINES

To determine cytokine production, GRX cells were cultured for 24 h and 10 days. The supernatants were collected and stored at -20ºC for later analysis. Multiple soluble cytokines (IL-12 (p70), IFN-γ, IL-10 and MCP-1) were simultaneously measured by flow cytometry using the Cytometric Bead Array (CBA) Mouse Inflammation Kit (BD Biosciences). Acquisition was performed with a FACSCanto II flow cytometer (BD Biosciences). Quantitative results were generated using FCAP Array v1.0.1 software (Soft Flow Inc., Pecs, Hungary). The detection limit was 20 to 5 000 pg/mL.

2.7. STATISTICAL ANALYSIS

All data are expressed as mean ± SD. Comparisons were performed according to unpaired Student's t test. A level of $P < 0.05$ was considered statistically significant. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA).

3. RESULTS

To further identify the antiproliferative action of capsaicin, we investigated the effect of capsaicin on programmed cell death and cell cycle in GRX cells, a murine hepatic stellate cell line which presents a myofibroblast transitional phenotype. We also assessed the effect of capsaicin on inflammatory cytokines release during lipocyte induction. The dose and duration of capsaicin used for these experiments were based on our previous work [6].

3.1. CAPSAICIN INHIBITS HSC PROLIFERATION THROUGH CELL CYCLE ARREST

As shown in Figure 1, in the first 24 h capsaicin significantly reduced the number of viable cells by approximately 32% compared with untreated control ($P < 0.05$). This finding raised the question of apoptosis pathway in capsaicin-treated cells. The potential effect of capsaicin on apoptosis was analyzed by two different methods, one detecting apoptotic cells by measuring the translocation of phosphatidylserine to the outer cell membrane surface and a second measuring the impact on mitochondrial transmembrane potential ($\Delta\Psi$m).

Flow cytometric analysis of the Annexin V labeling assay did not detect apoptosis in the capsaicin treated cells. As shown in Figure 2, the number of apoptotic cells did not increase compared to control experiments. To assess effects of capsaicin on mitochondrial injury, we analyzed the $\Delta\Psi$m of treated cells. Changes of $\Delta\Psi$m were determined by JC-1 staining of GRX cells treated

with capsaicin for 24 h. Capsaicin did not provoke significant loss of $\Delta\Psi$m (Figure 3).

Since capsaicin, in the dose tested, reduced proliferation of HSC, which was not accompanied by programmed cell death, it was thought that capsaicin might induce cell cycle arrest in HSC. To investigate the capsaicin-dependent mechanism involved in the decrease of cell number, we analyzed the cell cycle by flow cytometry. The analysis showed that the antiproliferative effect of capsaicin was associated to a G0/G1 cell cycle arrest (Figure 4). The percentage of cells in phase G0/G1 increased from 31.2% in control cells to 44.7% in cells treated with capsaicin ($P < 0.05$). No modification was observed in the other phases.

3.2.   CAPSAICIN UPREGULATES ANTIFIBROTIC CYTOKINES

We had previously proposed that capsaicin might de-activate HSC by decreasing profibrotic and proinflammatory mediators. HSCs de-differentiate when exposed to capsaicin for 10 days [6]. Thus, to complement our studies we decided to investigate soluble agents involved in HSC activation. Multiple inflammatory cytokines (IL-12 (p70), IFN-γ, IL-10 and MCP-1) were assessed in culture supernatants by CBA. All cytokines were analyzed after 24 hours and 10 days of 100 μM capsaicin exposure.

IL-12, known to be the trigger of other cytokines [9], had its bioactive form (p70) secretion increased only after 10-day exposure to capsaicin ($P < 0.01$) (Figure 5 A). Consequently, the release of IFN-γ increased 2 times ($P < 0.01$). Capsaicin had no significant effect on IFN-γ secretion in 24 h (Figure 5

B). In addition, anti-inflammatory cytokine IL-10 levels were substantially unaffected in the first 24 h. On the other hand, IL-10 release had a 4.3-fold increase after 10-day treatment with capsaicin ($P < 0.05$) (Figure 5 C). As shown in Figure 5 D, chemotaxis via MCP-1 was increased in the first 24 h ($P < 0.05$) and then, drastically reduced up to 9-fold ($P < 0.01$) in day 10.

4. DISCUSSION

The murine GRX cell line presents several characteristics of activated HSCs. They are in a transitional state between quiescent lipocyte and fully activated myofibroblast [10]. It has been previously demonstrated that GRX cells can be induced to express the lipocyte phenotype with an overall increase of lipid storage by a variety of phytochemicals [6, 11-13]. Capsaicin, a naturally occurring alkaloid from capsicum fruits is known to have antiproliferative and anti-inflammatory properties [14]. Several studies support that capsaicin can induce apoptosis or inhibit proliferation or cell cycle arrest in a variety of cell lines, especially tumor cells [15-17]. Our previous work showed that capsaicin de-activates HSCs by inhibiting proliferation and suppressing fibrotic mediators probably via PPARγ pathway [6]. Since PPARγ is implicated in growth arrest and apoptosis [18], we thought that capsaicin, as a PPARγ-ligand, may act via apoptosis pathway. To test whether capsaicin-induced growth inhibition is due to programmed cell death, flow cytometry analysis of apoptosis was conducted. In the early stages of apoptosis changes occur at the cell surface, one of these alterations is the externalization of phosphatidylserine (PS) [19]. Furthermore, mitochondrial dysfunction has been shown to participate and perhaps be central to the apoptotic pathway. Mitochondrial disintegration not only leads to a depolarization of the transmembrane potential ($\Delta\Psi$m) but also causes the release of proapoptotic factors. In some apoptotic systems, loss of $\Delta\Psi$m may be an earlier event in the apoptotic process [20]. According to our findings, capsaicin does not induce change in plasma membrane structure or disruption

of mitochondrial membrane potential. An alternative cellular pathway for capsaicin to induce growth arrest in GRX cells could be a cell cycle arrest. Indeed, capsaicin induces the cell arrest at the stage G0/G1, supporting cells that are in G0 phase account for the major part of non-growing and non-proliferative cells while cells in G1 precede S phase for DNA replication [21].

