UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

Efeitos do resveratrol sobre as células estreladas hepáticas.

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Orientador: Prof[a]. Dr[a]. Fátima T. Costa Rodrigues Guma

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas - Bioquímica, como requisito parcial à obtenção do grau de Doutor em Bioquímica.

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“A alegria está na luta, na tentativa, no sofrimento envolvido e não na vitória propriamente dita”.

Mahatma Gandhi

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APRESENTAÇÃO

Esta tese está apresentada da seguinte maneira:

PARTE I

Introdução contendo referências da literatura que fundamentam este trabalho.

Objetivos gerais e específicos do presente trabalho.

PARTE II

Capítulo I: Artigo publicado no periódico *Molecular and Celular Biochemistr.*

Capítulo II: Manuscrito a ser submetido ao periódico *Liver International.*

Capítulo III: Resultados complementares

PARTE III

Discussão contendo a interpretação dos resultados apresentados na presente tese.

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PARTE I

I.1 RESUMO

As células estreladas hepáticas (HSC) são pericitos específicos do fígado, são células armazenadoras de gordura e produzem componentes de matriz extracelular. As HSCs ao serem ativadas, proliferam, perdem as gotas lipídicas e aumentam a secreção de matriz extracelular. O controle da modulação fenotípica e da proliferação das HSCs é de suma importância para entendermos a fibrose hepática. Esta pesquisa foi realizada com a linhagem celular GRX (representativa das HSCs murinas) obtida a partir de reações fibrogranulomatosas inflamatórias. Estudos anteriores, em nosso laboratório, pesquisaram a ação de diferentes drogas sobre a modulação destas células, tais como: retinol, pentoxifilina, indometacina e insulina. Tem sido relatado que o resveratrol (3,4,5 trihidroxiestilbeno) (RSV) tem ação antioxidante, antiinflamatória, antiproliferativa bem como tem capacidade de ativar ou inibir a transcrição de enzimas envolvidas na regulação de vários eventos celulares, por exemplo metabolismo de lipídeos. Por isso, procuramos utilizar o RSV, um polifenol natural presente na casca das uvas e conseqüentemente em grande quantidade de vinhos tintos, neste modelo celular. As células GRX foram cultivadas (12, 24 e 120 h) em meio DMEM com 5% SFB acrescido ou não de RSV (100 nM, 1µM ou 100 µM) e retinol (RHO) (5µM). Nossos estudos demonstraram que tratamento por 120 h com RSV (100nM e 1µM) inibiu a proliferação das células GRX diminuindo em 35% o número de células. Analisando o efeito de 1µM de RSV sobre o ciclo celular e a apoptose, verificamos que após 120 h de tratamento houve um aumento das células na fase S e Sub-G1. Constatou-se condensação e fragmentação nuclear, sugerindo um possível efeito pró-apoptótico do RSV sobre a célula GRX. Observou-se que o tratamento com RSV (100 nM) por 5 dias não induziu a formação de gotas lipídicas nas células GRX. Porém ao acrescentar RSV+RHO, constatou-se que o RSV não interferiu na síntese e acúmulo de lipídios, provocado pelo RHO (5µM). Propondo que a lipogênese neste modelo de tratamento possa estar associada com a ação da sirtuina (deacetilase NAD-dependente) e do PPARγ (receptores ativados por proliferadores de peroxissomos), determinou-se a expressão do mRNA destas duas proteínas. Observou-se que o RSV (100 nM) aumenta a expressão do mRNA da sirtuina 1 (SIRT1) e diminui a expressão de PPARγ. Porém, o tratamento com RSV+RHO provocou uma redução na expressão da SIRT1, alterando a relação PPARγ/SIRT1, promovendo a síntese de lipídios. Nossos dados mostraram que após 24h de tratamento, o RSV não alterou a incorporação de acetato [C^{14}] em triacilglicerídios (TG), enquanto que, o RHO ou RSV+RHO aumentaram a incorporação do marcador nestes lipídios. O depósito de TG permaneceu constante, porém a síntese diminuiu com o tempo de tratamento. Acredita-se que estes compostos atuam por mecanismos moleculares diferentes modulando o metabolismo de lipídios. Sugerimos, de acordo dados da literatura, que o RHO atua via receptor nuclear LXR formando um heterodímero com PPARγ, favorecendo a lipogênese. Por outro lado, o RSV ativa a SIRT1, que reprime o PGC1α (coativador do PPARγ). Logo, a inibição do PPARγ pelo RSV inibe consequentemente a lipogênese. Concluímos que o resveratrol apresenta um efeito anti-proliferativo, pró-apoptótico sem induzir o fenótipo lipocítico nas células GRX. Desta forma, se mantém em equilíbrio, no espaço de Disse, as células mesenquimais quiescentes e ativadas, contribuindo para restabelecer a homeostase hepática.

I.2 ABSTRACT

The hepatic stellate cells (HSC) are liver-specific pericytes, as well as fat-storing cells and they are also responsible for the extracellular matrix components production. When activated, HSCs lose lipid droplets and increase extracellular matrix secretion. The phenotypical modulation and the HSCs proliferation control have paramount importance to understand the hepatic fibrosis. This research has been carried out with the GRX cell line (HSCs murine representative) which was obtained from inflammatory fibrogranulomatous reactions. Previous studies in our laboratory investigated the action of different drugs on the modulation of these cells, such as: retinol, pentoxifilin, indomethacin and insulin. Resveratrol (RSV) has been referred to as having antioxidant, antiinflammatory and antiproliferative action (3,4,5 trihidroxiestilbeno), as well as the capacity to activate or inhibit the transcription of enzymes involved in the regulation of several cell events, as, for example, lipid metabolism Therefore, we used RSV, which is a natural polyphenol found in the grapes skin and, consequently, in large amounts of red wine, in this cell model. The GRX cells were cultivated (12, 24 and 120 h) in medium DMEM with 5% SFB added RSV or not (100 nM, 1μM or 100 μM) and retinol (RHO) (5μM). Our studies demonstrated that a 120 h treatment with RSV (beginning with 100 nM) inhibited GRX cells proliferation, thus decreasing the cell number in 35%. Assessing the effect of 1μM RSV on the cell cycle and the apoptosis, we found that, after this treatment period, there was an increase of cells in phases S and Sub-G1. Nuclear condensation and fragmentation were observed, which suggested a probable pro-apoptotic RSV effect on the GRX cell. The RSV (100 nM) 120 h treatment did not induce lipid droplets formation in the GRX cells. However, RSV did not interfere in the synthesis or accumulation of lipids caused by RHO (5μM), when RSV+RHO were added. Considering that the lipogenesis in this treatment model might be associated with sirtuin action (deacetilase NAD-dependent) and PPARγ (receptors activated by peroxisome proliferators), the mRNA expression of these two proteins was determined. RSV (100 nM) proved to increase sirtuin mRNA expression 1 (SIRT1) and decrease PPARγ.expression. However, the treatment with the RSV+RHO caused a reduction in the SIRT1 expression thus alterating the relation PPARγ/SIRT1, which promoted the lypid synthesis. Our data showed that after the 24-hour treatment RSV did not change acetate incorporation [C^{14}] in triacylglicerides (TG), whilst RHO or RSV+RHO increased the marker incorporation in these lipids. The TG deposit remained constant, but the synthesis diminished during the treatment period. It appears that these compounds act through different molecular mechanisms when modulating lipids metabolism. According to literature data, we suggest that RHO acts via LXR nuclear receptor, thus forming an heterodímer with PPARγ, which favors lypogenesis. On the other hand, RSV activates SIRT1, which inhibits PGC1α (PPARγ coactivator). Therefore, the PPARγ inhibition by RSV consequently inhibits lypogenesis. We concluded that RSV presents pro-apoptotic, anti-proliferative effect, without inducing lypocytic phenotype in the GRX cells. By this means the mesenquimal activated quiescent cells keep their balance at the Disse space, thus contributing with the hepatic homeostasis restoration.

I.3 LISTA DE ABREVIATURAS

AGL – Ácido Graxo Livre

AH109A – Células de Hepatoma (Ascites)

A431 – Linhagem Celular de Carcinoma Epidermal

AMPK – Proteína Quinase Ativada por AMP

BCL-2 – Proteína Oncogênica Inibidora de Apoptose

CaCo-2 – Linhagem Celular de Carcinoma Epidermal

CDKs – Ciclinas Dependentes de Quinases

CEMC7H2 – Linhagem de Leucemia Linfocítica

C/EBP – Proteína Estimuladora de Ligação a CCAAT

C6 – Células de Glioma

ECM – Matriz Extracelular

ERK1/2 – Proteínas Quinases Reguladas por Sinais Extracelulares

Ero1-Lα – Oxidoredutase de Reticulo Endoplasmático

FAK – Quinase de Adesão Focal

Fox01 – Fator de Transcrição Associado a Supressão de Tumores

GRX – Célula Estrelada Hepática - murina

GW647 – Agonista do PPARα

GW501516 – Agonista do PPARδ/β

HepG2 – Células de Carcinoma Hepatocelular

HSCs – Células Estreladas Hepáticas

HL-60 – Células de Leucemia Promielítica

JB6 – Linhagem Celular de Carcinoma Epidermal

Jurkat – Célula-T Leucêmica

INDO – Indometacina

INK4A – Inibidor de CDKs

INS – Insulina

Ku70 – Fator de Transcrição Associado ao Ciclo Celular

LDL – Lipoproteína de Baixa Densidade

LNCap – Linhagem Celular de Câncer de Próstata

L1210 – Células de Leucemia murina

LXR – Receptores X do fígado (Liver X receptors)

MAPK – Proteína Quinase Ativada por Mitógenos

MCF-7 – Linhagem celular de câncer de mama

MIO – Miofibroblasto

MMP – Metaloproteinases

Molt4 – Células de Leucemia Linfoblástica

NF-κB – Fator Nuclear Kappa -B

p53 – Proteína Supressora de Tumores (via regulação do ciclo celular)

P16 – Proteína envolvida Regulação do Ciclo Celular

PGC-1α – Coativador-1 do PPARγ

PKC – Proteína Quinase C

PPRE – Elemento Responsivo ao PPAR

PTX – Pentoxifilina

RSV – Resveratrol

RHO – Retinol

RR – Ribonucleotídeo Redutase

RXR – Receptor do 9-cis Ácido Retinóico

α- SMA – alfa-actina de músculo Liso

SIRT1 – Sirtuína - 1

SREBP – Proteína 1c Ligadora do Elemento Regulado por Esteróis

3T3-L1 – pré-adipócitos

TG – Triacilglicerídios

TIMP – inibidor de metaloproteínases de matriz tecidual

TZD – Tiazolidinedionas

U937 – Células de Leucemia Monocítica

I.4 INTRODUÇÃO

I.4.1. Célula Estrelada Hepática

A fibrose é a principal fisiopatologia decorrente de uma injúria crônica ao fígado, é um processo comum na insuficiência hepática e é responsável por muitas complicações clínicas no estágio final das doenças deste órgão. Inicialmente representa um processo passivo, pelo qual, a morte celular leva à condensação do estroma preexistente (Hartroft et al., 1951). A fibrose foi redefinida pela World Health Organization em 1978 como "*a presença de excesso colágeno devido formação de novas fibras*" (Anthony et al., 1978). Atualmente, é conhecida como fazendo parte de um processo dinâmico e contínuo de remodelagem de matriz extracelular (ECM) em um quadro de injúria crônica hepática, levando a um excessivo acúmulo de várias proteínas extracelulares, proteoglicanas e carboidratos (Wheeler et al., 2001; Bataller e Brenner, 2005). Embora, o processo já tenha sido parcialmente elucidado, as células estreladas hepáticas (HSCs) tem sido responsabilizadas pela fibrogênese, indepedente de, sua base etiológica (Wheeler et al., 2001; Bridle et al., 2003). O entendimento dos mecanismos moleculares fisiopatológicos que envolvem estas células é fundamental para o desenvolvimento de terapias antifibrogênicas (Moreira, 2007).

As HSCs, conhecidas como células de "Ito", lipocíticas ou perisinusoidais específicas do fígado, encontradas em um estado quiescente no espaço de Disse (entre os hepatócitos e os sinusóides) são armazenadoras de gordura (Wake, 1971). Durante a lesão hepática crônica, as HSCs se diferenciam para um fenótipo miofibroblástico ativado que se caracteriza por uma acentuada

proliferação e secreção de componentes de matriz extracelular (Blonhoff e Wake, 1991; Bissel, 1992) como colágeno do tipo I, II, IV e V, laminina e elastina, com a predominância do colágeno do tipo I (Battaller e Brenner, 2005). A ativação das HSCs é acompanhada pela perda das gotas lipídicas ricas em vitamina A, por uma reorganização e expressão de proteínas de citoesqueleto, como α- actina de músculo liso (α- SMA) e desmina (figura 1) (Kisseleva e Brenner, 2006).

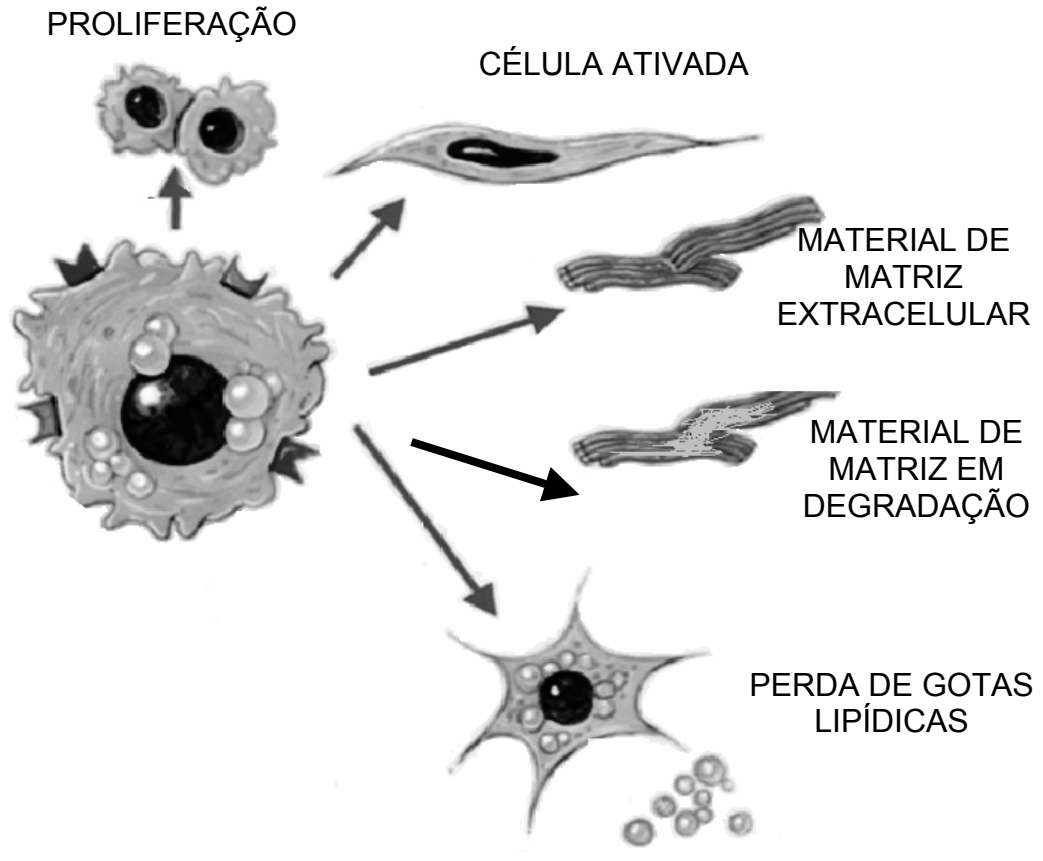


FIGURA 1. Célula Estrelada Hepática em um processo de injúria do fígado. Modificado de Friedman, (2000)

As HSCs participam do reparo e da regeneração da homeostase da matriz extracelular hepática, desta forma, a capacidade das HSCs de expressarem um duplo fenótipo é um aspecto intrigante (Blonhoff e Wake,

1991; Bissel, 1992). Estudos detalhados sobre o fenótipo expresso por células estreladas quiescentes ou ativadas mostraram que estas representam uma população celular heterogênea, em termos de sua função e expressão de proteínas citoesqueléticas (Guma et al., 2001).

Para estudos *in vitro* a linhagem celular GRX parece ser um modelo interessante, apresenta características morfológicas e bioquímicas das culturas primárias do tecido conjuntivo hepático humano (Monteiro e Borojevic,1987). A linhagem GRX é representativa das HSCs murinas, foi obtida a partir de reações fibrogranulomatosas inflamatórias induzidas em fígado de camundongo, após infecção por *Schistosoma mansoni* (Borojevic et al., 1985). Expressa dois fenótipos, o miofibroblástico e o lipocítico (Borojevic et al., 1985) semelhante à célula estrelada hepática *in vivo*. As células com o fenótipo miofibroblástico apresentam morfologia estrelada, poligonal ou alongada, secretam colágeno do tipo I e laminina, apresentam extensivos desmossomos e estão apoiadas à membrana basal. Em condições de cultura padrão, esta linhagem é altamente proliferativa e após atingir confluência, as células se sobrepõem em camadas não uniformes formando “vales e montanhas”, característica de linhagens de músculo liso. Sob determinadas condições diminuem a proliferação e acumulam gotas lipídicas (Borojevic et al., 1985).

Estudos anteriores, em nosso laboratório, pesquisaram a ação de diferentes drogas: sobre as células GRX: retinol (RHO), pentoxifilina (PTX), indometacina (INDO) e insulina (INS). Estas quando tratadas com RHO diminuíram a proliferação e a produção de colágeno, aumentaram a adesão ao substrato e a síntese de fibronectina, mostrando uma reorganização do citoesqueleto com o acúmulo progressivo de gotas lipídicas, correspondendo

ao fenótipo quiescente *in vivo* (Margis e Borojevic, 1989; Borojevic et al., 1990; Guaragna et al., 1991).

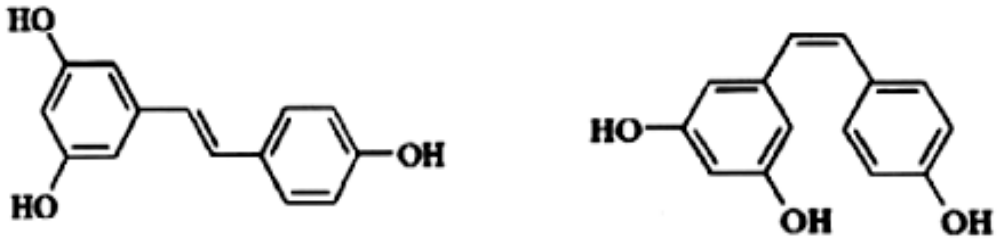
A PTX, potente inibidor da metil-xantina fosdiesterase, testado como antifibrogênico e antioxidante, diminui a proliferação das células GRX, entretanto necessita estar associada ao RHO para induzir a transformação para o fenótipo lipocítico (Cardoso et al., 2003). A indometacina, um inibidor da ciclooxigenase-1, promove a diferenciação do fenótipo miofibroblástico para lipocítico, quando associada ou não a insulina. Esta última, a INS, aumenta muito a proliferação desta linhagem celular, o que impede sua transformação em lipócito. Somente a partir de 20 dias de tratamento com INS se tem depósito intracelular de triacilglicerídios. Estes resultados sugerem uma estreita necessidade de diminuição de proliferação para alterações fenotípicas (Guaragna et al., 1991; Cardoso et al., 2003)**.**

I.4.2 Resveratrol (RSV)

O composto polifenólico resveratrol (3,5,4' triidroxi-trans-estilbeno) é um fitoquímico que ocorre naturalmente e pode ser encontrado em setenta e duas espécies de plantas. Entre as espécies cultivadas para alimento existem as videiras (uvas), amendoeiras (amendoim) e várias ervas (chá verde, chá branco) (Jang et al., 1997). A função fisiológica exata não é conhecida, mas o RSV pode ter um papel de proteção à planta contra infecções por fungo e resistência às doenças (Joe et al., 2002). Este polifenol é sintetizado pela enzima RSV sintase, principalmente na casca da uva, a partir de três moléculas de malonil-CoA e uma de 4-coumaroil-CoA (King et al., 2006).

Baliga et al., 2005, relatam que a concentração de RSV encontra-se em torno de (1,5-3 mg/L) em vinho tinto e (50-100 μg/g em cascas de uvas secas).

Nos chás verde e branco (*Polygonum cuspidatum*) a concentração é de aproximadamente (0,524 mg/g – 1,65 mg/g) provavelmente estas são as principais fontes deste polifenol nas dietas (Baur e Sinclair, 2006). Por outro lado, os níveis de RSV nos vinhos variam devido à composição do solo, exposição ao sol, infecção por fungos e quanto ao processo de fabricação e conservação dos vinhos (Soleas et al., 1997). O RSV é um estilbeno encontrado nas isoformas *trans-resveratrol*, *cis-resveratrol* e ainda como *resveratrol-glicosídeo*, mas sua maior atividade biológica esta relacionada à forma *trans* (figura 2) (Waterhouse, 2002). A biodisponibilidade do RSV, após administração oral, tanto em humanos quanto em animais é bastante baixa. A metabolização do RSV envolve os processos de glucuronidação e sulfatação (Walle et al., 2004). Alguns estudos com animais sugerem que mesmo as pequenas doses biodisponíveis são suficientes para a sua atividade quimiopreventiva (Tessitore et al., 2000; Banerjee et al., 2002; Li et al., 2002).



Trans-resveratrol **Cis-resveratrol**

Figura 2. Estrutura química dos isômeros trans-resveratrol e cis-resveratrol (Signorelli et al., 2005)

Estudos mostram que o RSV livre nas concentrações menores que 2μM, pode ser detectável no sangue de humanos, após 1h da ingestão oral de alimentos e/ou bebidas e que o fígado é um dos maiores sítios de concentração do RSV (Walle et al., 2004; Meng et al., 2004). Os hepatócitos

apresentam um duplo papel, além de ser o maior sítio de acúmulo do RSV, são responsáveis pela sua bio-conjugação e eliminação (Notas et al., 2006).

O RSV é absorvido, metabolizado e em torno de 75% é excretado via fezes e urina (Wenzel et al., 2005). Em particular, no plasma a forma sulfatada é menor que a forma glucuronida, experimentos usando sulfotransferases recombinantes mostraram que esta forma encontra-se predominantemente no intestino (Walle et al., 2004).

A albumina parece ser um dos transportadores plasmático do RSV (Jannin et al., 2004). O transporte de RSV via membrana plasmática ocorre por difusão passiva e facilitada (mediado por carreadores ainda não conhecidos) (Lançon et al., 2004).

A ação antioxidante, anti-inflamatória, antiproliferativa do RSV e sua habilidade em ativar e inibir a transcrição e a atividade de enzimas na regulação de vários eventos celulares, associado à carcinogênese, tem sido alvo de várias pesquisas (Joe et al, 2002).

Estudos mostram que o RSV inibe a agregação plaquetária, podendo ser um dos mecanismos pelo qual o vinho tinto exerce um efeito cardioprotetor (Soleas et al., 1997; Wang et al., 2002). Um possível efeito protetor do *trans-resveratrol* na oxidação da lipoproteína de baixa densidade (LDL), resultando em uma atividade antitrombogênica vascular foi mostrado por Chen et al., (2007).

Lu and Serrero, 1999 mostraram que o RSV age como um antagonista do estrógeno, levando à inibição do crescimento das células MCF-7 (linhagem celular de câncer de mama). Bowers et al., (2000) mostrou que além da ação antagonista, o RSV exerce uma ação agonista ao estrógeno em células MCF-7

dependendo do tipo de receptor e dos elementos responsivos ao estrógeno. O RSV também está associado à reorganização de citoesqueleto, aumentando a formação de filipódios e uma diminuição no número de adesões focais e atividade da FAK (quinase de adesão focal), exercendo um efeito inibitório sobre a migração celular em câncer de mama (Azios et al., 2005).

I.4.2.1 Resveratrol e proliferação celular

As propriedades quimiopreventivas do RSV foram primeiramente, relatadas por Jang e colaboradores em 1997. Que demonstraram a sua ação inibitória nos três maiores estágios da carcinogênese: iniciação, promoção e progressão celular. O RSV reduziu o desenvolvimento de lesões pré-neoplásicas em cultivos *in vitro* de glândulas mamárias de camundongo e inibiu a tumorogênese em modelo de câncer de pele de camundongo (Jang *et al.,* 1997). Vários estudos demonstraram o efeito antiproliferativo do RSV em células epiteliais de mama humana (Mgbonyebi et al., 1998; Subbaramaiah et al., 1999; Pozo-Guisado et al., 2002 e Dubuisson et al., 2002), em células de epiderme de camundongo (JB6) e em linhagem celular de carcinoma epidermal (CaCo-2 e A431) (Huang et al.,1999 e Ahmad et al., 2001).

O RSV, também reduz a viabilidade celular e capacidade de síntese de DNA de células de leucemia promielocítica de humanos (HL-60), induzindo apoptose via BCL-2 (proteína oncogênica, isolada das células-B de linfoma, inibidora da apoptose, localizada principalmente na membrana externa mitocondrial, retículo endoplasmático e envoltório nuclear) (Surh et al., 1999).

Em 2000, Kampa e colaboradores mostraram que muitos dos polifenóis presentes no vinho, incluindo o RSV, inibem a proliferação de linhagens celulares de câncer de próstata em humanos. Em 2002, Narayanan et al.,

sugeriram que a modulação de várias vias de sinalização pelo RSV, estaria envolvida na inibição do crescimento de muitas linhagens celulares de câncer de próstata LNCaP.

Carbo et al. (1999), demonstraram que o RSV diminuiu significativamente a proliferação de células tumorais em ratos Wistar machos, inoculados com ascites do hepatoma (AH-130 Yoshida) e que esta resposta pode estar associada a uma parada no ciclo celular na fase G2/M e a indução de apoptose. Outros estudos mostraram que altas concentrações de RSV (100 e 200 μM) inibem a proliferação e a invasão de células AH109A. Entretanto, uma concentração intermediária (50μM) apresentava um menor efeito anti proliferativo e concentrações na faixa de 25μM somente suprimiam a invasão celular. Os autores sugerem que a atividade anti-invasiva do RSV é independente da atividade antiproliferativa (Kozuki et al., 2001).

Em 2004, Olson e colaboradores, mostraram que o RSV inibe dois estágios cruciais da ativação de fibroblastos cardíacos: proliferação e diferenciação, que são parâmetros fisiológicos determinantes da fibrose cardíaca, diminuindo consideravelmente a produção total de componentes de ECM no miocárdio.

Em 1998, Kawada e colaboradores mostraram que o RSV é um potente inibidor da proliferação das células estreladas hepáticas em cultura. Notas et al. (2006) e Stervbo et al. (2006) demonstraram em células de carcinoma hepatocelular (HepG2) que o tratamento com concentrações variáveis de RSV (10^{-12} - 10^{-6}M), produzia um efeito antiproliferativo, que era dose e tempo dependente. Já, Kirimlioglu e colaboradores (2008) sugeriram que em ratos submetidos à hepatectomia, o RSV pode ser responsável pelo processo de

regeneração, exercendo uma ação antiproliferativa e próapoptotica sobre os hepatócitos.

I.4.2.2 Resveratrol e ciclo celular

O ciclo celular é definido como o intervalo entre duas divisões mitóticas sucessivas resultando na produção de duas células-filhas. O ciclo é dividido em quatro fases: G1, S, G2 (interfase) e fase M (Mitose), caso a progressão celular seja interrompida na fase S, a célula permanece no estado de repouso denominado fase G0. O ciclo celular é controlado pelas ciclinas e pelas proteínas quinases dependentes de ciclinas (CDKs) (Lu et al., 2005).

As células de leucemia promielocítica (HL-60) tratadas com RSV apresentaram uma inibição do crescimento celular, indução de apoptose, parada do ciclo celular nas fases S-G2 e diferenciação mielomonocítica (Clement et al., 1998; Ragione et al., 1998). Outros pesquisadores sugerem que o RSV induz a parada no ciclo celular na fase S por redução na síntese de DNA (Ragione et al., 1998; Ahmad et al., 2001).

Os efeitos na progressão do ciclo celular podem também ser explicados pela inibição direta da atividade da ribonucleotideo redutase (RR) (Fontecave et al., 1998) e da DNA polimerase (Sun et al., 1998). A RR existe em todas as células vivas e catalisa o passo limitante na síntese dos deoxiribonucleotídeos necessários para síntese do DNA. Outros estudos mostraram que o RSV inibe a RR em células L1210 de leucemia murina (Fontecave et al., 1998). A ribonucleotídeo redutase é um alvo promissor na terapia do câncer e doenças causadas por protozoários (Cory 1988; Louie et al., 1999; Melo et al., 2000; Finch et al., 2000). Em fibroblastos normais e células de fibrosarcoma foi

observado que a inibição da síntese de DNA foi induzida diretamente pela interação específica do RSV com a DNA polimerase (Stivala et al., 2001).

A linhagem celular de leucemia linfocítica (CEM-C7H2) apresenta uma deficiência funcional de p53 (proteína responsável pela proliferação celular) e de p16 (proteína do ciclo celular de mamíferos, supressora das atividades de CDKs). Estas células, quando tratadas com RSV sofrem uma parada do ciclo celular na fase S e entram em apoptose. Quando as mesmas células foram transfectadas com p16/INK4A o tratamento com RSV levou a uma parada no ciclo celular em G0/G1 e reduziu significativamente a percentagem de células apoptóticas (Bernhard et al., 2000; Stewart et al., 2003; Ghazizadeh et al., 2005).

Altas concentrações de RSV induzem um aumento significativo do número de células MCF-7 nas fases G0/G1, diminuindo nas demais fases do ciclo celular (Pozo-Guisado et al., 2004). No entanto, em linhagens de câncer de próstata, tratadas com diferentes concentrações de RSV, é observado um aumento moderado do número de células na fase S (Sgambato, 2001), sugerindo que, em linhagens de câncer de próstata, o RSV interrompe a transição da fase G1/S do ciclo celular.

I.4.2.3 Resveratrol e apoptose

Apoptose é a morte celular geneticamente programada. O processo de apoptose caracteriza-se por: 1- retração e condensação celular; 2- perda de adesão intercelular; 3- fragmentação da cromatina; 4- fragmentação celular em corpos apoptóticos. As caspases, enzimas da família de cisteína-aspartato proteases, são fundamentais na iniciação e no desenvolvimento deste processo (Earnshaw et al., 1999).

Em 2002, Roman e colaboradores, mostraram que o RSV induz apoptose envolvendo mecanismos dependentes e independentes de caspases em células-B de leucemia crônica. Em células de glioma (C6), Zhang W e colaboradores (2006) mostraram que o RSV induz apoptose *in vitro*, não ocorrendo o mesmo em fibroblastos 3T3, indicando a caspase-3 como o principal mediador neste processo.

Estudos com uma variedade de linhagens celulares de câncer humano, demonstraram que o RSV é capaz de induzir apoptose dependente de p53 via ERK1/2 mediada por proteínas supressora de oncogênese (Lin et al., 2008).

Linhagens celulares de câncer humano: MCF7 (carcinoma mamário), HepG2 (hepatoblasma), U937 (leucemia monocítica), Molt4 (leucemia linfoblástica) e Jurkat (célula-T leucêmica), apresentaram diferentes graus de indução de apoptose pelo RSV dependendo da via sinalização ativada (Nigro et al., 2007).

Células de carcinoma hepatocelular (HepG2) tratadas com concentrações variáveis de RSV, além do efeito antiproliferativo, são induzidas a apoptose (Stervbo et al., 2006).

Muitos trabalhos mostram o efeito do RSV sobre o ciclo celular e apoptose, por mecanismos que envolvem a modulação de ciclinas, regulação da p16 e p53, rotas mediadas por PKC e dependentes de MAPK (Delmas et al., 2002; Chen et al., 2004; Shih et al., 2004; Zhang P et al., 2006)

I.4.3 Sirtuína (SIRT1)

A SIRT1 (sirtuína 1) é um gene ortólogo de mamífero, homólogo ao Sir2, gene regulador do silenciamento da informação, encontrado em leveduras. É uma deacetilase NAD^+-dependente agindo como um grande sensor metabólico

dos níveis de NAD^+. Modula o metabolismo e a sobrevivência celular, desta forma, está envolvido na senescência e apoptose (Xiuyun Hou et al., 2008). A superexpressão da SIRT1 atenua a adipogênese em células 3T3-L1 (pré-adipócitos), reprimindo a expressão do PPARγ (Picard et al., 2004). Em hepatócitos, o RSV diminui o acúmulo de lipídios, induzidos por alta concentração de glicose. Este efeito protetor do RSV pode estar associado à ativação da SIRT1 e AMPK (proteína quinase ativada por AMP) (Xiuyun Hou et al., 2008).

Em pré-adipócitos 3T3-L1 a ativação da SIRT1 pelo RSV reduz a secreção de adiponectina (proteína secretada pelos adipócitos, que regula a homeostasia dos lipídios e da glicose e potencia a ação da INS a nível hepático) (Qiang et al., 2007). Entretanto, não foi observado efeito sobre a expressão de adipsina (proteína secretada pelo tecido adiposo com função imunológica). Através da inibição genética e farmacológica da SIRT1, Xiuyun Hou e colaboradores (2008) mostraram que os polifenóis estimulam a AMPK e suprimem o acúmulo de lipídios pelo menos em parte pela ativação da SIRT1.

I.4.4 Receptores ativados por proliferadores de peroxissomo (PPARs)

Os PPARs (receptores ativados por proliferadores de peroxissomos) são fatores de transcrição da família de receptores nucleares, envolvidos com a modulação do metabolismo (Tavares et al., 2007). Os PPARs apresentam três isoformas distintas : PPAR α, PPAR β e PPAR γ. Estes receptores têm sido alvo de estudos para o tratamento de doenças metabólicas (Isseman et al., 1990). São identificados inúmeros ligantes, tais como: ácidos graxos e derivados de eicosanóides e prostaglandinas. As tiazolidinedionas (TZD),

derivadas do ácido linoléico e prostaglandinas, são ligantes específicos dos PPARs, promovendo a adipogênese, aumentando a sensibilidade à INS e diminuindo os ácidos graxos livres circulantes (AGL) (Costa e Duarte, 2006).

Picard e colaboradores (2004) demonstraram que o PPARγ promove a adipogênese em pré-adipócitos-3T3-L1 e que este efeito pode ser revertido após tratamento com RSV, correlacionando inversamente a expressão do PPARγ com a da SIRT1. Também foi observado que o RSV apresenta efeito sobre a expressão do PPARγ e da SIRT1 em células de câncer de colorretal (Ulrich et al., 2006). Em fígado normal, a expressão do PPARγ é mínima em hepatócitos, porém o acúmulo de triacilglicerídios nestas células tem sido associado a um aumento da expressão do PPARγ. Isto sugere que o PPARγ desempenha um papel estimulador da lipogênese (Chao et al., 2000).

Estudando o efeito do retinol e da indometacina sobre a modulação de fenótipo da linhagem GRX, nosso grupo de pesquisa observou que estes compostos estimulam a expressão dos mRNA dos PPAR γ e α e diminuem a expressão do mRNA do PPARβ. Estes resultados sugerem que o RHO e a INDO induzem mudança fenotipica (miofobroblasto para lipócito) por inibirem os genes envolvidos na oxidação e dissipação de energia, assim como aumentam a expressão dos genes da lipogênese. Desta forma, a modulação do PPARγ, durante o processo de ativação das HSCs pode ter um efeito antifibrótico por induzir a manutenção do fenótipo quiescente (Guimarães et al., 2007).

I.5 OBJETIVOS

I.5.1Objetivo geral

Investigar o efeito do RSV sobre os mecanismos moleculares envolvidos na proliferação e diferenciação das células estreladas hepáticas (linhagem GRX).

I.5.2 Objetivos específicos

a) Analisar a ação de concentrações crescentes de RSV (1nM a 1μM) sobre a proliferação, viabilidade e ciclo celular da linhagem GRX;
b) Avaliar o efeito do RSV sobre a transformação fenotípica nas células GRX (miofibroblasto para lipócito);
c) Avaliar o efeito do RSV sobre a expressão mRNA de PPARγ, PPARα e SIRT1;
d) Avaliar o efeito do RSV sobre a incorporação de acetato em lipídios.

PARTE II

CAPÍTULO I

Artigo publicado no periódico *Molecular Cellular Biochemistry*

Resveratrol inhibits cell growth by inducing cell cycle arrest in activated hepatic stellate cells.

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Resveratrol inhibits cell growth by inducing cell cycle arrest in activated hepatic stellate cells

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Abstract Resveratrol (RSV) exerts anti-proliferative and pro-apoptotic actions in different cell lines. Hepatic stellate cells (HSCs) are major fibrogenic cell types that contribute to collagen accumulation during chronic liver disease. In the present study, the inhibitory effects of RSV on cell proliferation, cell cycle, and apoptosis were evaluated in the mouse hepatic stellate cell line GRX. Cells treated with 1 nM-1 lM of RSV demonstrated a decrease in cell growth of about 35% after 5 days. GRX cells, treated with RSV (100 nM or 1 lM), were analyzed by flow cytometry; RSV induced an increase in the number of GRX cells in the S- and sub-G1 phases. The increase in sub-G1 phase cells and the nuclear condensation and fragmentation shown by DAPI staining identified a possible pro-apoptotic effect of RSV on GRX cells. Furthermore, the RSV anti-proliferative effects could be explained by an S-phase accumulation caused by a decrease in the progression through the cell cycle or an inhibition of S or G2 phase transition. It is notable that these RSV actions are mediated at nanomolar levels, compatible with the concentrations of free RSV in biological fluids after ingestion of polyphenol-rich foods, suggesting a possible effect of these foods as an adjuvant treatment in chronical liver diseases.

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Keywords Hepatic stellate cells _ Resveratrol _ Cell cycle _ Apoptosis

Introduction

Liver fibrosis is a dynamic and sophisticated regulated wound healing response to chronic hepatocellular damage that represents a major medical problem with significant morbidity and mortality. This fibrotic process results from the accumulation of extracellular matrix (ECM) proteins including collagens, proteoglycans, and glycoproteins [1]. Hepatic stellate cells (HSCs) (previously known as Ito cells, lipocytes, or fat storing cells) are liver-specific pericytes which were identified as a major source of collagen in pathologic fibrosis following their activation to miofibroblast-like cells [2].