HSCs are important source of cytokines in the liver resulted from autocrine and paracrine signaling [22]. Increased production and activity of cytokines are essential for maintenance of HSC activation and liver fibrogenesis [23]. Innate immune mechanisms are important to defense from microorganism but a secondary consequence of this response may also affect liver fibrogenesis [1]. Recent evidence suggests that some innate immunity components also play a role in regulating HSC activation [9]. IL-12 is a cytokine that coordinates innate and adaptative response and its major action is the induction of other cytokines, specially IFN-γ [24]. IFN-γ is a proinflammatory cytokine that is capable to reduce HSC activation, cell growth and differentiation. IFN-γ antifibrotic effects in the liver are via impairment of TGF-β signaling, including the blockage of proliferation and induction of apoptosis [9, 25]. In the current investigation, we demonstrated that capsaicin increases production of IL-12 and consequently, IFN-γ. The last result is in accordance with our previous work that shows a decrease in TGF-β secretion by HSCs exposed to capsaicin for 10 days [6]. These findings suggest that capsaicin inhibits TGF-β synthesis via up-regulation of IFN-γ.

The anti-inflammatory IL-10 that downregulates the immune response is also synthesized by activated HSCs. As a key factor for reducing perpetuation of fibrogenesis, it suppresses inflammation through several mechanisms,

including the reduction of proinflammatory cytokines [26]. In this study we showed that IL-10 levels increased supporting the inhibitory effect of IL-10 on fibrogenesis.

In another interaction, the activation of HSCs may be provoked by activated Kupffer cells that lead to increased NF-κB activity and subsequent secretion of proinflammatory cytokines such as TNF-α and MCP-1. HSCs in turn, respond to this stimulation secreting MCP-1 and then amplifying acute phase response [27]. MCP-1 is a chemoattractant implicated in direct migration of activated HSCs to the site of injury. However, it is not produced by quiescent cells [28]. Recent data indicate that MCP-1 expression can be down-regulated by agonists of PPARγ [29]. Capsaicin-treated HSCs had their secretion of MCP-1 profoundly decreased (~90%) suggesting that after 10 days MCP-1 is unable to induce GRX cell migration.

In conclusion, the present study showed that capsaicin inhibits HSC proliferation by inducing cell cycle arrest. Furthermore, it up-regulates antifibrotic cytokines and inhibits cell chemotaxis, demonstrating that capsaicin may suppress HSC activation via antiproliferative and antifibrotic effects.

5. ACKNOWLEDGEMENT

6. REFERENCES

[1]   Hernandez-Gea V, Friedman SL. Pathogenesis of liver fibrosis. Annu Rev Pathol 2011;6:425-56.

[2]   Kershenobich Stalnikowitz D, Weissbrod AB. Liver fibrosis and inflammation. A review. Ann Hepatol 2003;2:159-63.

[3]   Day SA, Lakner AM, Moore CC, Yen MH, Clemens MG, Wu ES, et al. Opioid-like compound exerts anti-fibrotic activity via decreased hepatic stellate cell activation and inflammation. Biochem Pharmacol 2011;81:996-1003.

[4]   Solís-Herruzo J, Solís-Muñoz P, Muñoz-Yagüe T, García-Ruiz I. Molecular targets in the design of antifibrotic therapy in chronic liver disease. Rev Esp Enferm Dig 2011;103:310-23.

[5]   Kim CS, Park WH, Park JY, Kang JH, Kim MO, Kawada T, et al. Capsaicin, a spicy component of hot pepper, induces apoptosis by activation of the peroxisome proliferator-activated receptor gamma in HT-29 human colon cancer cells. J Med Food 2004;7:267-73.

[6]   Bitencourt S, Mesquita F, Caberlon E, da Silva G, Basso B, Ferreira G, et al. Capsaicin induces de-differentiation of activated hepatic stellate cell Biochem Cell Biol 2012;in press.

[7]   Cossarizza A, Baccarani-Contri M, Kalashnikova G, Franceschi C. A new method for the cytofluorimetric analysis of mitochondrial membrane potential using the J-aggregate forming lipophilic cation 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1). Biochem Biophys Res Commun 1993;197:40-5.

[8]   Reers M, Smiley ST, Mottola-Hartshorn C, Chen A, Lin M, Chen LB. Mitochondrial membrane potential monitored by JC-1 dye. Methods Enzymol 1995;260:406-17.

[9]   Kong X, Horiguchi N, Mori M, Gao B. Cytokines and STATs in Liver Fibrosis. Front Physiol 2012;3:69.

[10]   Borojevic R, Guaragna RM, Margis R, Dutra HS. In vitro induction of the fat-storing phenotype in a liver connective tissue cell line-GRX. In Vitro Cell Dev Biol 1990;26:361-8.

[11]   Souza IC, Martins LA, Coelho BP, Grivicich I, Guaragna RM, Gottfried C, et al. Resveratrol inhibits cell growth by inducing cell cycle arrest in activated hepatic stellate cells. Mol Cell Biochem 2008;315:1-7.

[12]   Teodoro AJ, Perrone D, Martucci RB, Borojevic R. Lycopene isomerisation and storage in an in vitro model of murine hepatic stellate cells. Eur J Nutr 2009;48:261-8.

[13]   Braganca de Moraes CM, Melo DA, Santos RC, Bitencourt S, Mesquita FC, Santos de Oliveira FD, et al. Antiproliferative effect of catechin in GRX cells. Biochem Cell Biol 2012;90:575-84.

[14]   Gupta SC, Kim JH, Prasad S, Aggarwal BB. Regulation of survival, proliferation, invasion, angiogenesis, and metastasis of tumor cells through modulation of inflammatory pathways by nutraceuticals. Cancer Metastasis Rev 2010;29:405-34.

[15] Surh YJ. Anti-tumor promoting potential of selected spice ingredients with antioxidative and anti-inflammatory activities: a short review. Food Chem Toxicol 2002;40:1091-7.

[16] Takahata K, Chen X, Monobe K, Tada M. Growth inhibition of capsaicin on HeLa cells is not mediated by intracellular calcium mobilization. Life Sci 1999;64:PL165-71.

[17] Hsu CL, Yen GC. Effects of capsaicin on induction of apoptosis and inhibition of adipogenesis in 3T3-L1 cells. J Agric Food Chem 2007;55:1730-6.

[18] Zhang F, Kong D, Lu Y, Zheng S. Peroxisome proliferator-activated receptor-gamma as a therapeutic target for hepatic fibrosis: from bench to bedside. Cell Mol Life Sci 2012.

[19] van Engeland M, Nieland LJ, Ramaekers FC, Schutte B, Reutelingsperger CP. Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. Cytometry 1998;31:1-9.

[20] Ly JD, Grubb DR, Lawen A. The mitochondrial membrane potential (deltapsi(m)) in apoptosis; an update. Apoptosis 2003;8:115-28.

[21] Vermeulen K, Van Bockstaele DR, Berneman ZN. The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. Cell Prolif 2003;36:131-49.

[22] Friedman SL. Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. Physiol Rev 2008;88:125-72.

[23] Friedman SL. Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. J Biol Chem 2000;275:2247-50.

[24] Watford WT, Moriguchi M, Morinobu A, O'Shea JJ. The biology of IL-12: coordinating innate and adaptive immune responses. Cytokine Growth Factor Rev 2003;14:361-8.

[25] Saile B, Eisenbach C, Dudas J, El-Armouche H, Ramadori G. Interferon-gamma acts proapoptotic on hepatic stellate cells (HSC) and abrogates the antiapoptotic effect of interferon-alpha by an HSP70-dependant pathway. Eur J Cell Biol 2004;83:469-76.

[26] Iimuro Y, Brenner DA. Matrix metalloproteinase gene delivery for liver fibrosis. Pharm Res 2008;25:249-58.

[27] Lee UE, Friedman SL. Mechanisms of hepatic fibrogenesis. Best Pract Res Clin Gastroenterol 2011;25:195-206.

[28] Li JT, Liao ZX, Ping J, Xu D, Wang H. Molecular mechanism of hepatic stellate cell activation and antifibrotic therapeutic strategies. J Gastroenterol 2008;43:419-28.

[29] Marra F. Chemokines in liver inflammation and fibrosis. Front Biosci 2002;7:d1899-914.

7. FIGURE CAPTIONS

FIGURE 1 – Effect of capsaicin on GRX cells viability. Cells were treated with capsaicin (100 µM) for 24 h and cell viability assessed by direct cell count. Data represent the mean ± SD (n = 3). Results were expressed as cell number. * $P <$ 0.05 as compared with control. CPS, capsaicin.

FIGURE 2 – Flow cytometric scatter plot of FITC-annexin V/PI stained GRX (A) control cells and (B) capsaicin-treated cells for 24 h. The lower left quadrant show the viable cells, which are negative for annexin V and PI. The lower right quadrant represents the apoptotic cells, annexin V positive and PI negative. The upper right quadrant contains the late apoptotic or dead cells that are positive for annexin V and PI. Representative experiment out of three.

FIGURE 3 – Flow cytometric scatter plot of the capsaicin impact on ΔΨm. GRX cells non-treated (A) or treated with capsaicin (B) for 24 h. The upper quadrant represents the polarized cells and the lower quadrant the depolarized cells. Representative experiment out of three.

FIGURE 4 – Effect of 24-hour capsaicin treatment on the GRX cell cycle measured by flow cytometry. Data represent the mean ± SD (n = 3). Results

were expressed as percentage of cell number. * $P < 0.05$ as compared with control. CPS, capsaicin.