Indeed, much of the knowledge on the cell and molecular biology of liver fibrosis has been gained through animal models and from in vitro models employing culture activated HSC isolated from rat, mouse, or human liver. From these, in vitro models grew a large body of information characterizing stellate cell activation, cytokine signaling, intracellular pathways regulating liver fibrogenesis, production of extracellular matrix proteins, and development of antifibrotic drugs [3].

In this study, we used the GRX cell line, established from hepatic fibrogranulomatous reactions, which mobilizes adjacent stellate cells. Under standard conditions, these cells express a transitional myofibroblast phenotype and present morphological and biochemical aspects of hepatic connective tissue [4-6]. It has been previously demonstrated that the GRX cell line can be induced, in vitro, to express alternative phenotypes. The lipocyte

phenotype can be induced by retinol, indomethacin, or b-carotene treatment, with a decrease in proliferation and collagen production and progressive accumulation of fat droplets, corresponding to the frequently termed quiescent fat storing phenotype, in vivo [7-9]. These cells are also responsive to cytokines, such as TNFa, increasing oxidative stress levels, and ECM production [10-12].

Resveratrol (3,5,4 ,-trihydroxystilbene—RSV) is a polyphenolic compound found in the skins of red fruits. It is thought that the presence of RSV in red wine explains, in part, the "French paradox" and may be responsible for many of the health benefits ascribed to the consumption of red wine. Resveratrol has been proposed to exhibit anti-oxidative, anti-proliferative, and anti-inflammatory properties [13, 14]. This harmless compound exhibits chemopreventive activities in multiple animal and in vitro models with less defined molecular mechanisms [15, 16].

Accumulating data from many cell line studies indicate that RSV possesses strong anti-proliferative and apoptosis-inducing properties. S-phase or G1-phase arrest was mainly observed during RSV-induced apoptosis, but the apoptosis inducing effects of RSV appeared diverse in different cells. These conflicting data regarding RSV-induced biological effects may be due to the specific cell type and cellular environment [17].

Thepurposeofthepresentinvestigationwastostudytherole of RSV with respect to the modulation of cell proliferation, cell cycle, and stimulation of apoptosis in activated HSCs.

Material and methods

Cell culture and treatments

The murine HSC cell line, GRX, was established by Borojevic [5] and kindly provided by the Cell Bank of Rio de Janeiro (HUCFF, UFRJ, RJ). Cells were routinely maintained in Dulbecco's Modified Minimum Essential Medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FBS, Cultilab, Campinas, Br) and 2 g/l HEPES buffer, pH 7.4 under 37°C and 5% CO_2 conditions. The cells were plated (5 9 10 / ml) in 12, 24, or 96-well plates and cultured for 24 h to reach 60-70% of confluence before RSV treatment. Resveratrol (Sigma Inc., Saint Louis, MO, USA) was dissolved in ethanol (Merck, Darmstadt, Germany) to a stock concentration of 100 mM and diluted in culture medium to final concentrations of 1, 10, 100 nM, and 1 lM, just before use. The GRX cells were treated with the above-mentioned RSV concentrations for 5 days and the treatment medium was daily changed. Each concentration group included four wells. The routinely cultured cells were used as normal controls.

Cell growth assays

Bromodeoxyuridine incorporation into DNA

Cell proliferation was measured 5 days after RSV treatment by the extent of 5-bromo-2-deoxyuridine (BrdU) incorporation into DNA. BrdU, a thymidine analog that is incorporated into proliferating cells mainly during the S-phase of DNA synthesis is detected by peroxidase-conjugated mouse monoclonal anti-BrdU antibody, followed by the addition of o-phenylenediamine substrate. The optical density (OD) was measured using a spectrophotometric microtiter plate reader (Spectra Max 190, Molecular Devices), at 492 nm wavelength [18].

Tritiated thymidine incorporation assay

GRX cells treated, or not, for 5 days with RSV (at the above described concentrations) were incubated (24 h, 1 lCi/ml) with [6-^{3}H] thymidine ([3H]dT) (specific activity 23.0 Ci/nmol, Amersham Biosciences, Hillerod, Denmark). Subsequently, the medium was removed, and 10% of trichloroacetic acid (TCA) was added to each well. The cell pellet was then dissolved in 200 ll of 0.1 N NaOH, the incorporated DNA radioactivity was determined by scintillation counting [19]. The protein content was measured according to Peterson [20].

Cell viability assays

Colorimetric MTT assay

MTT (3-(4,5-dimethylthiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) (Sigma Inc., Saint Louis, MO, USA) is a yellow tetrazolium salt that is reduced to a blue formazan. The MTT assay assesses cell viability by measuring cellular redox environment [21, 22]. Pre-confluent GRX cells were incubated with variable concentrations of RSV for 5 days. Cells were then incubated with 1 mg/ml MTT for 2 h at 37°C. Blue crystals were dissolved in dimethylsulfoxide (DMSO, Sigma Inc., Saint Louis, MO, USA). OD was measured using a spectrophotometric microtiter plate reader (Spectra Max 190, Molecular Devices) at 570 nm and 630 nm.

Lactate dehydrogenase (LDH) assay

The cytotoxicity of RSV was also evaluated by determination of LDH activity in the culture medium. LDH activity was measured by a colorimetric assay (Kit from Doles, Brazil). Cells were treated or not with RSV (100 nM-1 lM) for 5 days. Subsequently, the medium was removed after centrifugation, LDH in the supernatant of the conditioned

media was determined as medium LDH. Attached cells were lysed completely by 1 ml of 5% FBS/DMEM with Triton X-100 (0.1%). After centrifugation, LDH in the supernatant was determined as cellular LDH. LDH in DMEM with 5% FBS was determined as contamination arising from FBS and subtracted from medium LDH values. Results were shown as the percentage of total LDH, i.e., [medium LDH/(medium LDH + cellular LDH)] 9 100 [23].

Cell cycle analysis by flow cytometry

Flow cytometry analysis [24] was used to assess the cell cycle phase distribution and the percentage of fragmented nuclei. Briefly, after treatment with 100 nM or 1 lM of RSV for 5 days, cells were harvested by trypsinization and counted. Samples of 1 9 10^6 cells were fixed in 70% ethanol at 4°C overnight. After centrifugation, the cell pellet was washed in ice-cold PBS, resuspended in hypotonic propidium iodide solution (50 lg/ml propidium iodide, 0.1% sodium citrate, 0.1% Triton X-100, 0.1 mg/ml RNAse A) and incubated in the dark at room temperature for 30 min. The DNA content was then analyzed using a FACScan Calibur flow cytometer equipped with a Modfit 2.0 software (Beckton Dickinson, San Jose, USA). The number of cells having a subdiploid DNA content was taken as a measurement of apoptotic cells.

Identification of apoptotic cells after DAPI nuclei staining

The nuclear morphology of cells was studied using the cell-permeable DNA dye DAPI. GRX cells grown on coverslips were treated with 1 lM RSV for 5 days, fixed in 4% (v/v) paraformaldeyde for 30 min, washed in PBS, and permeabilized with 0.5% Triton X-100 in PBS 3 times for 10 min. The DAPI nuclear dye was added at 0.1 lg/ml in 0.9% NaCl for 5 min. Coverslips were mounted with Fluorsave (Calbiochem, San Diego,CA, USA). Cells were analyzed and photographed with a Nikon inverted microscope using a TE-FM Epi-Fluorescence accessory. Apoptotic cells were morphologically defined by nuclear shrinkage and chromatin condensation or fragmentation.

Statistical analysis

Data were expressed as mean ± standard error. $P \backslash 0.05$ was considered significant. Statistical comparisons were performed using ANOVA. For post-hoc testing, the Duncan test was used. All analyses were performed with the SPSS statistical package (SPSS Inc., Chicago, IL, USA).

Results

Resveratrol inhibits cell growth and interferes with cell viability in GRX cells

The anti-proliferative effects of RSV were assessed by incubating pre-confluent GRX cells with RSV at indicated concentrations for 5 days. Proliferation of GRX cells measured by BrdUrd assay was significantly decreased by about 35% at all concentrations of RSV, compared to control (Table 1). This effect was confirmed by a thymidine incorporation assay, where the decrease in GRX cell proliferation was dose dependent (Table 1).

In addition, the effects of RSV on cell viability were determined by MTT assay and by measuring LDH activity in extracellular medium. The results show that, only concentrations above 10 lM significantly decreased cell viability (Data not shown). In view of these results, in subsequent experiments, we used the concentrations of 100 nM and 1 lM.

Resveratrol induces S-phase cell cycle arrest in GRX cells

The effect of RSV on cell proliferation could be due to its actions on cell cycle and the initiation of programmed cell death. In order to explore this possibility, we treated exponentially growing GRX cells with RSV and assayed its effects on cell cycle. The untreated cells showed the expected pattern for continuously growing cells, whereas the cells treated with 100 nM or 1 lM RSV for 5 days showed a 50% increase in the number of GRX cells in S-phase (Fig. 1).

Table 1 Effect of RSV on GRX cell growth. GRX cells were treated with variable concentrations of RSV for 5 days and the proliferation measured by BrdUrd or [3H]dT incorporation assay

RSV [lM]	Control	0.001	0.01	0.1	1
BrdU-DNA (Immunoactivity)	0.57 ± 0.020^{a}	0.41 ± 0.024^{b}	0.36 ± 0.023^{b}	0.39 ± 0.017^{b}	0.36 ± 0.011^{b}
^{3}H Thymidine (cpm/lg protein)	16.9 ± 0.94^{a}	15.1 ± 0.33^{a}	13.7 ± 1.17^{b}	12.0 ± 1.01^{b}	ND

Results are expressed as the means ± S.E.M. of three independent experiments. Different letters indicate statistically significant differences between groups $P \backslash 0.02$. ND, not determined

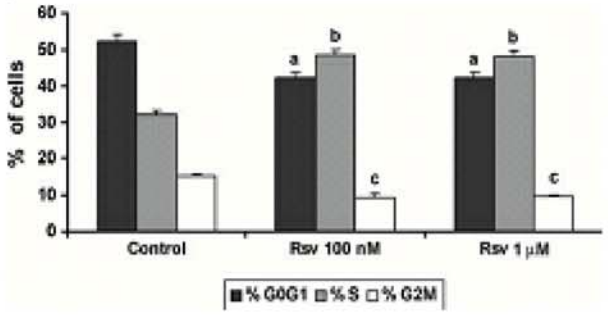

Fig. 1 Effect of RSV on GRX cell cycle. Exponentially growing GRX cells were treated with RSV for 5 days at indicated concentrations and their effects on cell cycle were assayed as described in the "Material and methods" section. Data represent the mean ± S.E.M. of three independent experiments performed in triplicate. (a), (b), and (c) significantly different from control G0/G1, S, and G2 + M, respectively, P \ 0.02

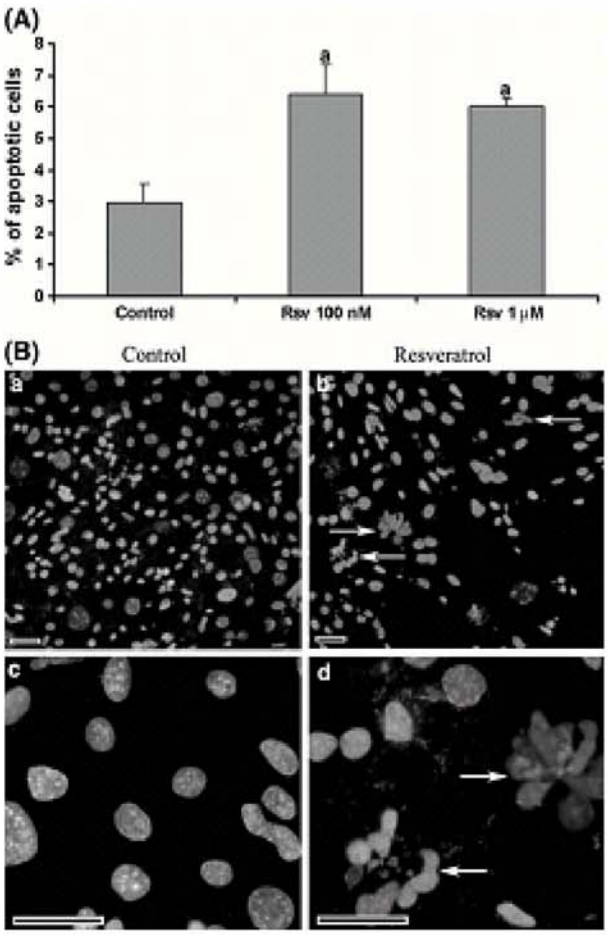

Fig. 2 Influence of RSV on apoptosis, as analyzed by subdiploid cell population and nuclear morphology of GRX cells. GRX cells were treated with RSV at indicated concentrations for 5 days. (A) The number of cells having a subdiploid DNA content was taken as a measurement of apoptotic cells, as described in the "Material and methods" section. Values are expressed as means ± S.E.M. of three independent experiments performed in triplicate. (a) significantly different from control,P \ 0.02. (B) Representative images of four experimental conditions. DAPI staining was used to visualize nuclear morphology under the fluorescence microscope after exposure to RSV at 1 lM (b, d) for 5 days, compared to the control cells (a, c). The apoptotic cells demonstrated nuclear fragmentation or partially condensed nuclei without fragmentation (arrows). Scale bar: 50 lm

Resveratrol stimulates apoptosis in GRX cells

Cells with DNA content below G1 phase, defined as a hypodiploid sub-G1 peak, were regarded as apoptotic cells. The results shown in Fig. 2A demonstrate that RSV treatment (100 nM or 1 lM) induced a two-fold increase in the number of sub-G1 GRX cells, as compared with controls. The ability of RSV to modulate apoptosis was also studied by examination of nuclear morphology. After treatment, cells were stained with DAPI and nuclear morphology visualized under a fluorescence microscope (Fig. 2B). RSV (1 lM, 5 days), induced an increase in the number of GRX nuclei with a condensed and fragmented morphology, indicating stimulation of apoptosis (Fig. 2B, c and d).

Discussion

Resveratrol (3,5,4 -trihydroxy-trans-stilbene, RSV) is a naturally occurring polyphenol synthesized by a variety of plant species in response to injury, UV irradiation, and fungal attack [25]. Accumulating evidences indicate that an inverse relationship exists between RSV and its striking inhibition of diverse cellular events associated with cell growth [17, 26, 27]. RSV has now been recognized as a promising candidate for chemopreventive effects [17].

The major fibrogenic cell type HSC contributes to collagen accumulation during chronic liver disease. Liver regeneration, driven by hepatocyte proliferation is necessary for tissue repair and survival after acute liver injury and chronic hepatic disease, such as fibrosis and cirrhosis [28]. Induction of hepatocyte proliferation depends on cross-talk between hepatocytes and non parenchymal-liver cells, such as HSCs [28]. At sites of injury, HSC differentiate to myofibroblasts and secrete ECM and growth factors that support hepatocyte proliferation [29]. Apoptosis of activated HSC is a key factor in regression of liver fibrosis. Activated HSC are more susceptible to apoptosis and can undergo spontaneous cell death or receptor-mediated death, caused by serum deprivation or cytokine signaling [30].

Putative anti-fibrogenic drugs mainly include agents able to reduce inflammation, agents able to reduce HSC activation, agents with a pro-apoptotic potential for HSC, agents with antioxidant effects, and agents able to increase fibrillar ECM degradation. Numerous studies have attributed a beneficial role in a number of pathologies to RSV-

rich foods and beverages. In order to further understand the biochemical mechanisms of this molecule, a number of studies have been carried out with very high concentrations of RSV: In the present study, we used concentrations compatible with those found in biological fluids after consumption of RSV-rich foods and beverages, in an attempt to understand in vitro (with all the limitations of cell culture systems) the beneficial actions of the agent found in vivo by using physiologically relevant RSV concentrations [26].

With the increasing interest in developing antifibrotic therapies, there is a need for cell lines that preserve the in vivo phenotype of HSCs to elucidate pathways of hepatic fibrosis. In this context, the GRX cell line represents an interesting and efficient study tool [6, 9, 10, 12, 31-35]. In standard culture conditions, GRX cells have a myofibroblast-like phenotype that resembles activated HSCs.

The treatment of GRX cells for 5 days with variable concentrations of RSV resulted in a decrease in cell growth of about 35%. These findings are in agreement with previous studies performed in HL-60 [26], HepG2 cells [26], and C6 glioma [17, 36]. Kawada et al. [19] demonstrated that RSV dose-dependently suppressed the incorporation of [^{3}H]thymidine in rat HSC, but the value of median inhibitory concentration for RSV was 37 lM. Our results show that RSV inhibits GRX cell growth at concentrations that are lower that than those found in the majority of studies dealing with RSV effects in cells, but compatible with those reported previously in HepG2 [17]. As discussed above, we chose to use low doses of the agents that are compatible with reported concentrations of RSV after ingestion of polyphenol-rich foods [37].

In vitro studies have shown that RSV reduces cell proliferation and modulates the cell cycle in different cell lines. G1-arrest has been observed in human epidermoid carcinoma A431 cells [38], human leukemia U937 cells [39], Hep G2 cells [40], and esophageal adenocarcinoma Bic 1 [41]. Resveratrol blocked the S-G2 transition in HL-60 [42] and [17] induced a more than twofold increase in number of C6 cells in S phase. In agreement with these findings, S-phase arrest was also seen in human neuroblastoma SH-SY5Y and in Neuro-2a neuroblastoma cells [43, 44]. In rat HSCs, 100 lM of RSV reduced the level of cyclin D1, a cell cycle-related protein [19]. Since cyclin D1 regulates the activity of cdk4 and cdk6, resulting in the phosphorylation of retinoblastoma gene product Rb p110 and the successive G1-to S-phase transition, its reduction might cause G1 arrest of the cells. In general, the effect of RSV on cell cycle distribution is concentration-dependent, where lower RSV concentrations cause accumulation of the cells in S-phase and higher concentrations cause accumulation of the cells in the G0/G1 phase or G2 + M phases [27]. This may indicate that RSV has more than one target inhibiting cell division, which may be modulated at different concentrations [45].

The S-phase arrest produced by RSV is often accompanied by inhibition in [^{3}H] thymidine incorporation. RSV, at 20 lM, induced S-phase accumulation in HL-60 with a 30% inhibition of cell growth. In contrast, in HepG2, 40 lM RSV induced a maximal S-phase accumulation without a significant decrease in [^{3}H] thymidine incorporation. Here, we demonstrate that treatment of GRX cells with small doses of RSV (100 nM and 1 lM) induced a 35% inhibition in [^{3}H] thymidine incorporation and a 50% increase in S-phase cells. These S-phase arrests indicate that RSV at 100 nM and 1 lM did not induce a decrease in cyclin D1 expression like described by Kawada et al. [19] for rat HSC. Our results could be explained by the hypothesis proposed by Delmas et al. [16], in which S-phase accumulation is suggested to be caused by a decrease in the progression through the cell cycle or an inhibition of S or G2 phase transition. Furthermore, our results are in agreement with observations that RSV inhibits the production of polyamines by interfering with ornithine decarboxylase [46], which in turn catalyses the rate-limiting step in polyamine production.

In addition to its cell cycle arresting properties, another essential effect of RSV is its apoptosis-inducing ability. RSV has been previously shown to trigger apoptosis in leukemia, mammary, myeloma, epidermoid, embryonal rhabdomyosarcoma, and glioblastoma cell lines [38, 47]. The doses required for RSV apoptosis induction (100-200 lM range) are often higher than those used to induce growth inhibition and cell cycle arrest [41]. Interestingly, in GRX cells, small doses of RSV (100 nM and 1 lM) resulted in twice the number of sub-G1 cells. An obvious increase in the number of GRX nuclei with a condensed and fragmented morphology is visualized by DAPI-staining, indicating the stimulation of apoptosis by RSV.

Taken together, our observations indicate that GRX cells, when treated with RSV, are induced to enter into the S phase, but subsequent progression through the S phase is limited by the inhibitory effect of RSV on DNA synthesis. Therefore, as suggested by Kuwajerwala [45], this unique ability of RSV to exert opposing effects on two important processes in cell cycle progression, induction of S phase, and inhibition of DNA synthesis, indicate that RSV could modulate apoptotic and anti-proliferative effects in GRX. In this context, more studies are necessary to demonstrate these effects in liver fibrosis.

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References

1. Pinzani M, Rombouts K (2004) Liver fibrosis: from the bench to clinical targets. Dig Liver Dis 36:231-242
2. Friedman SL, Roll FJ, Boyles J, Bissell DM (1985) Hepatic lipocytes: the principal collagen-producing cells of normal rat liver. Proc Natl Acad Sci USA 82:8681-8685
3. Gutierrez-Ruiz MC, Gomez-Quiroz LE (2007) Liver fibrosis: searching for cell model answers. Liver Int 27:434-439
4. Pinzani M, Gentilini P (1999) Biology of hepatic stellate cells and their possible relevance in the pathogenesis of portal hypertension in cirrhosis. Semin Liver Dis 19:397-410
5. Borojevic R, Monteiro AN, Vinhas SA, Domont GB, Mourao PA, Emonard H, Jr.Grimaldi G, Grimaud JA (1985) Establishment of a continuous cell line from fibrotic schistosomal granulomas in mice livers. In vitro Cell Dev Biol 21:382-390
6. Guma FCR, Mello TG, Mermelstein CS, Fortuna VA, Wofchuk ST, Gottfried C, Guaragna RM, Costa ML, Borojevic R (2001) Intermediate filaments modulation in an in vitro model of the hepatic stellate cell activation or conversion into the lipocyte phenotype. Biochem Cell Biol 79:409-417
7. Margis R, Borojevic R (1989) Retinoid-mediated induction of the fat-storing phenotype in a liver connective tissue cell line (GRX). Biochim Biophys Acta 1011:1-5
8. Borojevic R, Guaragna RM, Margis R, Dutra HS (1990) In vitro induction of the fat-storing phenotype in a liver connective tissue cell line-GRX. In vitro Cell Dev Biol 26:361-368
9. Martucci RB, Ziulkoski AL, Fortuna VA, Guaragna RM, Guma FC, Trugo LC, Borojevic R (2004) Beta-carotene storage, conversion to retinoic acid, and induction of the lipocyte phenotype in hepatic stellate cells. J Cell Biochem 92: 414-423
10. da Silva FM, Guimaraes EL, Grivicich I, Trindade VM, Guaragna RM, Borojevic R, Guma FC (2003) Hepatic stellate cell activation in vitro: cell cycle arrest at G2/M and modification of cell motility. J Cell Biochem 90: 387-396
11. Chiarini LB, Borojevic R, Monteiro AN (1994) In vitro collagen synthesis by liver connective tissue cells isolated from schistosomal granulomas. Braz J Med Biol Res 27: 1193-1197
12. Guimaraes EL, Franceschi MF, Grivicich I, Dal-Pizzol F, Moreira JC, Guaragna RM, Borojevic R, Margis R, Guma FC (2006) Relationship between oxidative stress levels and activation state on a hepatic stellate cell line. Liver Int 26:477-485
13. Donnelly LE, Newton R, Kennedy GE, Fenwick PS, Leung RH, Ito K, Russell RE, Barnes PJ (2004) Anti-inflammatory effects of resveratrol in lung epithelial cells: molecular mechanisms. Am J Physiol Lung Cell Mol Physiol 287:L774-783
14. Baur JA, Sinclair DA (2006) Therapeutic potential of resveratrol: the in vivo evidence. Nat Rev Drug Discov 5:493-506
15. Kundu JK, Surh YJ (2004) Molecular basis of chemoprevention by resveratrol: NF-kappaB and AP-1 as potential targets. Mutat Res 555:65-80
16. Delmas D, Lancon A, Colin D, Jannin B, Latruffe N (2006) Resveratrol as a chemopreventive agent: a promising molecule for fighting cancer. Curr Drug Targets 7:423-442
17. Zhang W, Fei Z, Zhen HN, Zhang JN, Zhang X (2007) Resveratrol inhibits cell growth and induces apoptosis of rat C6 glioma cells. J Neurooncol 81:231-240
18. Muir D, Varon S, Manthorpe M (1990) An enzyme-linked immunosorbent assay for bromodeoxyuridine incorporation using fixed microcultures. Anal Biochem 185:377-382
19. Kawada N, Seki S, Inoue M, Kuroki T (1998) Effect of antioxidants, resveratrol, quercetin, and N-acetylcysteine, on the functions of cultured rat hepatic stellate cells and Kupffer cells. Hepatology 27:1265-1274
20. Peterson GL (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem 83:346-356
21. Hayon T, Dvilansky A, Shpilberg O, Nathan I (2003) Appraisal of the MTT-based assay as a useful tool for predicting drug chemosensitivity in leukemia. Leuk Lymphoma 44:1957-1962
22. Hansen MB, Nielsen SE, Berg K (1989) Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J Immunol Methods 119: 203-210
23. Chen A, Zhang L, Xu J, Tang J (2002) The antioxidant (-)-epigallocatechin-3-gallate inhibits activated hepatic stellate cell growth and suppresses acetaldehyde-induced gene expression. Biochem J 368:695-704
24. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C (1991) A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods 139:271-279
25. Bhat KPL, Kosmeder JW II, Pezzuto JM (2001) Biological effects of resveratrol. Antioxid Redox Signal 3:1041-1064
26. Notas G, Nifli AP, Kampa M, Vercauteren J, Kouroumalis E, Castanas E (2006) Resveratrol exerts its antiproliferative effect on HepG2 hepatocellular carcinoma cells, by inducing cell cycle arrest, and NOS activation. Biochim Biophys Acta 1760: 1657-1666
27. Stervbo U, Vang O, Bonnesen C (2006) Time- and concentration-dependent effects of resveratrol in HL-60 and HepG2 cells. Cell Prolif 39:479-493
28. Taub R (2004) Liver regeneration: from myth to mechanism. Nat Rev Mol Cell Biol 5:836-847
29. Balabaud C, Bioulac-Sage P, Desmouliere A (2004) The role of hepatic stellate cells in liver regeneration. J Hepatol 40: 1023-1026
30. Kisseleva T, Brenner DA (2006) Hepatic stellate cells and the reversal of fibrosis. J Gastroenterol Hepatol 21Suppl 3:S84-87
31. de Aguirres AB, Mello PA, Andrade CM, Breier AC, Margis R, Guaragna RM, Borojevic R, Guma FC, Trindade VM (2007) Variations of ganglioside biosynthetic pathways in the phenotype conversion from myofibroblasts to lipocytes in murine hepatic stellate cell line. Mol Cell Biochem 303:121-130
32. Andrade CM, Trindade VM, Cardoso CC, Ziulkoski AL, Trugo LC, Guaragna RM, Borojevic R, Guma FC (2003) Changes of sphingolipid species in the phenotype conversion from myofibroblasts to lipocytes in hepatic stellate cells. J Cell Biochem 88:533-544
33. Mermelstein CS, Guma FC, Mello TG, Fortuna VA, Guaragna RM, Costa ML, Borojevic R (2001) Induction of the lipocyte phenotype in murine hepatic stellate cells: reorganisation of the actin cytoskeleton. Cell Tissue Res 306:75-83
34. Vicente CP, Fortuna VA, Margis R, Trugo L, Borojevic R (1998) Retinol uptake and metabolism, and cellular retinol binding protein expression in an in vitro model of hepatic stellate cells. Mol Cell Biochem 187:11-21
35. Vicente CP, Guaragna RM, Borojevic R (1997) Lipid metabolism during in vitro induction of the lipocyte phenotype in hepatic stellate cells. Mol Cell Biochem 168:31-39
36. dos Santos AQ, Nardin P, Funchal C, de Almeida LM, Jacques-Silva MC, Wofchuk ST, Goncalves CA, Gottfried C (2006) Resveratrol increases glutamate uptake and glutamine synthetase activity in C6 glioma cells. Arch Biochem Biophys 453: 161-167
37. Bertelli A, Bertelli AA, Gozzini A, Giovannini L (1998) Plasma and tissue resveratrol concentrations and pharmacological activity. Drugs Exp Clin Res 24:133-138

38. Ahmad N, Adhami VM, Afaq F, Feyes DK, Mukhtar H (2001) Resveratrol causes WAF-1/p21-mediated G(1)-phase arrest of cell cycle and induction of apoptosis in human epidermoid carcinoma A431 cells. Clin Cancer Res 7:1466-1473
39. Park JW, Choi YJ, Jang MA, Lee YS, Jun DY, Suh SI, Baek WK, Suh MH, Jin IN, Kwon TK (2001) Chemopreventive agent resveratrol, a natural product derived from grapes, reversibly inhibits progression through S and G2 phases of the cell cycle in U937 cells. Cancer Lett 163:43-49
40. Kuo PL, Chiang LC, Lin CC (2002) Resveratrol- induced apoptosis is mediated by p53-dependent pathway in Hep G2 cells. Life Sci 72:23-34
41. Joe AK, Liu H, Suzui M, Vural ME, Xiao D, Weinstein IB (2002) Resveratrol induces growth inhibition, S-phase arrest, apoptosis, and changes in biomarker expression in several human cancer cell lines. Clin Cancer Res 8:893-903
42. Ragione FD, Cucciolla V, Borriello A, Pietra VD, Racioppi L, Soldati G, Manna C, Galletti P, Zappia V (1998) Resveratrol arrests the cell division cycle at S/G2 phase transition. Biochem Biophys Res Commun 250:53-58
43. Rigolio R, Miloso M, Nicolini G, Villa D, Scuteri A, Simone M, Tredici G (2005) Resveratrol interference with the cell cycle protects human neuroblastoma SH-SY5Y cell from paclitaxel-induced apoptosis. Neurochem Int 46:205-211
44. Chen C, Zhang J, Li J, Huang J, Yang C, Huang G, Shi J (2004) Hydrodynamic-based in vivo transfection of retinoic X receptor-alpha gene can enhance vitamin A-induced attenuation of liver fibrosis in mice. Liver Int 24:679-686
45. Kuwajerwala N, Cifuentes E, Gautam S, Menon M, Barrack ER, Reddy GP (2002) Resveratrol induces prostate cancer cell entry into s phase and inhibits DNA synthesis. Cancer Res 62: 2488-2492
46. Schneider Y, Vincent F, Duranton B, Badolo L, Gosse F, Bergmann C, Seiler N, Raul F (2000) Anti-proliferative effect of resveratrol, a natural component of grapes and wine, on human colonic cancer cells. Cancer Lett 158:85-91
47. Clement MV, Hirpara JL, Chawdhury SH, Pervaiz S (1998) Chemopreventive agent resveratrol, a natural product derived from grapes, triggers CD95 signaling-dependent apoptosis in human tumor cells. Blood 92:996-1002

CAPÍTULO II

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Lipid accumulation in a HSC cell line is linked to the PPARs and SIRT1 ratio

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Lipid accumulation in a HSC cell line is linked to the PPARγ and SIRT1 ratio.

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Running Title: PPARγ/SIRT1 ratio and HSC lipid accumulation.

ABSTRACT:

BACKGROUND: Hepatic stellate cell (HSC) is a key step in the development of liver fibrosis. PPARγ expression decreased markedly with the activation of HSC. Sirtuins (SIRT) are highly conserved NAD^+-dependent protein deacetylases; SIRT1 has recently been shown to suppress PPAR_ activity. Resveratrol (RSV) is a polyphenol compound and in mammals it possesses the ability to activated SIRT1, reducing fat accumulation, increasing free fatty acid release and inhibiting the adipogenesis. GRX cell line represents the HSC activated phenotype and could be induced to quiescent phenotype by retinol. *AIMS:* The possible involvement of RSV and retinol (RHO) on SIRT1 and PPARs expression was evaluated in this study. *METHODS*: The quiescent phenotype differentiation process was visualized by light microscopy after Oil red O staining. Expression of Sirt1 and PPARγ was detected with qRT-PCR. Lipid synthesis was monitored by acetate incorporation and triacylglicerides (TG) quantification was used as an index of lipid accumulation. *RESULTS:* In our experimental model RSV stimulated SIRT1 mRNA. RSV did not induce the lipocyte phenotype because the PPARγ activity was repressed. *CONCLUSIONS*: The impact of the SIRT1 increasing on GRX leads to the repression of genes involved in PPARγ-mediated fat storage and can result in the maintenance of myofibroblast phenotype. Consequently, we hypothesized that the ratio between PPARγ/SIRT1 mRNA could be considered directly involved in the homeostasis of liver connective tissue, and may actively participate in pathogenesis of hepatic fibrosis or cirrhosis.

Key Words: Hepatic stellate cells, GRX cell line, Resveratrol, PPARγ, SIRT1, Lipid accumulation

INTRODUCTION:

Hepatic fibrosis, a precursor of liver cirrhosis, is a consequence of severe liver damage occurred in many patients with chronic liver disease and involves the abnormal accumulation of extracellular matrix (ECM) (1), the discovery of stellate cell-activation from a quiescent vitamin A-storing cell to a proliferative myofibroblast remains among the most informative discoveries to understanding the mechanistic basis of hepatic fibrosis progression and regression (2). HSCs activation is a highly pleiotropic process and dramatic phenotypic changes require global reprogramming of HSCs gene expression that, in turn, must be orchestrated by long-term changes in the expression and/or activity of key transcription factors of the HSCs genome (3). The authors hypothesized the importance of the study on the function of transcription factors that are active in quiescent HSCs, and showed that PPARγ expression decreased markedly with the activation of HSCs. Their study also showed that PPARγ plays an important role in maintaining the quiescent phenotype. In a previous study we have demonstrated PPARγ mRNA was increased in quiescent like phenotype of GRX cells, a murine HSC cell line (4).

PPARα, PPARδ/β, and PPARγ form a subgroup of the nuclear receptor superfamily and, upon ligand binding, regulate the expression of several genes mainly involved in lipid metabolism (5). PPARs, which are activated by long-chain polyunsaturated fatty acids, are also one of the few examples of nutrient-regulated transcription factors. Hence, it has been suggested that these receptors play a central role in sensing nutrient levels and in modulating their metabolism. PPARα promotes fatty acid metabolism by inducing the expression of genes involved in the transport of fatty acids within the cell and

the mitochondria, as well as those coding for many enzymes of the β-oxidation pathway (6). The functions of PPARδ/β remain largely mysterious. In vitro, activation of PPAR δ/β in adipocytes and skeletal muscle cells promotes fatty acid oxidation and utilization (7). PPARγ is expressed primarily in adipose tissue and promotes fat storage by increasing adipocyte differentiation and transcription of a number of lipogenic proteins (8, 9). It was reported that PPARγ expression is ten-fold higher in hepatic tissues than in peripheral-blood mononuclear cells, but the functional activities of PPARγ in the liver remain largely unknown (3).

Sirtuins are highly conserved NAD^+-dependent protein deacetylases (10-12). SIRT1 also regulates fat metabolism and glucose homeostasis by interacting with the nuclear receptor PPARγ through nuclear receptor corepressor (N-CoR) to repress adipogenesis (13), modifying the PPARγ coactivator PGC-1α to regulate hepatic glucose homeostasis (14, 15) and regulating levels of insulin secreted by pancreatic β cells (16, 17).

Putative anti-fibrogenic drugs mainly include agents able to reduce inflammation, agents able to reduce HSC activation, agents with a pro-apoptotic potential for HSC, agents with antioxidant effects, and agents able to increase fibrillar ECM degradation. Numerous studies have attributed a beneficial role in a number of pathologies to resveratrol (RSV; 3,5,4'-trihydroxystilbene), a naturally occurring phytoalexin found in red wines and grape juice, which has shown to reduce the synthesis of lipids in rat liver (18) and 3T3-L1 adipocytes (13), inhibit the synthesis of eicosanoids in rat leukocytes (19) interfere in arachidonate metabolism (20), and inhibit the activity of some protein kinases (21). RSV decreased proliferation and induced apoptosis and cell cycle arrest

in various cell lines. In rat HSCs, 100 μM of RSV reduced the level of cyclin D1, a cell cycle-related protein (22). Recently, we demonstrated that treatment with nanomolar concentrations of RSV cell proliferation, induced an S phase arrest and has pro-apoptotic effects in activated GRX cells (23).

In this study, we demonstrated that the expression of lipocyte phenotype in GRX cells is dependent of the relation between the PPARγ / SIRT1 transcripts and the correlation of this modulation with the lipids metabolism of GRX cells.

MATERIAL AND METHODS

Cell culture and treatments:

The murine HSC cell line, GRX, was established by Borojevic (24) and kindly provided by the Cell Bank of Rio de Janeiro (HUCFF, UFRJ, RJ). Cells were routinely maintained in Dulbecco's Modified Minimum Essential Medium (DMEM, Invitrogen, Carlsbad, CA,USA) supplemented with 5% fetal bovine serum (FBS, Cultilab, Campinas, Br) and 2 g/L HEPES buffer, pH 7.4 under 37^{o}C and 5% CO_2 conditions. The cells were plated (5 x 10^{4}cells/mL) and cultured for 24 h to reach 60-70% of confluence before retinol (RHO) and/or resveratrol (RSV) treatment. In the experimental series, GRX cells were treated with the 5 μM RHO, 100 nM RSV or with both (RSV+RHO) for 12h, 24h or 120 h, the routinely cultured cells were used as normal controls.

All-trans-retinol (RHO) was dissolved in ethanol (0.1% final concentration) (both from Sigma Chemical Company, St Louis, MO, USA). The concentration of *all-trans*-retinol in the stock solution was determined by ultra-violet absorption at 325 nm, using the molar extinction coefficient (ε) of 52.770 $cm^{-1}M^{-1}$, (4, 25).

Resveratrol (RSV) (Sigma Inc., Saint Louis, MO, USA) was dissolved in ethanol (Merck, Darmstadt, Germany) to a stock concentration of 100 mM and diluted in culture medium to final concentrations of 100 nM, just before use. Intracellular lipid droplets were identified by standard staining with Oil-Red-O (Sigma) (23).