FIGURE 5 – Flow cytometric analyses of IL-12 (p70) (A), IFN-γ (B), IL-10 (C) and MCP-1 (D) in cell supernatant of 24-hour and 10-day treatments. Data represent the mean ± SD (n = 4). Cytokines levels were expressed as picograms per 1 x 10^5 cells. * $P < 0.05$, ** $P < 0.01$ as compared with control.
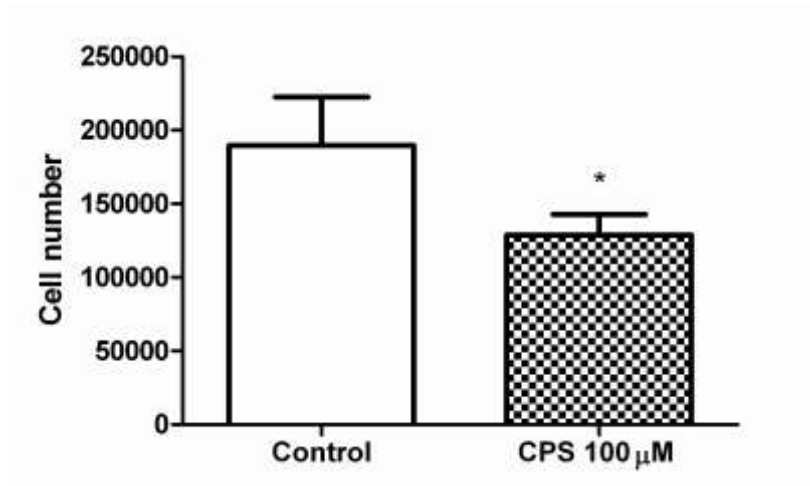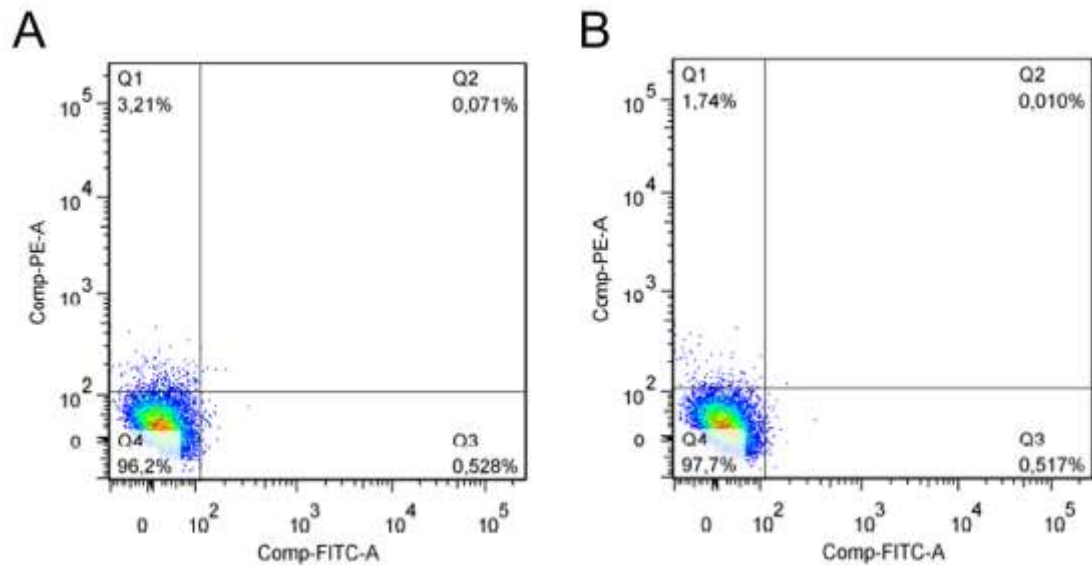
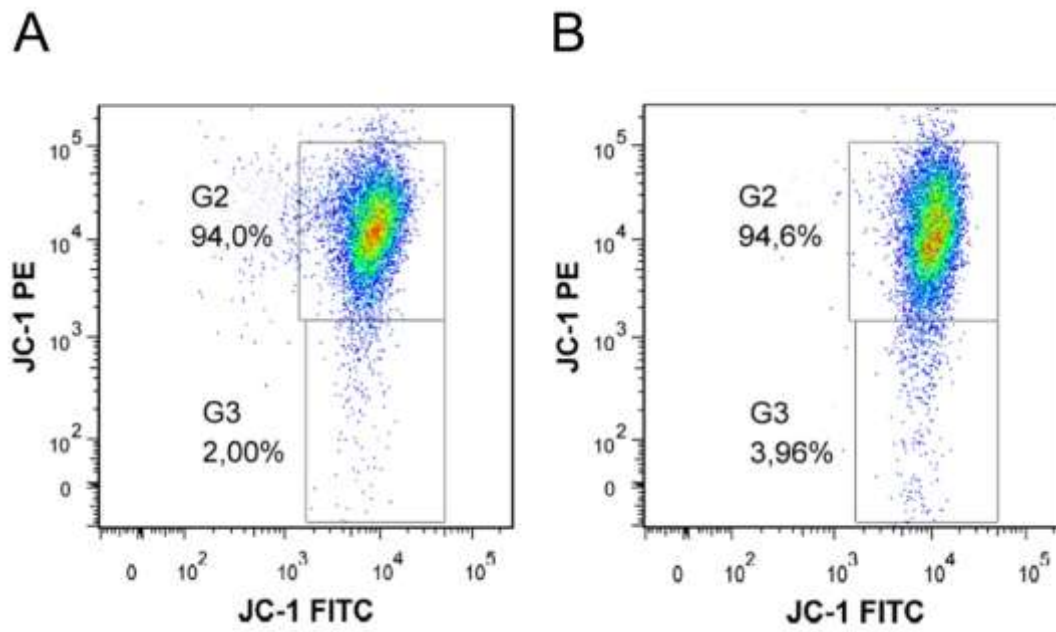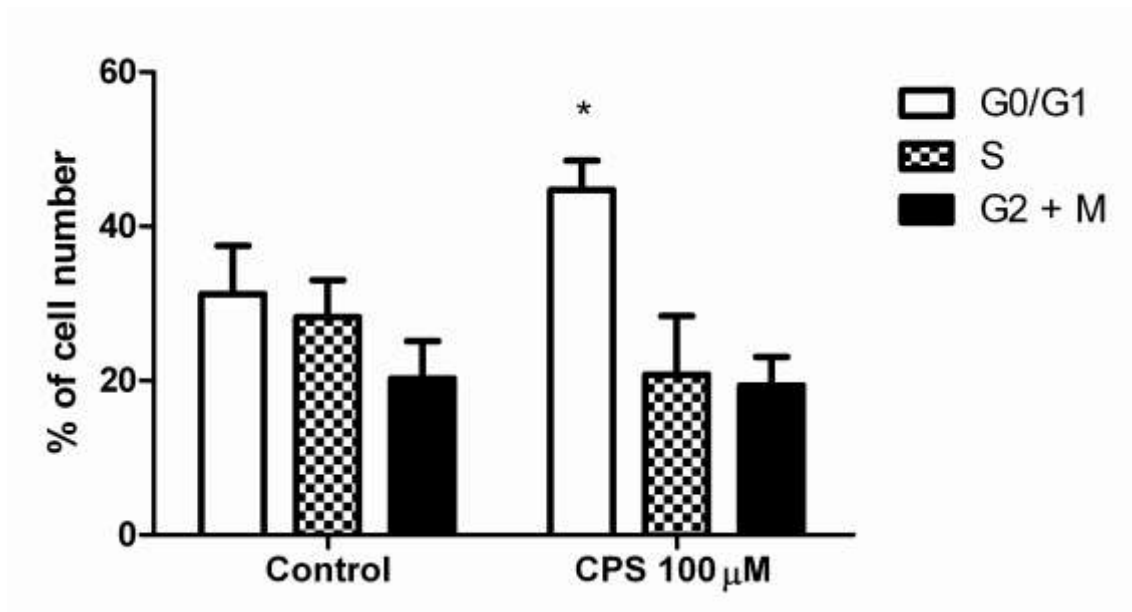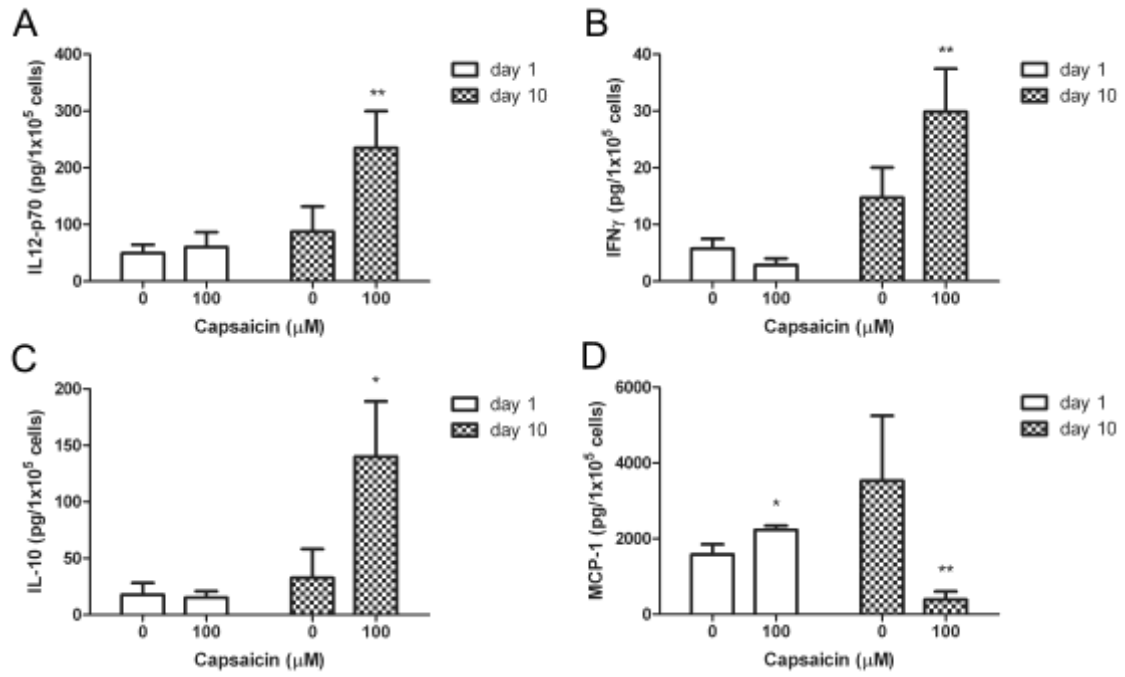FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4

FIGURE 5

CAPÍTULO 4

1. CONSIDERAÇÕES FINAIS
2. CONCLUSÕES

1 CONSIDERAÇÕES FINAIS

O entendimento da fisiologia das células estreladas hepáticas (HSCs) é de extrema importância para o desenho de estratégias terapêuticas no intuito de reduzir a fibrose hepática. Abordagens que inibam a ativação e proliferação das HSCs que visem diferentes alvos talvez sejam mais vantajosas e efetivas que drogas direcionadas a apenas um alvo na cascata fibrogênica (12). Alguns produtos naturais possuem múltiplas ações sobre as HSCs. Devido ao alto consumo e a fácil disponibilidade desses fitoterápicos, muitos estudos têm sido realizados (33, 42). Dessa forma, o crescente interesse nos alimentos funcionais e nutracêuticos, aliado ao alto consumo de pimenta em nossa região sustentam a importância deste trabalho.

Considerando as ações da capsaicina descritas em outras linhagens celulares, este trabalho teve como objetivo verificar os efeitos dessa molécula sobre a linhagem GRX, modelo murino de HSC, visando estudar seus efeitos hepatoprotetores. Os objetivos principais foram: verificar o efeito da capsaicina na inativação e diferenciação das HSCs e estudar sua ação sobre a proliferação celular.

O presente estudo baseou-se em evidencias de pesquisas anteriores realizadas em nosso laboratório com o suco total e frações da pimenta dedo-de-moça (*Capsicum baccatum*), uma variedade de pimenta vermelha (43). Nosso trabalho confirmou a atividade antifibrótica e antiprolifertiva do princípio ativo da pimenta.

Na primeira parte da pesquisa, buscamos as concentrações não citotóxicas da capsaicina. Utilizando tratamentos nas doses de 10 µM, 50 µM, 100 µM, 200 µM e 400 µM, vimos que essa molécula apresentava citotoxicidade nas doses de 200 µM e 400 µM (dados não apresentados). A partir daí, seguimos com as doses não-citotóxicas. Como exposto no Capítulo 1, essas doses apresentaram efeito inibitório sobre a proliferação celular, além de induzir a reversão fenotípica das células GRX. A reversão pôde ser evidenciada através da visualização ao microscópio e confirmada pela quantificação de lipídios. Além disso, a expressão de mRNA de PPARγ apresentou um aumento considerável nas células tratadas, mostrando que a rota lipogênica é ativada pela capsaicina. O PPARγ quando ligante-ativado possui um papel fundamental na reversão da fibrose hepática devido a sua sinalização multidirecional (16) e o fato da capsaicina possuir ação sobre esse fator de transcrição tornou esse trabalho ainda mais atraente.

Outro aspecto interessante a ser considerado é o fato da ação antiproliferativa da capsaicina não estar ligada à apoptose. De acordo com estudos anteriores que revelam a ação apoptótica da capsaicina em células tumorais, nós acreditávamos que o mesmo pudesse ocorrer nas células GRX. Porém, os resultados apresentados no Capítulo 2 mostraram que o princípio ativo da pimenta atua na parada do ciclo celular, impedindo o crescimento das células. A capsaicina interfere exatamente no ponto do ciclo em que ocorrem paradas estratégicas antes da replicação de DNA. A primeira parada é chamada de G1 e está relacionada ao momento em que as células estão se preparando para a síntese de DNA. A segunda pode ocorrer quando as células que estão em G1 entram em um estado de repouso conhecido como G0.

Curiosamente, as células que estão nessa fase do ciclo representam a maior parte das células não-proliferativas (44).

Sabemos que o estado inflamatório das HSCs é um fator importante para o início e manutenção da fibrogênese e ele está associado às múltiplas interações existentes entre as citocinas e a biologia das HSCs. A amplificação da resposta inflamatória local pelas HSCs e os efeitos pro-fibrogênicos diretos das citocinas podem estabelecer um círculo vicioso que leva ao dano crônico e ao reparo (45). Por isso, decidimos aprofundar o conhecimento da ação da capsaicina sobre as citocinas. Avaliamos a liberação de algumas citocinas pró e anti-inflamatórias (IL-12, IFN-γ, MCP-1 e IL-10) pelas células GRX durante dois períodos de exposição, um curto (24 h) e outro prolongado (10 dias). Nossos resultados mostraram que a capsaicina aumenta significativamente a produção de citocinas com ação antifibrótica, evidenciando que este efeito pode estar relacionado com a desativação e inibição do crescimento celular. Um ponto interessante desses resultados é a importante diminuição dos níveis de MCP-1, que além de sugerir uma diminuição da migração das GRX, sugere que as células sofrem uma reversão fenotípica, já que essa quimiocina não é sintetizada pelas HSCs em estado quiescente (24).

Podemos finalizar este estudo dizendo que a capsaicina inicia o processo de desativação das HSCs primeiramente com a inibição da proliferação através da parada do ciclo celular. Depois, inibindo a secreção de agentes pró-fibróticos e ativando mediadores anti-inflamatórios e antifibróticos. Ao final, a capsaicina provoca a reversão fenotípica das células.

Um problema existente no desenvolvimento da terapêutica ideal é a dificuldade de encontrar um agente que seja altamente seletivo às HSCs (12).

A capsaicina, após todos os resultados apresentados neste trabalho, mostra-se como uma possível candidata. Cabe salientar que mais estudos devem ser realizados para aprofundar o conhecimento das propriedades antiproliferativas e antifibróticas da capsaicina. Isso se torna pertinente para o desenvolvimento de novos agentes no combate às doenças crônicas de fígado.

2   CONCLUSÕES

a) Nosso estudo mostra uma atividade antifibrótica e antiproliferativa da capsaicina em células GRX, linhagem utilizada como modelo *in vitro* de fibrose hepática.

b) A capsaicina altera o fenótipo das GRX, levando-as novamente ao seu estado quiescente. Essa mudança ocorre basicamente devido as ações antifibróticas da molécula.

c) A capsaicina reduz a fibrogênese através da inibição da síntese de TGF-β e consequente diminuição da expressão de colágeno tipo I e da secreção de colágeno total.

d) O efeito antifibrótico da capsaicina está relacionado à sua ação PPAR-ligante.

e) A capsaicina diminui o crescimento das células GRX, porém a diminuição não se dá através da apoptose e sim pela parada do ciclo celular.

f) A capsaicina aumenta a produção de citocinas antifibróticas e anti-inflamatórias.

g) A capsaicina inibe a migração das células GRX através da diminuição da secreção de MCP-1.