RNA extraction, cDNA synthesis and Real-time PCR

RNA was isolated using the TRIzol Reagent (Invitrogen, Carlsbad, California, USA). Approximately 2 µg of total RNA were added to each cDNA synthesis reaction using the SuperScript-II RT pre-amplification system (Invitrogen). Reactions were performed at 42ºC for 1 h using the primer T23V (5' TTT TTT TTT TTT TTT TTT TTV). PCR amplification was carried out using specific primer pairs designed Oligo Calculator version 3.02 (http://basic.nwu.edu/biotools/oligocalc.html) and synthesized by RW-Genes (RJ, Brazil). The sequences of the primers used are listed in Table 1. Total RNA and cDNA were generated as described in RT-PCR analysis. Real-time PCRs were carried out in an Applied-Biosystem 7500 real-time cycler and performed in quadruplicate. Reaction settings were composed of an initial denaturation step of 5 min at 94ºC, followed by 40 cycles of 10 s at 94ºC, 15 s at 60ºC, 15 s at 72ºC and 35 s at 60ºC for data acquisition; samples were kept for 2 min at 40ºC for annealing and then heated from 55 to 99ºC with a ramp of 0.1ºC/sec to acquire data to produce the denaturing curve of the amplified products. Real-time PCRs were carried out in a 20 µl final volume composed of 10 µl of each reverse transcription sample diluted 50 to 100 times, 2 µl of 10 times PCR buffer, 1.2 µl of 50 mM $MgCl_2$, 0.4 µl of 5 mM dNTPs, 0.4 µl of 10 µM primer pairs, 3.95 µl of water, 2.0 µl of SYBRgreen (1:10,000 Molecular Probe), and

0.05 µl of Platinum Taq DNA polymerase (5 U/µl) (Invitrogen). The primers used for real time PCRs were the same used in RT-PCR analysis. All results were analyzed by the $2^{-\Delta\Delta CT}$ method (26) . β-actin was used as the internal control gene for all relative expression calculations (27).

Lipid Synthesis

Lipid synthesis was monitored by acetate incorporation. Cells were mantained in standard or treatment medium (RSV, RHO, RSV+RHO) for 24 h or 120 h and than incubated with 0.1 uCi/mL [14C] acetate (Amersham) for 16 h. After incubation, lipids were extracted from the cell layer by method of (28) Folch *et al*. (195..). The chloroform phase was dried under nitrogen and analyzed by TLC on G-60 silica-gel aluminium plates with hexane: ethyl ether: acetic acid (90:10:1; v/v/v). Radioactive lipids were visualized by exposition of a radiographic film at -70°C, and their relative content was determined by densitometric scanning of the X-ray film in a CS 930 Shimadzu UV/vis densitometer. The standards were stained by Comassie Brilliant Blue R-250 (Sigma) (29).

Quantification of triacylglicerides content

Lipid content was quantified using commercially available assay reagent. In brief, GRX grown in 24-well plates were treated as described above in Cell Cultures and Treatments, and on 120 h, cells were assayed for lipid content according to the manufacturer's instructions (Triglycerides Liquiform Labtest, MG, Brasil). The experiments were performed with at least 6 replicates per treatment and repeated 3 times.

Protein quantification

The protein sediments obtained after lipid extractions were dissolved in 1.0 N NaOH and measured as described by Peterson (30) using bovine serum albumin as standard

Statistical Analysis

Data represent mean ± standard error of mean (SEM). Statistical analysis was performed by ANOVA followed by Duncan post hoc, using the statistical program, SPSS 10.0 for Windows. The values were considered statistically different when $p<0.05$.

RESULTS:

Effect of RSV in the induction of GRX differentiation to lipocyte:

Through Oil red O staining, we could see that GRX cells treated with RHO and RVS+RHO were filled with fat droplets and acquired characteristics of lipocytes. In comparison, cells that were treated only with RSV were similar to untreated cells (Myo) and didn`t accumulate fat droplets (Figure 1).

Effect of RSV in the time-spatial expression of PPARγ and Sirt1 mRNA during the GRX differentiation to lipocyte:

In parallel with the establishment of lipocyte phenotype retinol produced an increase in PPARγ transcripts (4). Here we demonstrated that RHO treatment increased PPARγ mRNA as soon as 12 h. At 24 h all culture groups presented an increase in the PPARγ mRNA, but in the groups treated with RHO and RHO+RSV there was a two-fold increase when compared to Myo and RSV groups. After 5 days (120 h) the expression was not affected by RSV treatment and decreased significantly in RHO plus RSV-treated cells as compared with

RHO treated group (figure 2). By Western blot we observed the expression of the PPARγ protein in all experimental groups (data not shown).

Under all experimental conditions the expression of SIRT1 mRNA increases gradually with the culture time. Specifically, after 120 h Sirt1 mRNA expression in the RSV treatment was significantly higher than in the other groups. At this treatment time, Sirt1 mRNA expression in the RSV+RHO-treated cells was lower than the other groups (Figure 2). These results indicate that, like demonstrated by other cells, SIRT1 mRNA expression could be induced by RSV in GRX cells.

The figure 2b showed the relationship between PPARγ and SIRT1 mRNA expression. After 120 h this relation demonstrated the prevailing of the SIRT1 transcripts upon the PPARγ transcripts in Myo and RSV treated cells. In all other times and experimental groups the transcription of PPARγ was predominant above SIRT1.

RSV effect on lipogenesis and lipid accumulation

The lipid synthesis was analyzed through incorporation of [^{14}C] acetate after 24 h and 5 days treatment. The results showed in figure 3-A demonstrated that in RHO and RSV+RHO-treated cells the incorporation of acetate in triacylglicerol was increased after 24h and the same rate of incorporation was observed in RHO group after 120 h treatment (figure 3-B) After 120 h, besides the lower acetate incorporation in the RSV+RHO group, the triacylglicerol accumulation was similar to RHO (Figures 3-B and 4). This last result is in accordance with the results of Oil red O stain presented in figure 1.

DISCUSSION

PPARs are important for the regulation of lipid and glucose metabolism, cell proliferation and differentiation, adipogenesis, and inflammatory signaling in numerous tissues (31). GRX cell represent liver connective myofibroblasts, that may switch between two phenotypes: myofibroblasts and lipocytes. The discovery that PPARγ expression is down modulated during HSC activation suggests that its ligands exert antifibrotic effects and highlights a possible involvement of this transcription factor in liver fibrosis. SIRT1 plays a role in a wide variety of processes, including stress resistance, metabolism and differentiation. Resveratrol activate SIRT1, which reduce fat accumulation, increasing free fatty acid release and inhibiting the adipogenesis. The possible involvement of RSV and RHO on SIRT1 and PPARs expression was evaluated in this study.

GRX cells cultivated in standard culture medium or treated with RSV by 120 h presented myofibroblast phenotype (Figure 1). At this time SIRT1 mRNA expression was increased related to PPARγ. After this time, RSV down regulated PPARγ, that returning to basal levels (Figure 2a). The '*de novo*' synthesis of TG represents only 10 % of total acetate incorporation and the cells do not accumulate lipids (Figures 1 e 3a). When the cells where treated RHO or RSV+RHO for 120 h the mRNA-PPARγ was increased significantly. The ratio PPARγ/SIRT1 was increased when the cells where treated only with RHO or associated with RSV (Figure 2b). RHO is the responsible for lipogenesis because it increased significantly the incorporation of acetate radioactive in TG (figure 3). After 120 h, the cells treated with combinated drugs did not stimulate the '*de novo*' synthesis of TG, however the lipids depots was increased and the

phenotype was changed. We believe that the PPARγ mRNA transcription stimulation at 24 h of RSV+RHO treatment (Figure 2a) was responsible for the phenotype switch and lipid accumulation as showed in Figures 1, 3 and 4.

The PPARα mRNA expression was not modulated by RHO or by RSV in GRX cells (date not shown). This was already observed by Guimarães (4) that related modulation of this nuclear receptor only by indomethacin and not by RHO. These results are indicative, that in GRX cells treated with RHO or RSV+RHO the lipid homeostasis was not dependent of PPARα action.

After 24 h the treatment with RHO and RSV+RHO induced an overexpression of PPARγ transcription. At this time, the lipogenesis was intensified; the synthesis of lipids was increased and approximately 50% of acetate was incorporated in TG (Figure 3a). Furthermore, the ratio PPARγ/SIRT1 mRNA was maximal after 24 h and returned to the basal expression after 120 h treatment (Figure 2b), when lipocytes have already constituted the lipid droplets (Figure 1). These results are in accordance with Vicente (32), who showed that several enzymes of lipid metabolism are upregulated in early stages of GRX cells lipocyte induction. The ratio PPARγ/SIRT1 mRNA expression was definitively important for lipocyte induction in our model.

Ours results demonstrated that resveratrol did not activate PPARγ mRNA expression and the lipogenesis in GRX cells (Figure 1 and Figure 3). These results corroborate with others authors, who showed that RSV did not promote the differentiation of pre-adipocytes into adipocytes (33). We hypothesized that RSV bound to SIRT1 and controlled transcriptional co-regulators such as PGC1α or by directly interacting with transcription factors modulating PPARγ.

Then, the impact of the SIRT1 increasing on GRX leads to the repression of genes involved in PPARγ-mediated fat storage and can consequently result in the maintenance of myofibroblast phenotype.

Consequently, the ratio between PPARγ/SIRT1 mRNA could be considered directly involved in the homeostasis of liver connective tissue, and may actively participate in pathogenesis of hepatic fibrosis or cirrhosis. The controls involved in the switch between lipocyte and myofibroblast phenotypes, occurring either under normal or pathologic conditions. These results suggest that PPARγ is possibly a key regulator in the early stages of lipocyte differentiation. Soon, drugs that modulate PPARγ can control the HSC phenotypic transformation.

Recently studies have shown that PPARγ heterodimerizes with the retinoid-X receptor (RXR) and binds to DNA at the peroxisome proliferator-response element (PPRE) activating transcription of enzymes that controlling the capacity to storage lipids in the adipocytes (34) and hepatocytes (35). PPARγ-RXR can be activated by ligands specific for either receptor, the presence of both ligands can result in a cooperative effect on the transactivation of target genes. The results suggest that the cooperative effects of PPARγ and RXR-specific ligands may occur at the level of selective coactivator recruitment (36). Endogenous PPARγ ligands such as fatty acids or their metabolites and RXR ligands such as 9-cis-retinoic acid naturally exist in cells. Thus these naturally occurring ligands would lead to a basal activity often observed for PPARγ without the addition of exogenous ligand (36). It could explain the level of mRNA PPARγ expression in GRX cell in early stages of culture. But when

the GRX cells were treated with ROH-RXR ligand, it potentiated the effect of these endogenous PPAR ligands.

These results should open a new paradigm of a novel cross-talk of nutritional transcription factors in energy metabolism where the nuclear concentrations of each receptors and ligands are crucial for nutritional regulation of metabolism lipid.

PPARγ is now shown to serve as a key transcription factor for HSC quiescence as it is considered as master regulated for adipogenesis. If HSC is similar to adipocytes, HSC quiescence should be facilitated by a group of adipogenic transcription factors besides PPARγ.

Sirtuins, a family of proteins**,** interact with PGC-1α, a coactivator that regulates cellular metabolism. SIRT1 regulates the activity of a variety of transcription factors and transcriptional co-regulators such as PPARγ. SIRT1 binds to PGC-1α, and inactivates PPARγ. This leads to the repression of genes involved in PPARγ-mediated fat storage and consequently results in mobilization of free fatty acid.

SIRT1 exerts at least some of its effects *in vivo* by modulating positively LXRs (37). SIRT1 deacetylates LXR and promote cholesterol efflux and lipid homeostasis in cells (37). In our cellular model, SIRT1 mRNA expression increases after treatment with RSV, but decreases in the presence of RHO. Our findings raise a possibility that SIRT1 may have a role in modulating transcription factors, such as PPARγ and LXR.

A role for the selective recruitment of coactivator either co-repressor complexes by specifics ligands must be considered to reverse the morphological and biochemical characteristics of activated HSC.

In conclusion, in our model RSV stimulated SIRT1 mRNA after 120 h of treatment. RSV did not induce the lipocyte phenotype because the PPARγ activity was repressed. Moreover, when associated with RHO, a RXR ligand, RSV did not block the action of RHO because RSV could modulate LXR that forms a heterodimer with RXR. Consequently the LXR-RXR-PPARγ recruited nuclear receptor coactivators, modulating lipogenesis (fat storage) in HSC and lipids homeostasis.

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REFERENCES:

1. CHENG Y, PING J, XU H D, FU H J, ZHOU Z H. Synergistic effect of a novel oxymatrine-baicalin combination against hepatitis B virus replication, alpha smooth muscle actin expression and type I collagen synthesis in vitro. World J Gastroenterol 2006; 12(32): 5153-9.
2. FRIEDMAN S L. Mechanisms of hepatic fibrogenesis. Gastroenterology 2008; 134(6): 1655-69.
3. CHENG Y, PING J, XU L M. Effects of curcumin on peroxisome proliferator-activated receptor gamma expression and nuclear translocation/redistribution in culture-activated rat hepatic stellate cells. Chin Med J (Engl) 2007; 120(9): 794-801.
4. GUIMARAES E L, FRANCESCHI M F, ANDRADE C M, GUARAGNA R M, BOROJEVIC R, MARGIS R, et al. Hepatic stellate cell line modulates lipogenic transcription factors. Liver Int 2007; 27(9): 1255-64.
5. DESVERGNE B. PPARdelta/beta: the lobbyist switching macrophage allegiance in favor of metabolism. Cell Metab 2008; 7(6): 467-9.
6. PETERS J M, CATTLEY R C, GONZALEZ F J. Role of PPAR alpha in the mechanism of action of the nongenotoxic carcinogen and peroxisome proliferator Wy-14,643. Carcinogenesis 1997; 18(11): 2029-33.
7. WANG Y X, LEE C H, TIEP S, YU R T, HAM J, KANG H, et al. Peroxisome-proliferator-activated receptor delta activates fat metabolism to prevent obesity. Cell 2003; 113(2): 159-70.
8. TEBOUL M, ENMARK E, LI Q, WIKSTROM A C, PELTO-HUIKKO M, GUSTAFSSON J A. OR-1, a member of the nuclear receptor superfamily that interacts with the 9-cis-retinoic acid receptor. Proc Natl Acad Sci U S A 1995; 92(6): 2096-100.
9. SONG C, KOKONTIS J M, HIIPAKKA R A, LIAO S. Ubiquitous receptor: a receptor that modulates gene activation by retinoic acid and thyroid hormone receptors. Proc Natl Acad Sci U S A 1994; 91(23): 10809-13.
10. IMAI S, ARMSTRONG C M, KAEBERLEIN M, GUARENTE L. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature 2000; 403(6771): 795-800.
11. LANDRY J, SUTTON A, TAFROV S T, HELLER R C, STEBBINS J, PILLUS L, et al. The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases. Proc Natl Acad Sci U S A 2000; 97(11): 5807-11.
12. SMITH J S, BRACHMANN C B, CELIC I, KENNA M A, MUHAMMAD S, STARAI V J, et al. A phylogenetically conserved NAD+-dependent protein deacetylase activity in the Sir2 protein family. Proc Natl Acad Sci U S A 2000; 97(12): 6658-63.
13. PICARD F, KURTEV M, CHUNG N, TOPARK-NGARM A, SENAWONG T, MACHADO DE OLIVEIRA R, et al. Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-gamma. Nature 2004; 429(6993): 771-6.
14. NEMOTO S, FERGUSSON M M, FINKEL T. SIRT1 functionally interacts with the metabolic regulator and transcriptional coactivator PGC-1{alpha}. J Biol Chem 2005; 280(16): 16456-60.
15. RODGERS J T, LERIN C, HAAS W, GYGI S P, SPIEGELMAN B M, PUIGSERVER P. Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. Nature 2005; 434(7029): 113-8.

16. BORDONE L, MOTTA M C, PICARD F, ROBINSON A, JHALA U S, APFELD J, et al. Sirt1 regulates insulin secretion by repressing UCP2 in pancreatic beta cells. PLoS Biol 2006; 4(2): e31.
17. MOYNIHAN K A, GRIMM A A, PLUEGER M M, BERNAL-MIZRACHI E, FORD E, CRAS-MENEUR C, et al. Increased dosage of mammalian Sir2 in pancreatic beta cells enhances glucose-stimulated insulin secretion in mice. Cell Metab 2005; 2(2): 105-17.
18. ARICHI H, KIMURA Y, OKUDA H, BABA K, KOZAWA M, ARICHI S. Effects of stilbene components of the roots of Polygonum cuspidatum Sieb. et Zucc. on lipid metabolism. Chem Pharm Bull (Tokyo) 1982; 30(5): 1766-70.
19. KIMURA Y, OHMINAMI H, ARICHI H, OKUDA H, BABA K, KOZAWA M, et al. Effects of Various Coumarins from Roots of Angelica dahurica on Actions of Adrenaline, ACTH and Insulin in Fat Cells. Planta Med 1982; 45(7): 183-7.
20. RAGAZZI E, FROLDI G, FASSINA G. Resveratrol activity on guinea pig isolated trachea from normal and albumin-sensitized animals. Pharmacol Res Commun 1988; 20 Suppl 5: 79-82.
21. RAYALAM S, DELLA-FERA M A, YANG J Y, PARK H J, AMBATI S, BAILE C A. Resveratrol potentiates genistein's antiadipogenic and proapoptotic effects in 3T3-L1 adipocytes. J Nutr 2007; 137(12): 2668-73.
22. KAWADA N, SEKI S, INOUE M, KUROKI T. Effect of antioxidants, resveratrol, quercetin, and N-acetylcysteine, on the functions of cultured rat hepatic stellate cells and Kupffer cells. Hepatology 1998; 27(5): 1265-74.
23. SOUZA I C, MARTINS L A, COELHO B P, GRIVICICH I, GUARAGNA R M, GOTTFRIED C, et al. Resveratrol inhibits cell growth by inducing cell cycle arrest in activated hepatic stellate cells. Mol Cell Biochem 2008; 315(1-2): 1-7.
24. BOROJEVIC R, MONTEIRO A N, VINHAS S A, DOMONT G B, MOURAO P A, EMONARD H, et al. Establishment of a continuous cell line from fibrotic schistosomal granulomas in mice livers. In Vitro Cell Dev Biol 1985; 21(7): 382-90.
25. MARGIS R, BOROJEVIC R. Retinoid-mediated induction of the fat-storing phenotype in a liver connective tissue cell line (GRX). Biochim Biophys Acta 1989; 1011(1): 1-5.
26. LIVAK K J, SCHMITTGEN T D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25(4): 402-8.
27. GUIMARAES E L, FRANCESCHI M F, GRIVICICH I, DAL-PIZZOL F, MOREIRA J C, GUARAGNA R M, et al. Relationship between oxidative stress levels and activation state on a hepatic stellate cell line. Liver Int 2006; 26(4): 477-85.
28. FOLCH J, LEES M, SLOANE STANLEY G H. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 1957; 226(1): 497-509.
29. BOROJEVIC R, GUARAGNA R M, MARGIS R, DUTRA H S. In vitro induction of the fat-storing phenotype in a liver connective tissue cell line-GRX. In Vitro Cell Dev Biol 1990; 26(4): 361-8.
30. PETERSON G L. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem 1977; 83(2): 346-56.
31. KERSTEN S, DESVERGNE B, WAHLI W. Roles of PPARs in health and disease. Nature 2000; 405(6785): 421-4.

32. VICENTE C P, GUARAGNA R M, BOROJEVIC R. Lipid metabolism during in vitro induction of the lipocyte phenotype in hepatic stellate cells. Mol Cell Biochem 1997; 168(1-2): 31-9.
33. RAYALAM S, DELLA-FERA M A, BAILE C A. Phytochemicals and regulation of the adipocyte life cycle. J Nutr Biochem 2008; 19(11): 717-26.
34. JUVET L K, ANDRESEN S M, SCHUSTER G U, DALEN K T, TOBIN K A, HOLLUNG K, et al. On the role of liver X receptors in lipid accumulation in adipocytes. Mol Endocrinol 2003; 17(2): 172-82.
35. SCHULTZ J R, TU H, LUK A, REPA J J, MEDINA J C, LI L, et al. Role of LXRs in control of lipogenesis. Genes Dev 2000; 14(22): 2831-8.
36. YANG W, RACHEZ C, FREEDMAN L P. Discrete roles for peroxisome proliferator-activated receptor gamma and retinoid X receptor in recruiting nuclear receptor coactivators. Mol Cell Biol 2000; 20(21): 8008-17.
37. LI X, ZHANG S, BLANDER G, TSE J G, KRIEGER M, GUARENTE L. SIRT1 deacetylates and positively regulates the nuclear receptor LXR. Mol Cell 2007; 28(1): 91-106.