REFERÊNCIAS

1. Hernandez-Gea V, Friedman SL. Pathogenesis of liver fibrosis. Annu Rev Pathol. 2011 Feb 28;6:425-56.

2. Gressner AM. The cell biology of liver fibrogenesis - an imbalance of proliferation, growth arrest and apoptosis of myofibroblasts. Cell Tissue Res. 1998 Jun;292(3):447-52.

3. Mormone E, George J, Nieto N. Molecular pathogenesis of hepatic fibrosis and current therapeutic approaches. Chem Biol Interact. 2011 Sep 30;193(3):225-31.

4. Friedman SL. Hepatic fibrosis -- overview. Toxicology. 2008 Dec 30;254(3):120-9.

5. Starr SP, Raines D. Cirrhosis: diagnosis, management, and prevention. Am Fam Physician. 2011 Dec 15;84(12):1353-9.

6. Kisseleva T, Brenner DA. Fibrogenesis of parenchymal organs. Proc Am Thorac Soc. 2008 Apr 15;5(3):338-42.

7. Gressner OA, Weiskirchen R, Gressner AM. Biomarkers of hepatic fibrosis, fibrogenesis and genetic pre-disposition pending between fiction and reality. J Cell Mol Med. 2007 Sep-Oct;11(5):1031-51.

8. Shirakami Y, Lee SA, Clugston RD, Blaner WS. Hepatic metabolism of retinoids and disease associations. Biochim Biophys Acta. 2012 Jan;1821(1):124-36.

9. Iimuro Y, Brenner DA. Matrix metalloproteinase gene delivery for liver fibrosis. Pharm Res. 2008 Feb;25(2):249-58.

10. Li JT, Liao ZX, Ping J, Xu D, Wang H. Molecular mechanism of hepatic stellate cell activation and antifibrotic therapeutic strategies. J Gastroenterol. 2008;43(6):419-28.

11. Lee UE, Friedman SL. Mechanisms of hepatic fibrogenesis. Best Pract Res Clin Gastroenterol. 2011 Apr;25(2):195-206.

12. Wu J, Zern MA. Hepatic stellate cells: a target for the treatment of liver fibrosis. J Gastroenterol. 2000;35(9):665-72.

13.   Elsharkawy AM, Oakley F, Mann DA. The role and regulation of hepatic stellate cell apoptosis in reversal of liver fibrosis. Apoptosis. 2005 Oct;10(5):927-39.

14.   Elias-Miro M, Jimenez-Castro MB, Mendes-Braz M, Casillas-Ramirez A, Peralta C. The Current Knowledge of the Role of PPAR in Hepatic Ischemia-Reperfusion Injury. PPAR Res. 2012;2012:802384.

15.   Fajas L, Debril MB, Auwerx J. Peroxisome proliferator-activated receptor-gamma: from adipogenesis to carcinogenesis. J Mol Endocrinol. 2001 Aug;27(1):1-9.

16.   Zhang F, Lu Y, Zheng S. Peroxisome proliferator-activated receptor-gamma cross-regulation of signaling events implicated in liver fibrogenesis. Cell Signal. 2012 Mar;24(3):596-605.

17.   Tsukamoto H, Zhu NL, Wang J, Asahina K, Machida K. Morphogens and hepatic stellate cell fate regulation in chronic liver disease. J Gastroenterol Hepatol. 2012 Mar;27 Suppl 2:94-8.

18.   Tyagi S, Gupta P, Saini AS, Kaushal C, Sharma S. The peroxisome proliferator-activated receptor: A family of nuclear receptors role in various diseases. J Adv Pharm Technol Res. 2011 Oct;2(4):236-40.

19.   Guimaraes EL, Franceschi MF, Andrade CM, Guaragna RM, Borojevic R, Margis R, et al. Hepatic stellate cell line modulates lipogenic transcription factors. Liver Int. 2007 Nov;27(9):1255-64.

20.   Sauvant P, Cansell M, Atgie C. Vitamin A and lipid metabolism: relationship between hepatic stellate cells (HSCs) and adipocytes. J Physiol Biochem. 2011 May 31.

21.   Friedman SL. Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. Physiol Rev. 2008 Jan;88(1):125-72.

22.   Gressner AM, Weiskirchen R, Breitkopf K, Dooley S. Roles of TGF-beta in hepatic fibrosis. Front Biosci. 2002 Apr 1;7:d793-807.

23.   Saile B, Eisenbach C, Dudas J, El-Armouche H, Ramadori G. Interferon-gamma acts proapoptotic on hepatic stellate cells (HSC) and abrogates the antiapoptotic effect of interferon-alpha by an HSP70-dependant pathway. Eur J Cell Biol. 2004 Sep;83(9):469-76.

24.   Marra F, Romanelli RG, Giannini C, Failli P, Pastacaldi S, Arrighi MC, et al. Monocyte chemotactic protein-1 as a chemoattractant for human hepatic stellate cells. Hepatology. 1999 Jan;29(1):140-8.

25.   Bataller R, Brenner DA. Liver fibrosis. J Clin Invest. 2005 Feb;115(2):209-18.

26.   Fallowfield JA, Kendall TJ, Iredale JP. Reversal of fibrosis: no longer a pipe dream? Clin Liver Dis. 2006 Aug;10(3):481-97, viii.

27.   Solís-Herruzo J, Solís-Muñoz P, Muñoz-Yagüe T, García-Ruiz I. Molecular targets in the design of antifibrotic therapy in chronic liver disease. Rev Esp Enferm Dig. 2011;103(6):310-23.

28.   Zhang F, Kong D, Lu Y, Zheng S. Peroxisome proliferator-activated receptor-gamma as a therapeutic target for hepatic fibrosis: from bench to bedside. Cell Mol Life Sci. 2012 Jun 15.

29.   Braganca de Moraes CM, Melo DA, Santos RC, Bitencourt S, Mesquita FC, Santos de Oliveira FD, et al. Antiproliferative effect of catechin in GRX cells. Biochem Cell Biol. 2012 May 10.

30.   Kim HK, Yang TH, Cho HY. Antifibrotic effects of green tea on in vitro and in vivo models of liver fibrosis. World J Gastroenterol. 2009 Nov 7;15(41):5200-5.

31.   Shin E, Lee C, Sung SH, Kim YC, Hwang BY, Lee MK. Antifibrotic activity of coumarins from Cnidium monnieri fruits in HSC-T6 hepatic stellate cells. J Nat Med. 2011 Apr;65(2):370-4.

32.   Szuster-Ciesielska A, Plewka K, Daniluk J, Kandefer-Szerszen M. Betulin and betulinic acid attenuate ethanol-induced liver stellate cell activation by inhibiting reactive oxygen species (ROS), cytokine (TNF-alpha, TGF-beta) production and by influencing intracellular signaling. Toxicology. 2011 Feb 27;280(3):152-63.

33.   Al Mofleh IA. Spices, herbal xenobiotics and the stomach: friends or foes? World J Gastroenterol. 2010 Jun 14;16(22):2710-9.

34.   Bode AM, Dong Z. The two faces of capsaicin. Cancer Res. 2011 Apr 15;71(8):2809-14.

35.   Reyes-Escogido Mde L, Gonzalez-Mondragon EG, Vazquez-Tzompantzi E. Chemical and pharmacological aspects of capsaicin. Molecules. 2011;16(2):1253-70.

36.   Surh YJ, Lee SS. Capsaicin, a double-edged sword: toxicity, metabolism, and chemopreventive potential. Life Sci. 1995;56(22):1845-55.

37.   Reifschneider F. Capsicum: pimentas e pimentões no Brasil. Brasília: Embrapa comunicação para transferência de tecnologia/Embrapa hortaliças; 2000.

38.   Shin DH, Kim OH, Jun HS, Kang MK. Inhibitory effect of capsaicin on B16-F10 melanoma cell migration via the phosphatidylinositol 3-kinase/Akt/Rac1 signal pathway. Exp Mol Med. 2008 Oct 31;40(5):486-94.

39.   Takahata K, Chen X, Monobe K, Tada M. Growth inhibition of capsaicin on HeLa cells is not mediated by intracellular calcium mobilization. Life Sci. 1999;64(13):PL165-71.

40.   Kim CS, Park WH, Park JY, Kang JH, Kim MO, Kawada T, et al. Capsaicin, a spicy component of hot pepper, induces apoptosis by activation of the peroxisome proliferator-activated receptor gamma in HT-29 human colon cancer cells. J Med Food. 2004 Fall;7(3):267-73.

41.   Park JY, Kawada T, Han IS, Kim BS, Goto T, Takahashi N, et al. Capsaicin inhibits the production of tumor necrosis factor alpha by LPS-stimulated murine macrophages, RAW 264.7: a PPARgamma ligand-like action as a novel mechanism. FEBS Lett. 2004 Aug 13;572(1-3):266-70.

42.   Chen XW, Serag ES, Sneed KB, Zhou SF. Herbal bioactivation, molecular targets and the toxicity relevance. Chem Biol Interact. 2011 Jul 15;192(3):161-76.

43.   Scherer BS. Avaliação dos efeitos antifibróticos do suco total e frações da pimenta Capsicum baccatum na linhagem celular GRX. Porto Alegre: Pontifícia Universidade do Rio Grande do Sul; 2010.

44.   Vermeulen K, Van Bockstaele DR, Berneman ZN. The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. Cell Prolif. 2003 Jun;36(3):131-49.

45.   Marra F. Chemokines in liver inflammation and fibrosis. Front Biosci. 2002 Sep 1;7:d1899-914.

ANEXO

CARTA DE SUBMISSÃO
Biochemical Pharmacology

Ref.: Ms. No. BCP-D-12-00842
EFFECTS OF CAPSAICIN ON PROLIFERATION AND CYTOKINE RELEASE OF ACTIVATED
HEPATIC STELLATE CELLS

Dear Dr. Oliveira,

The above-titled submission has been assigned to
the Editorial Office as the editor responsible for the peer review process.

Feel free to contact the editorial office if you have questions or concerns. We appreciate you
choosing Biochemical Pharmacology.

Sincerely,

Lynn LeCount, CMA
Managing Editor
Biochemical Pharmacology Editorial Office
University of Kansas Medical Center
3901 Rainbow Boulevard, Mail Stop 4016
Kansas City, Kansas 66160 USA
Tel: +1-913-588-7533
Fax: +1-913-588-7373
E-mail: BP@kumc.edu

Biochemical Pharmacology