FIGURE LEGENDS:

Figure 1: Phase contrast images of GRX cells. **Myo**: GRX in standard culture medium; **RSV**: GRX after 120 h treatment with RVS 100 nM; **RHO**: GRX after 120 h treatment with RHO 5μM; **RSV+RHO**: GRX after 120 h treatment with RHO 5μM plus RVS 100 nM. **N** = nucleus; **LD** = lipid droplet stained with Oil Red O.

Figure 2: Relative mRNA expression of peroxisome proliferator-activated receptor gamma (**PPARγ**) or sirtuin 1 (**SIRT1**). **(a)** GRX-hepatic stellate cell cells were left untreated, maintaining the myofibroblast phenotype (**Myo**), or treated with 100nM resveratrol (**RSV**), or 5μM retinol (**RHO**), or 5μM retinol plus 100nM resveratrol (**RSV+RHO**) for 12 , 24 or 120 hours and the relative transcript levels were measured by real-time polymerase chain reaction. **(b)** represents the PPARγ/Sirt1 expression ratio. Data represent means ± SEM, means without a common letter (PPARγ) or capital letters (SIRT1) are statistically different, $p < 0.05$. ANOVA/ SNK post hoc.

Figure 3: [^{14}C] Acetate incorporation into phospholipids (PL) and triacylglycerols (TG)**.** GRX-hepatic stellate cell cells were left untreated, maintaining the myofibroblast phenotype (Myo), or treated with 100nM resveratrol (RSV), or 5μM retinol (RHO), or 5μM retinol plus 100nM resveratrol (RSV+RHO) for (A) 24 h or (B) 120 h. They were incubated for subsequent 16 h with radiolabeled acetate, lipids were extracted, and analyzed as described Material and Methods. Results are expressed as percentage (%) of total radioactivity incorporated and correspond to densitometric analysis of TLC-autoradiographic film of two experiments done in triplicate. Means without a common letter (PL) or symbol (TG) are statistically different, $P<0.05$. ANOVA/SNK post hoc.

Figure 4: Accumulation of triacylglicerol in GRX lipid droplets. Cells were treated as described Material and Methods for 120 h. Lipid content was quantified using commercially available assay reagent (Triglycerides Liquiform Labtest, MG, Brasil).The triacylglicerol content was detertermined Results are expressed as mg TG/ mg protein. Means without a common letter are statistically different, $P<0.05$. ANOVA/SNK post hoc.

Table 1: Primers used for real time PCR

	forward primer	reverse primer
PPARγ	5'-TGGAATTAGATGACAGTGACTTGG	5'-CTCTGTGACGATCTGCCTGAG
SIRT1	5'-GGCTTGAGGGTAATCAATACCTG	5'-AAACTTGGACTCTGGCATGTG
β-actin	5'-TATGCCAACACAGTGCTGTCTGG	5'-TACTCCTGCTTGCTGATCCACAT

FIGURE 1:

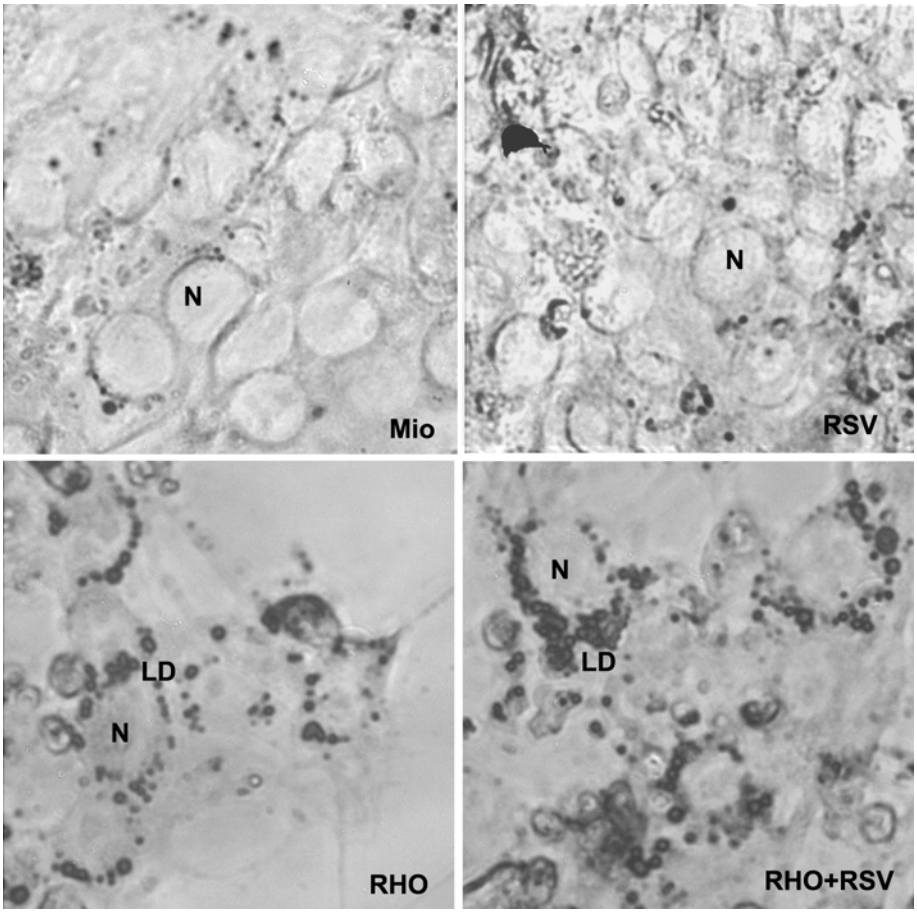
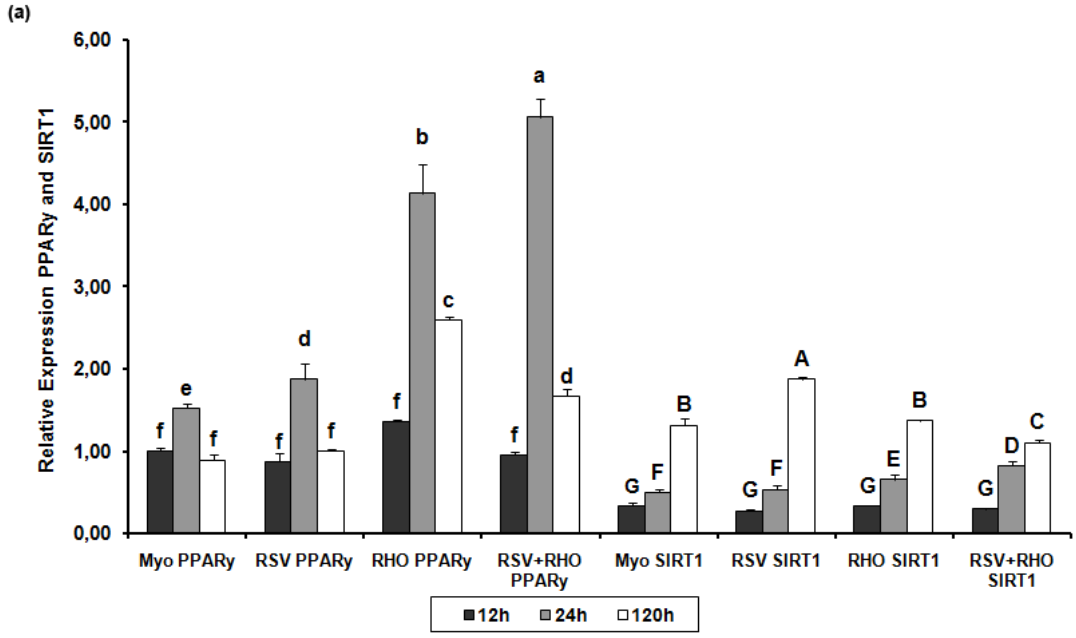


FIGURE 2:


(a)
Relative Expression PPARγ and SIRT1
6,00
5,00
4,00
3,00
2,00
1,00
0,00
Myo PPARγ
RSV PPARγ
RHO PPARγ
RSV+RHO PPARγ
Myo SIRT1
RSV SIRT1
RHO SIRT1
RSV+RHO SIRT1
12h
24h
120h

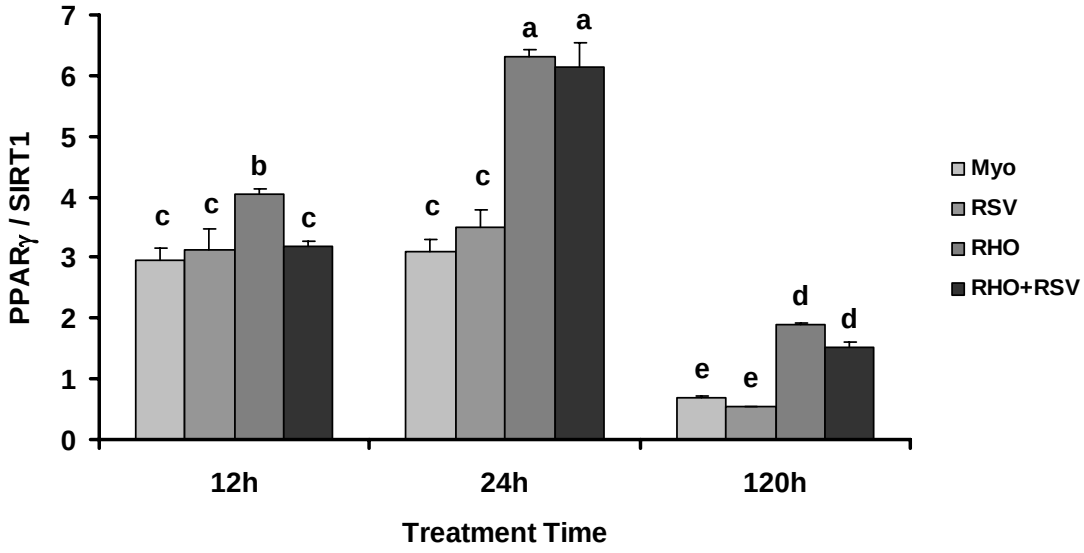

PPARγ / SIRT1
7
6
5
4
3
2
1
0
12h
24h
120h
Treatment Time
Myo
RSV
RHO
RHO+RSV

FIGURE 3:

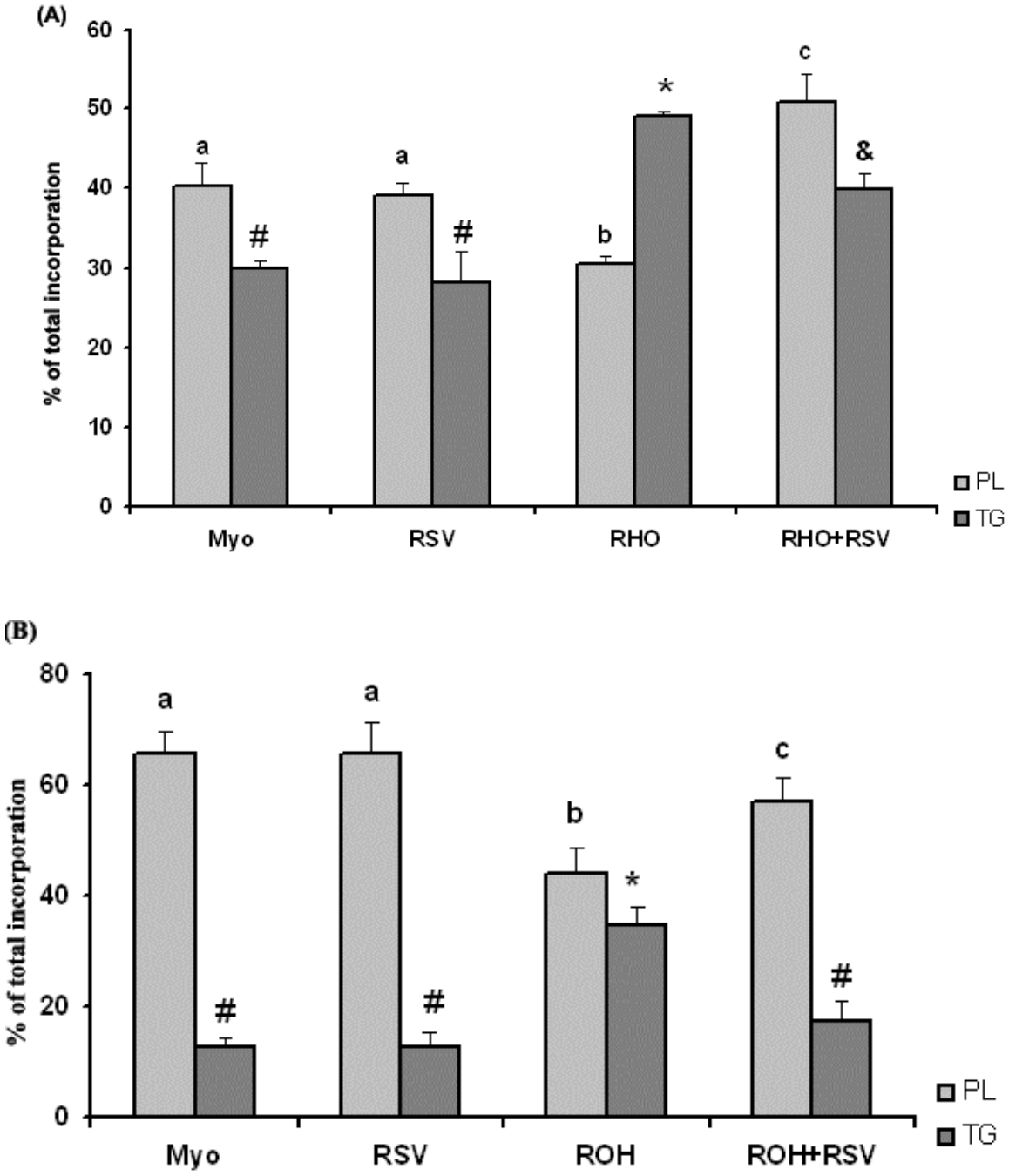
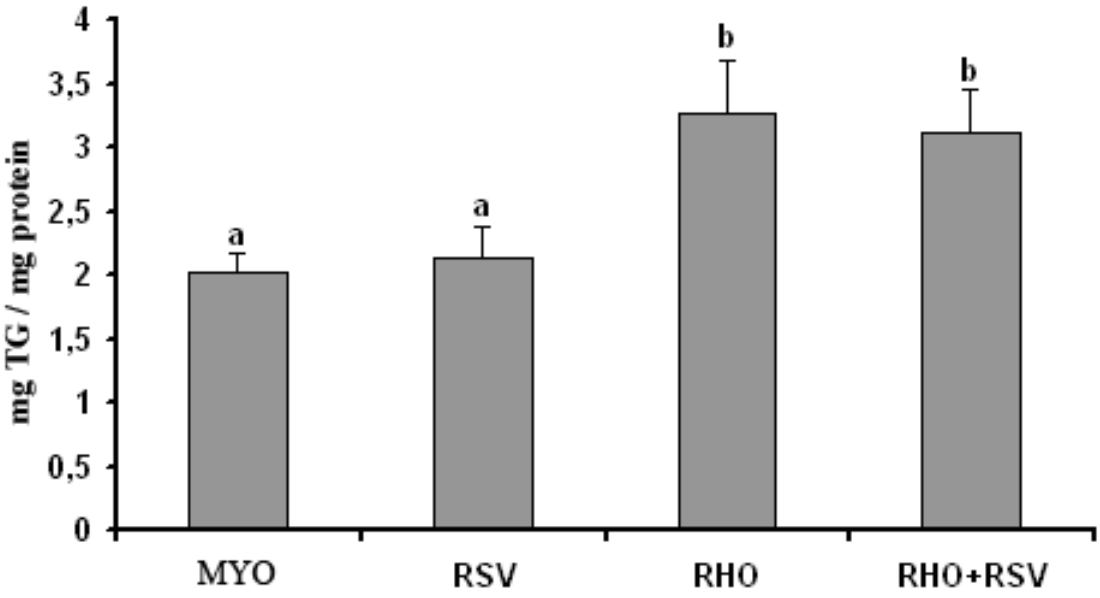

(A)
% of total incorporation
60
50
40
30
20
10
0
a
a
b
c
#
#
*
&
Myo
RSV
RHO
RHO+RSV
PL
TG
(B)
% of total incorporation
80
60
40
20
0
a
a
b
c
#
#
*
#
Myo
RSV
ROH
ROH+RSV
PL
TG

FIGURE 4:



CAPÍTULO III

RESULTADOS COMPLEMENTARES

Efeito do resveratrol, retinol e resveratrol associado ao retinol sobre a organização do citoesqueleto de actina nas células GRX.

Introdução

Estudos detalhados sobre o fenótipo expresso por células estreladas hepáticas quiescentes ou ativadas mostraram que estas representam uma população celular heterogênea, em termos de sua função e expressão de proteínas citoesqueléticas (Guma *et al*, 2001). A origem deste tipo celular é considerada miogênica devido à expressão de actina de músculo liso (α-SMA) (Pinzani, 1999). Os receptores de adesão de integrinas estabelecem conexão entre a matriz extracelular e a fibra citoesquelética da actina, emitindo movimentos e sinais bioquímicos através da membrana plasmática (Calderwood *et al*, 2000).

O presente estudo tem por objetivo mostrar o efeito do RSV, RHO e RSV+RHO sobre a organização do citoesqueleto de actina na linhagem celular GRX. Observamos que o tratamento com RSV provocou alterações no citoesqueleto de actina sem que ocorra a diferenciação diferenciação fenotípica.

Materiais e Métodos

Cultura celular

As células GRX foram obtidas do banco de células do Rio de Janeiro (Universidade Federal do Rio de Janeiro). As células foram mantidas em meio Dulbecco`s (DMEM, Sigma Chemical Company, St. Louis, MO, USA) suplementado com 5% soro fetal bovino (FBS) (Cultilab, Campinas, SP) e tampão 2 g/L HEPES, pH 7.4, sob uma atmosfera contendo 5% CO2.

Tratamento Celular

As células foram plaqueadas sobre lamínulas em placas de 24 poços (5 x 10^4 células/poço). Após, 24h as culturas foram tratadas por 120 h com meio suplementado com RSV (100 nM); RHO(5μM) ou RSV+RHO, nas mesmas doses,

Imunocitoquímica das células GRX

Após o tratamento as células foram fixadas em 4% de paraformaldeído tamponado por 30 min, permeabilizadas com 0,5% Triton X-100 em PBS, 3 vezes por 10 min. Incubadas com PBS contendo 3% albumina bovina (BSA) por 1 h à 37ºC para bloquear os sítios não específicos. Após as lamínulas foram incubadas com rodamina/faloidina (3.3 mM, 1:100 diluição em PBS) por , 20 min). As lamínulas foram montadas em *Fluorsave*. As imagens foram adquiridas em microscópio confocal (Carl Zeiss LSM5 Pascal laser scanning confocal microscope) com argon/helium/neon laser e em objetiva de 63x (1.4) com óleo de imersão.

Resultado e Conclusão

O RSV induz a fragmentação das fibras de actina (Figura 1). Na mesma figura pode-se observar que nos lipócitos induzidos por RHO + RSV a presença de fragmentos difusos ou granulares de actina na zona perinuclear e em torno das gotas lipídicas como acontece no tratamento apenas com RHO (Mermelstein et al. ,2001).

No fenótipo lipocítico as extensões citoplasmáticas são reduzidas e essa condição corresponde, sobretudo, a mudança na organização do citoesqueleto (Guma et al., 2001).

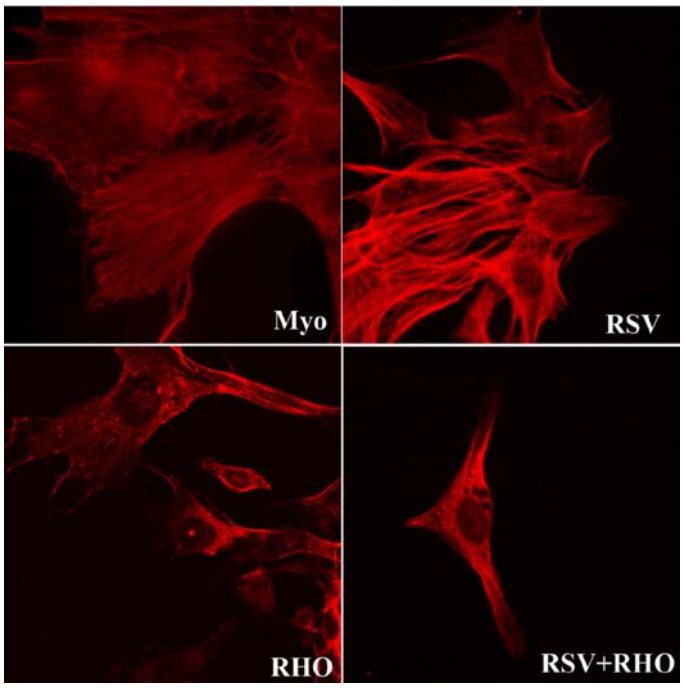


Figura 1. Efeito do resveratrol (RSV), retinol (RHO) e RSV associado ao RHO sobre a organização do citoesqueleto de actina nas células GRX evidenciado pela marcação com rodamina-faloidina. As células foram tratadas com 100nM RSV, RHO 5μM e RSV+RHO nas mesmas concentrações. Myo = miofibroblasto

PARTE III

III.1 DISCUSSÃO

O resveratrol (RSV) é uma fitoalexina com potente ação terapêutica. Nestes últimos anos o interesse por este composto tem aumentado e muitos estudos têm mostrado o efeito protetor do RSV em diferentes patologias (Baur e Sinclair, 2006).

O fígado tem sido alvo de muitas pesquisas que evidenciam o efeito protetor do RSV, pois os hepatócitos são os maiores sítios de bioacumulação, de bioconjugação e de eliminação deste composto. Portanto, é interessante elucidar seus efeitos na biologia da célula hepática (Notas et al., 2006). Estudos em culturas celulares indicam que o RSV pode modular várias vias metabólicas envolvendo crescimento celular, apoptose e inflamação (Athar et al., 2007).

Estudos epidemiológicos têm atribuído aos alimentos e bebidas ricas em RSV um papel benéfico em numerosas patologias. Muitas pesquisas são direcionadas para o câncer hepatocelular, e apesar das limitações do cultivo celular, estudos com células HepG2 tratadas com RSV em concentrações compatíveis com as encontradas nos alimentos e bebidas, tentam reproduzir *in vitro* a ação benéfica do RSV (Notas et al., 2006).

Em mamíferos o RSV tem sido associado à ativação de genes como o da sirtuína1 (SIRT1) levando a uma mobilização de ácidos graxos em tecido adiposo branco através da repressão do gene do PPARγ (Picard et al., 2004). Nos hepatócitos o RSV previne o acúmulo de gotas lipídicas (Baur e Sinclair, 2006). Em adipócitos 3T3-L1, está envolvido com a ativação da lipólise e com a indução da apoptose. Muitos autores têm sugerido que o resveratrol modula

processos metabólicos através do bloqueio da adipogênese. (Rayalam et al., 2007; Yang et a l, 2008).

A regeneração tecidual desencadeada pela proliferação dos hepatócitos é necessária para o reparo e sobrevivência do tecido hepático após uma injúria aguda, transplante ou em doenças crônicas, como cirrose e fibrose (Taub R., 2004). O estímulo para a proliferação dos hepatócitos depende da interação destes com outros tipos celulares hepáticos, onde se incluem as células estreladas hepáticas (HSCs) (Taub R., 2004). Alvo das injúrias, as HSCs diferenciam-se para miofibroblastos, secretam matriz extracelular (ECM) e fatores de crescimento que induzem a proliferação dos hepatócitos (Balabaud et al., 2004). Embora, a diferenciação das HSCs seja considerada um processo fundamental para a indução da regeneração hepática, os mecanismos moleculares que regulam a transição para o reparo e suporte das células no fígado ainda são pouco entendidos (Passino et al., 2007).

O nosso grupo de pesquisa tem estudado os mecanismos que levam à transformação fenotípica da célula GRX representativa das HSCs murinas que apresenta fenótipo miofibroblástoide, podendo ser induzida a expressar o fenótipo lipocítico semelhante as HSCs em estado quiescente. Considerando as ações do RSV descritas em outras linhagens celulares (Notas et al., 2006), este trabalho teve como objetivo analisar os efeitos deste composto sobre a linhagem GRX, visando estudar efeitos hepatoprotetores para esta fitoalexina.

Utilizando tratamentos de 1nM a 1μM, doses similares as que são biodisponibilizadas após a metabolização de alimentos no sistema gastrointestinal, mostramos que o RSV apresentou efeito inibitório sobre a

proliferação das células GRX, induziu um aumento no número de células na fase S do ciclo celular e um efeito pró-apoptótico (Capitulo I).

Observamos uma diminuição no crescimento celular em cerca de 35%, o que está em concordância com o descrito em linhagens hepáticas como HepG2 (Notas et al., 2006). Esses e outros autores mostraram que o efeito do RSV sobre a proliferação celular é devido a sua ação sobre o ciclo celular. Stervbo e colaboradores (2006) mostraram que a ação do RSV sobre a proliferação e o ciclo celular depende do tempo de tratamento e das concentrações usadas.

Como nas células GRX, Ragione e colaboradores (1998) também mostraram em células HL-60, que o RSV causa uma parada do ciclo celular na fase S. Os autores sugerem que esse efeito decorre de uma diminuição na síntese de DNA devido à inibição da ribonucleotideo redutase e da DNA polimerase.

Outro aspecto interessante a ser considerado é a ação do RSV em induzir a apoptose (Joe et al., 2002; Garvin et al., 2006; Zhang W et al., 2006 e Kern et al., 2007). Nossos resultados mostraram um maior percentual de células em apoptose quando tratadas por 120 h com RSV com 100 nM e 1 μM de RSV .

As células estreladas hepáticas são pericitos específicos que contribuem para a angiogênese durante o desenvolvimento, regeneração e injúria hepática. Dados na literatura mostram que a apoptose e/ou a reversão das células ativadas para o fenótipo quiescente podem ser alvo da terapia antifibrogênica (Friedman, 2008). Malhi e Gores (2008) sugerem que a modulação diferencial da apoptose nos hepatócitos e nas HSCs poderia resultar em benefícios

terapêuticos para a injuria hepática. Assim, tratamentos que reduzem a apoptose dos hepatócitos diminuiriam a inflamação enquanto a apoptose nas HSCs resultaria em redução da fibrogênese.

Alguns pesquisadores consideram a apoptose um potencial fator para as células estreladas hepáticas ativadas. Estas células são mais suscetíveis a este processo e sofrem morte celular espontânea, pela ativação de um receptor mediador de morte ou ainda morrem por privação de soro *in vitro* ou por sinalização de citoquinas (Battaler et al., 2005).

A senescência das células estreladas hepáticas pode ser um importante processo para diminuição da proliferação e favorecimento da regeneração das células parenquimais hepáticas. O aumento da atividade colagenolítica, através das metaloproteínases (MMP), é o principal mecanismo envolvido na resolução da fibrose (Battaler et al., 2005). Estas enzimas são secretadas por muitos tipos celulares e requerem modificação pós-translacional para se tornarem ativas. Uma vez ativadas elas podem ser inibidas por seus inibidores naturais como: TIMP (inibidor de MMP de matriz tecidual). As MMP de matriz, especificamente as colagenases intersticiais (MMP-1, 9 e 13) são as responsáveis pela clivagem do colágeno tipo1, mantendo a arquitetura hepática normal. Por outro lado, o aumento da expressão de TIMP-1 resulta na inibição das MMP, favorecendo a progressão da fibrose. Portanto, a apoptose das HSCs elimina não somente a maior fonte de produção de colágeno, mas também a maior fonte de produção de TIMP-1, levando ao aumento da atividade das MMP, colagenases intersticiais, e a subsequente degradação de ECM (Iridale et al., 2001). A reversão da fibrose hepática também pode estar associada à interleucina 10 (IL10) que é estimulada durante a ativação das

células estreladas gerando uma sinalização autócrina de *feedback* negativo limitando a migração das células para o reparo tecidual (Wang et al., 1998; Thompson et al., 1998). Desta forma, citoquinas solúveis podem reprimir a ativação das HSCs por induzirem a reconstituição da ECM subendotelial. Sabe-se também, que quando HSCs são cultivadas sobre componentes de membrana basal (substrato matrigel) permanecem quiescentes (Olaso et al., 2001).

Nas células GRX, o RSV, nas doses e tempos testados neste trabalho, não desencadeou a formação de gotas lipídicas, portanto não induziu a diferenciação para o fenótipo lipocítico. Entretanto, quando adicionado ao meio de cultura junto com agentes que sabidamente induzem o fenótipo lipocítico, como o retinol (RHO) (Capítulo II) e a indometacina (INDO) (dados não mostrados), não impediu a diferenciação celular, como mostrado na figura 3 (Capítulo II).

Com o intuito de entender o processo de diferenciação das células GRX miofibroblásticas para lipócito, (Guimarães et al. 2007) analisaram a expressão do mRNA de fatores de transcrição, tais como: PPARα, PPARδ/β e PPARγ. Estes fatores estão ligados à diferenciação de pré-adipócitos em adipócitos descritos recentemente por (Joosen et al. 2008). Em condições basais de cultura, quando apresentam fenótipo de miofibroblasto, as células GRX expressam os fatores de transcrição PPARα, PPARδ/β e PPARγ. Trabalhos anteriores mostram que após a indução da diferenciação lipocítica das células GRX com a INDO ou com RHO houve um aumento nos níveis do mRNA do PPARγ , enquanto os de PPARδ/β diminuíram. Já o tratamento com INDO levou, também, a um aumento na expressão do mRNA do PPAR-α (Guimarães

et al., 2007). Além disso, estas drogas modulam a expressão de outros genes adipogênicos, como: LXRα, C/EBPα e SREBP1 na linhagem GRX (Guimarães et al., 2007).

Os PPARs são importantes na regulação do metabolismo dos lipídios e da glicose, na proliferação e diferenciação celular, na adipogênese e em vários tecidos sinalizam processos inflamatórios (Kersten et al., 2000). Sua ação depende da formação de heterodímeros com o receptor do ácido 9-cis retinóico (RXR) (Werman et al., 1997).

Vários estudos mostram que o agonista do PPARα, o GW647 aumenta a oxidação de ácidos graxos e induz à expressão do mRNA de genes envolvidos na captação de ácidos graxos e β-oxidação (Muoio et al., 2002; Granlund et al., 2005). *In vitro*, a ativação do PPARδ/β promove a oxidação e utilização de ácidos graxos em adipócitos e células musculares esqueléticas (Reddy e Hashimoto, 2001). O agonista do PPARδ/β, o GW501516 atenua o aumento de tecido adiposo, por ativar os genes que promovem o catabolismo de lipídios e desacoplamento mitocondrial em músculo esquelético de camundongos submetidos à dieta rica em lipídios (Cho et al., 2008). O PPARγ promove a diferenciação para adipócito por induzir armazenamento de lipídios e a transcrição de várias proteínas lipogênicas (Tontonoz et al., 1994; Roosen et al., 2001).

O processo de diferenciação adiposa é complexo, envolvendo a modulação dos PPARγ, PPARα e PPARδ/β. Estes receptores nucleares podem promover a diferenciação celular, quando ativados isoladamente ou associados a diferentes indutores. A razão entre PPARγ e β pode ser controlada por agonistas comuns a ambos PPARs, regulando a adipogênese e estimulando a

oxidação de ácidos graxos (Cho et al., 2008). Também foi relatado que agonistas comuns aos PPARα e β promovem a hiperlipidemia, a resistência à insulina e aumentam o risco de aterosclerose (Cho et al., 2008). Além disso, foi demonstrado que o clofibrato, um ligante de PPARα, aumenta a diferenciação das células HL-60 induzidas pelo ácido retinóico e trans-retinol (Balakumar et al., 2007). Ligantes endógenos como os ácidos graxos insaturados, se ligam aos três PPARs, estimulando mais fortemente o PPARα, enquanto que os ácidos graxos saturados apresentam uma fraca ligação aos PPARs em geral (Barrera et al., 2008).

Outro grupo de receptores nucleares, os LXRs (Liver X receptors - receptores X do fígado), desempenha um papel crucial na regulação da síntese de ácidos biliares, lipogênese e no metabolismo de colesterol (Peet et al., 1998; Alberti et al., 2001). O LXRβ é expresso ubiquamente (Song et al., 1994; Teboul et al., 1995), enquanto o LXRα é expresso predominantemente em tecidos conhecidos com funções importantes no metabolismo de lipídios, como o fígado, intestino delgado, rim, músculo esquelético e tecido adiposo entre outros (Apfel et al., 1994; Repa et al., 2000). Recentemente, estudos mostraram que o LXRα controla a capacidade de estocar lipídios pelos adipócitos (Juvet et al., 2003) e hepatócitos (Schultz et al., 2000). Os mesmos autores também mostram que o LXRα é um gene alvo do PPARγ em adipócitos e em hepatócitos.

Considerando a importância de xenobióticos provenientes da dieta, que possam constantemente modular a expressão de genes, procuramos avaliar o mecanismo molecular da ação do RSV, sobre as células estreladas hepáticas.

Desta forma, avaliamos a expressão de mRNA de PPARs e da SIRT1 nestas células quando tratadas com RSV.

Nossos resultados mostraram que o RSV não alterou, na GRX, a expressão do mRNA do receptor nuclear PPARγ, envolvido na lipogênese. Estes resultados corroboram com os descritos anteriormente, que mostram que o RSV não promoveu a diferenciação de miofibroblasto em lipócito. Entretanto, quando adicionamos RHO juntamente com RSV, observamos um aumento significativo na expressão do mRNA do PPARγ, embora menor que o encontrado nas células tratadas somente com RHO (Figura 2; Capitulo II). Por outro lado, o tratamento por 120 h com 100 nM de RSV não modulou a expressão do mRNA do PPARα (dados não mostrados). Guimarães et al., (2007) observaram um aumento da expressão do mRNA do PPAR-α após a indução da diferenciação lipocítica das células GRX com a INDO.

Como vários trabalhos mostram que o RSV é um potente ativador da expressão do mRNA e atividade da SIRT1 e que esta reprime a atividade do PPARγ (Picard et al., 2004; Wood et al., 2004; Howitz et al., 2003; Yeung et al., 2004), resolvemos verificar o nível de expressão desse modulador de PPARγ nas células GRX tratadas com RSV.

A SIRT1 é uma deacetilase nuclear envolvida na modulação do PPARγ. Rodgers e colaboradores (2005) descreveram a relação da SIRT1 com o envelhecimento e também e com o metabolismo hepático da glicose, através da regulação do co-ativador 1 do PPARγ (PGC-1-alfa). Também foi descrito que em hepatócitos a SIRT1 está associada fisicamente com PGC-1α e que regula a atividade de vários fatores de transcrição e co-reguladores

transcricionais, tais como p53, Ku70, FoxO1, NF-kB, PPARγ e p300 (Leibiger e Berggren, 2006).

A SIRT1 está associada à modulação da expressão gênica em diferentes tecidos, tais como: fígado, cérebro, tecido adiposo, células beta do pâncreas e músculo. É regulada positivamente por NAD^+, por isso é dependente das flutuações das concentrações deste cofator. A atividade da SIRT1 responde ao "status" nutricional. No jejum, a SIRT1 inativa o PPARγ através da ligação aos co-repressores PGC-1-α e PGC-1-β, levando a repressão de genes induzidos pelo PPARγ e que estão envolvidos no armazenamento de lipídios. Qiang e colaboradores (2007) mostraram que a supressão da SIRT1 ativa PPARγ através da expressão do Ero1-Lα (oxidoredutase de retículo endoplasmátco), estimulando a secreção de adiponectina em adipócitos maduros.

Nossos resultados mostraram que a expressão do mRNA da SIRT1 aumenta com o tempo de cultura, independente do tratamento com RSV e/ou RHO. O RHO não tem ação sobre a expressão do mRNA da SIRT1. (Figura 2; Capitulo II), o tratamento com 100 nM de RSV por 120 h provocou um aumento significativo nos transcritos de SIRT1 em comparação com os outros grupos experimentais. Já, o tratamento conjunto com RSV+RHO, pelo mesmo tempo, fez com os níveis de expressão de SIRT1 fossem significativamente menores que os encontrados nos outros grupos.

Como já descrito anteriormente o RHO modulou positivamente o PPARγ provocando a lipogênese, constatada nas imagens mostradas na figura 1 (Capítulo II) e pela incorporação de [C^{14}]-acetato em triacilglicerídios (TG) (Figura 3A e B; Capitulo II).

Em nosso modelo celular, o RSV modulou positivamente a expressão do mRNA da SIRT1, mas não ativou a expressão do mRNA do PPARγ após 120 h (Figura 2; Capitulo II), consequentemente não estimulou a síntese e acumulo de TG (Figuras 3 e 4; Capitulo II). Sendo que, após 120 h, o aumento da expressão da SIRT1 no grupo RSV foi significativamente maior que nos outros grupos. Entretanto, quando tratamos as células com RSV+RHO por 120 h, verificamos que ocorreu diferenciação fenotípica, comprovada pela alteração na morfologia e pelo acúmulo de gotas lipídicas.

O tratamento com RSV por 24h induziu um aumento significativo da expressão do mRNA do PPARγ em todos os grupos experimentais, inclusive nos miofibroblastos. É interessante salientar, que esse aumento foi maior no grupo tratado com RSV+RHO. Após 120 h, os níveis de transcritos de PPARγ diminuem quando comparados aos encontrados após 24 h de tratamento; voltando aos níveis basais (12 h de tratamento) nos miofibroblastos e no grupo RSV. Nos grupos RHO ou RSV+RHO a diminuição da transcrição foi menor, mantendo os níveis de transcrição significativamente aumentados em relação aos grupos miofibroblastos e RSV.

A relação entre os níveis de transcrição de PPARγ e SIRT 1 (figura 2b, Capítulo II) indica que predominância da expressão de SIRT1 sobre a do PPARγ, como acontece nos grupos miofibroblastos e RSV (120 h), impede a indução do fenótipo lipocítico. Esta constatação é reforçada pelos experimentos mostrados nas figuras 1, 3 e 4 (Capítulo II).

Esses resultados nos levam a sugerir a existência de uma relação mínima gerando um balanço entre as expressões desses dois genes. Assim, o

RSV sozinho por não induzir o aumento de PPARγ e estimular a expressão da SIRT1 não desencadeia a diferenciação lipocítica nas células GRX.

Especificamente com o objetivo de entender o efeito do RSV sobre a síntese e acúmulo de lipídios das células GRX, monitoramos a incorporação de acetato radioativo em TG e o conteúdo destes. Após 24 h de tratamento com RHO ou com RSV+RHO verificamos um aumento significativo de incorporação de acetato em TG. A análise da relação PPARγ/SIRT1 (figura 2b, Capítulo II) reforça a hipótese da necessidade de predominância da expressão de PPARγ sobre a de SIRT1; em ambos os grupos experimentais a expressão do PPARγ é aproximadamente cinco vezes maior que a da SIRT1.

Os níveis de expressão de PPARγ diminuem com o aumento do tempo (120 h) de tratamento em todos os grupos experimentais. Por outro lado, a expressão da SIRT1 continua crescendo. Quando analisamos a incorporação de acetato em TG após 120 h de tratamento, verificamos que esta permanece aumentada somente no grupo RHO. No entanto, o conteúdo de TG é semelhante nos grupos RHO e RSV+RHO V (figuras1, 3 e 4, Capitulo II).

Nossos resultados colocam em discussão a possível relação da SIRT1 em modular fatores de transcrição, como PPARγ. Assim, o RSV pode alterar a razão PPARγ/SIRT1 regulando o metabolismo de lipídios, mas não participando do processo de transformação fenotípica da célula GRX.

III.2 CONCLUSÕES:

- ✓ O RSV apresenta uma ação anti-proliferativa e pró-apoptótica sobre as células GRX;

- ✓ O RSV pode apresentar uma ação quimioprotetora no fígado.

- ✓ O RSV não altera o fenótipo miofibroblástico destas células;

- ✓ O RSV provoca alteração na organização do citoesqueleto de actina;

- ✓ O RSV não impede a ação do RHO na lipogênese e na indução de fenótipo ;

- ✓ O tratamento simultâneo com RSV+RHO, mostra um efeito estimulador do RHO sobre a expressão do mRNA do PPARγ se sobrepondo ao efeito do RSV, resultando em lipogênese;

- ✓ Quando a expressão do mRNA da SIRT1 é favorecida pelo RSV, a lipogênese não é estimulada;

- ✓ O aumento da expressão do mRNA do PPARγ em relação a SIRT1 é indispensável para a lipogênese nesta linhagem;

REFERÊNCIAS

Ahmad N, Adhami V M, Afaq F, Feyes DK, and Mukhtar H (2001). Resveratrol causes waf-1/p21-mediated G1-phase arrest of cell cycle and induction of apoptosis in human epidermoid carcinoma A431 cells. *Clin. Cancer Res*., *7:* 1466–73.

Alberti S, Schuster G, Parini P, Feltkamp D, Diczfalusy U, Rudling M, Angelin B, Bjorkhem I, Pettersson S, J Â, Gustafsson (2001). Hepatic cholesterol metabolism and resistence to dietary cholesterol in LXRβ-deficient mice. *J. Clin. Invest.*, 107: 565-573.

Anthony PP, Ishak KG, Nayak NG, Poulsen HE, Scheuer PJ, Sobin LH (1978).The morphology of cirrhosis: recommendations on definition, nomenclature, and classification by a working group sponsored by theWorld Health Organization. *J Clin Pathol.,* 31:395–414.

Apfel R, Benbrook D, Lernhardt Ortiz MA, Salberg G, Pfahl M, (1994). A novel orphan receptor specific for a subset of thyroid hormone-responsive elements and its interaction with the retinoid/thyroid hormone receptor subfamily. *Mol. Cell. Biol*., 14: 7025-35.

Athar M, Back JH, Tang X, Kim, KH Kopelovich L, Bickers DR, Kim AL (2007). Resveratrol: A review of preclinical studies for human cancer prevention. *Toxicology and Applied Pharmacology*, 224: 274-283.

Azios NG and Dhamawardhane SF (2005). Resveratrol and Estradiol Exert Disparate Effects on Migration, Cell Surface Actin Structures, and Focal Adhesion Assembly in MDA-MB-231 Human Breast Cancer cells. *Neoplasia,* 7:2: 128-140.

Balabaud C, Sage PB, Desmoulière A (2004). The role of hepatic stellate cells in liver regeneration. *Journal of Hepatology,* 40: 1023-26.

Balakumar P, Rose M, Singh M, (2007). PPAR ligands: are they potential agents for cardiovascular disorders? *Pharmacology*, 80: 1-10.

Balliga MS and Katiyar SK (2005). Chemoprevention of photocarcinogenesis by selected dietary botanicals. *Photochemical & Photobiological Sciences,* 5:243-253.

Banerjee S, Bueso-Ramos C, Aggarwal BB, (2002) Suppression of 7,12-Dimethylbenz(a)anthracene-induced mammary carcinogenesis in rats by resveratrol: role of nuclear factor-kappa B, cyclooxygenase 2, and matriz metalloprotease 9, *Cancer Res.,* 62:4945-54.

Barrera G, Toaldo C, Pizzimenti S, Cerbone A, Pettazzoni P, Dianzani M, Ferreti C, (2008). The Role of PPAR Ligands in Controlling Growth-Related Gene Expression and their Interaction with Lipoperoxidation Products. *PPAR Research*, 2008:1-15.

Bataller R and Brenner DA, (2005). Liver fibrosis. *The Journal of Clinical Investigation,* 115:209–218.

Baur JA and Sinclair DA (2006). Therapeutic potential of resveratrol: the in vivo evidence. *Nature Reviews/Drugs Discovery*, 5:493-506.

Bernhard D, Tinhofer I, Tonko M, Hübi H, Ausserlechner MJ, Greil R, Kofler R, Csordas A (2000). Resveratrol causes arrest in the S-phase prior to Faz-independent apoptosis in CEM-C7H2 acute leukemia cells. *Cell Death and Differentiation*, 7:834-42.

Bissel DM, (1992) Lipocyte activation and hepatic fibrosis. *Gastroenterology,* 102:1803-05.

Blonhoff R, Wake K (1991) Perisinusoidal stellate cell os the liver : important roles in retinal metabolism and fibrosis. *FASEB J*., 5:271-277.

Borojevic R, Monteiro AN, Vinhas SA, Domont GB, Mourao PA, Emonard H, Grimaldi G, Jr., Grimaud JA (1985). Establishment of a continuous cell line from fibrotic schistosomal granulomas in mice livers. *In Vitro Cell Dev Biol.,* 21:382-390.

Borojevic R, Guaragna RM, Margis R, Dutra HS (1990) In vitro induction of the fat-storing phenotype in a liver connective tissue cell line-GRX. *In Vitro Cell Dev* Biol., 26:361-368.

Bowers JL, Tyulmenkov VV, Jernigan SC, Klinge CM, (2000). Resveratrol acts as a mixed agonist/antagonist for estrogen receptors α and β. *Endocrinology*, *141:*3657–3667.

Bridle KR, Crawford DHG, Ramm GA (2003). Identification and characterization of the hepatic stellate cell transferring receptor. *Am J Pathol.,* 162:1661–67.

Carbo N, Costelli P, Baccino FM, Lopez-Soriano FJ and Argiles JM (1999). Resveratrol, a natural product present in wine, decreases tumour growth in a rat tumour model. *Biochem. Biophys. Res. Commun*., 254: 739-43

Cardoso CCA, Paviani ER, Cruz LA, Guma FCR, Borojevic R and Guaragna RM, (2003). Effect of pentoxifilline on arachidonic acid metabolism, neutral lipid synthesis and accumulation during induction of the lipocyte phenotype by retinol in murine hepatic stellate cell. *Molecular and Cellular Biochemistry*, 254:37-46.

Clement MV, Hirpara JL, Chawdhury SH, and Pervaiz, S, (1998) Chemopreventive agent resveratrol, a natural product derived from grapes, triggers CD95 signaling-dependent apoptosis in human tumor cells. *Blood*, *92:* 996–1002.

Chao L, Samuels B M, Mason M M, Vinson C, Arioglu E, Gavrilova O, Reitman M, (2000). Adipose tissue is required for the antidiabetic, but not for the hypolipidemic, effect of thiazolidinediones. *J Clin. Invest*., 106(10)-1221-28.

Chen Y, Tseng SH, Lai HS, Chen WJ (2004). Resveratro-induced cellular apoptosis and cell cycle arrest in neuroblastoma cells and antitumor effects on neuroblastoma in mice. *Surgery*, 136:57-66.

Chen YJ, Wang JS and Chow SE, (2007). Resveratrol Protects Vascular Endothelial Cell from Ox-LDL-induced Reduction in Antithrmbogenic Activity. *Chinese Journal of Phisiology*, 50:22-28.

Cho MC, Lee K, Paik SG, Yoon DY, (2008). Peroxisome Proliferators-Activated Receptor (PPAR) Modulators and Metabolic Disorders. *PPAR Research*, 2008:679137-50.

Cory JG, (1988). Ribonucleotide reductase as a chemotherapeutic target. *Adv. Enzyme Regul.*, *27*: 437-455.

Costa JV and Duarte JS, (2006). Tecido adiposo e adipocinas (review). *Acta Med Port*, 19:251-256

Delmas D, Passily-Degrace P, Jannin B, Malki MM and Latruffe N, (2002). Resveratrol, a chemopreventive agent, disrupts the cell cycle control of human SW 480 colorectal tumor cells. *International Journal of Molecular Medicine*, 10:193-199.

Dubuisson JG, Dyess DL and Gaubatz JW, (2002). Resveratrol modulates human mammary epithelial cell O-acetyltransferase, sulfotransferase, and kinase activation of the heterocyclic amine carcinogen N-hydroxy-PhIP. *Cancer Lett*,182: 27-32.

Earnshaw WC, Martins LM and Kaufmann SH, (1999). Mammalian caspases:structure, activation, substrates,and functions during apoptosis. *Annu. Rev.Biochem*, 68:383–424

Finch RA, Liu MC, Grill SP, Rose WC, Loomis R, Vasquez KM, Cheng YC, Sartorelli AC, (2000).Triapine (3-aminopyridine -2-carboxaidehydethiosemicarbazone) : A potente inhibitor of ribonucleotide reductase activity with broad spectrum antitumor activity. *Biochem. Pharmacol., 59:* 981-983.

Fontecave M, Lepoivre M, Elleingand E, Gerez C, and Guittet O, (1998) Resveratrol, a remarkable inhibitor of ribonucleotide reductase. *FEBS Lett*., 421*: 277–279*.

Friedman SL, (2000). Molecular regulation of hepatic fibrosis, an integrated celluler response to tissue injury. *J Biol Chem*., 275:2247-2250.

Friedman SL, (2008). Mechanisms of Hepatic Fibrogenesis. *Gastroenterology*, 134:1658-69.

Garvin S, Öllinger K, Dabrosin C, (2006). Resveratrol induces apoptosis and inhibits angiogenesis in human breast cancer xenografts in vivo. *Cancer Letters*, 231:113-122.

Ghazizadeh M, Jin E, Shimizu H, Fujiwara M, Arai A, Ohaki Y, Takemura T,Kawanami O, (2005). Role of cdk4, $p16^{INK4}$, and RbExpression in the Prognosis of Bronchioloalveolar Carcinomas. *Respiration*, 72:68-73.

Granlund L, Larsen LN, Nebb HI, Pedersen JI, (2005). Effects of structural changes of fatty on lipid accumulation in adipocytes and primary hepatocytes. *Biochemica and Biophysica Acta*, 1687:23-30

Guaragna RM, Trugo L, Borojevic R, (1991). Neutral lipid synthesis and accumulation during *in vitro* induction of the lipocyte phenotype in hepatic conective tissue cells. *Biochem Biophys Acta*, 1085:29-39.

Guimarães EL, Francheschi MFS, Andrade CMB, Guaragna RM, Borojevic R, Margis R, Bernard EA and Guma FCR, (2007). Hepatic stellate cell modulates lipogenic transcription factors. *Liver International*, 27:1255-64.

Guma FCR, Mello TG, Mermelstein CS, Fortuna VA, Wofchuk ST, Gottfried C, Guaragna RM, Costa ML, Borojevic R, (2001). Intermediate filaments modulation in vitro model of the hepatic stellate cell activation or conversion into the lipocyte phenotype. *Biochem Cell Biol*, *79:409*-417.

Hartroft WS, Ridout JH (1951) Pathogenesis of cirrhosis produced by choline deficiency; escape of lipid from fatty hepatic cysts into the biliary and vascular systems. *Am J Pathol.* 27:951–989.

Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, Zipkin R, Chung E, Kisilewski A, Zhang LL, Scherer B and Sinclair DA, (2003). Small molecule activators of sirtuins extend Sacharomyces cerevisae lifespan. *Nature*, 425:191-196.

Huang C, Ma WY, Goranson A and Dong Z, (1999). Resveratrol suppresses cell transformation and induces apoptosis through a p53-dependent pathway. *Carcinogenesis (Lond.)*, 20: 237–242.

Iredale JP, (2001). Hepatic stellate cell behavior resolution of liver injury. *Semin. Liver Dis*. 21:427–36.

Isseman I, Green S, (1990). Activation of a member of the steroid hormone receptor superfamily by peroxissome proliferators. *Nature*, 347:645-649.

Jang M, Cai L, Udeani, GO, Slowing KV, Thomas CF, Beecher CW, Fong HH, Farnsworth NR, Kinghorn A D, Mehta RG, Moon RC, and Pezzuto JM,

(1997). Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science (Wash. DC)*, *275:* 218-220.

Jannin B, Menzel M, Berlot JP, Delmas D, Lançon A, Latruffe N, (2004). Transport of resveratrol, a cancer chemopreventive agent to cellular targets: plasmatic protein binding and cell uptake. *Biochemical Pharmacology*, 68:1113-18.

Joe A, Liu H, Suzuki M, Vural ME, Xiao D and Weinstein IB, (2002). Resveratrol Induces Growth Inhibition, S-phase Arrest, Apoptosis, and Changes in Biomarker Expression in Several Human Cancer Cell lines. *Clinical Cancer Research*, 8:893-903.

Joosen AMC, Barker AHF, Kersten S and Westerterp KR, (2008). The PPARγ ligand rosiglitazone influences traicylglycerol metabolism in nonobese males, without increasing the transcriptional activity of PPARγ in the subcutaneous adipose tissue. *Britsh Journal of Nutrition*, 99:487-493.

Juvet LK, Andresen SM, Schuster G, Dalen KT, Tobin KAR, Hollung K, Haugen F, Jacinto S, Ulven K, Bamberg K, Gustafsson JA, Nebb HI, (2003). On the role of Liver X receptor in lipid accumulation in adipocytes. *Mol. Endcrinol*., 17: 172-182.

Kampa M, Hatzoglou A, Notas G, Damianaki A, Bakogeorgou E, Gemetzi C, Kouroumalis E, Martin PM and Castanas E, (2000). Wine antioxidant polyphenols inhibit the proliferation of human prostate cancer cell lines. *Nutr Cancer* , 37: 223-233.

Kawada N, Seki S, Inque M and Kuroki T, (1998). Effect of Antioxidants, Resveratrol, Quercetin, and N-Acetylcysteine, on the Functions of Cultured Rat Hepatic Stellate Cells and Kuppfer Cells. *Hepatology*, 27:1265-1274.

Kern M, Pahlke G, Balavenkatraman KK, Böhmer FD and Marko D, (2007). Apple Polyfenols Affect Protein Kinase C Activity and the Onset of Apoptosis in Human Colon Carcinoma Cells. *J. Agric. Food Chem*., 55:4999-5006.

Kersten S, Desvergne B, Wahli W, (2000). Roles of PPARs in health and disease. *Nature* 405: 421–424.

King RE, Bomser JA, Min DB, (2006). Bioactivity of resveratrol. Comprehensive. *Reviews in Food Science and Food Safety*, 5: 65-69.

Kirimlioglu H, Yilmaz ES, Kirimlioglu V and Karabulut AB, (2008). Effect of Resveratrol and Melatonin on Oxidative Stress Enzymes Regeneration, and Hepatocyte Ultrastructure in Rats Subjected to 70% Partial Hepatectomy. *Transplantation Proceedings*, 40:285-289.

Kisseleva T and Brenner DA, (2006). Hepatic stellate cells and the reversal of fibrosis. *Journal of Gastroenterology and Hepatology*, 21:S84-S87.

Kozuki Y, Miura Y, Yagasaki K, (2001). Resveratrol supresses hepatoma cell invasion independently of its anti-proliferative action. *Cancer Letters*, 167: 151-156.

Lançon A, Delmas D, Osman H, The´not JP, Jannin B, Latruffe N, (2004). Human hepatic cell uptake of resveratrol: involvement of both passive diffusion and carrier-mediated process. *Biochem Biophys Res Commun*, 316:1132-37.

Leibiger IB, Berggren PO, (2006). Sirt1: a metabolic master switch that modulates lifespan. *Nature*, 12: 34-36.

Li ZG, Hong T, Shimada Y, Komoto I, Kawabe A, Ding Y, Kaganoi J, Hashimoto Y and Imamura M, (2002). Suppression of *N*-nitrosomethylbenzylamine (NMBA)-induced esophageal tumorigenesis in F344 rats by resveratrol. *Carcinogenesis*, 23**:**1531-36.

Lin K, Rockliffe N, Johnson GG, Sherrington PD, Pettitt AR, (2008). Hsp90 inhibition has opposing effects on wild-type and mutant p53 and induces p21 expression and cytotoxicity irrespective of p53/ATM status in chronic lymphocytic leukaemia cell. *Oncogene*, 27/17: 2445-55.

Louie AY, Meade TJ, (1999). Metal Complexes as Enzyme Inhibitors. *Chem. Rev., 99*:2711-34.

Lu R, Serrero G, (1999). Resveratrol, a natural derived from grape, exhibits antiestrogenic activity and inhibits the growth of human breast câncer cells. *Journal of Cellular Physiollogy*, 179:297-304

Lu X, Jung J in, Cho HJ, Lim DY, Lee HS, Chun HS, Kwon DY and Park JHY, (2005) . Fisetin Inhibits the Activities of Cyclin-Dependent Kinases Leading to Cell Cycle Arrest in HT-29 Human Colon Cancer Cells. *J. Nutr*., 135: 2884–90.

Malhi H and Gores GJ, (2008). Cellular and Molecular Mechanisms of Liver Injury. *Gastroenterology*, 134: 1655-69.

Margis, R, Borojevic R, (1989). Retinoid-mediated induction of the fat-storing phenotype in a liver connective tissue cell line (GRX). *Biochem Biophys Acta*, 1011: 1-5.

Melo EJT, Mayerhoffer RO, Souza W, (2000). Hydroxyurea inhibits intracellular *Toxoplasma gondii* multiplication. *FEMS Microbiol. Lett.*, *185*:79.

Meng X, Maliakal P, Lu H, Lee MJ and Yang CS, (2004). Urinary and plasma levels of grape juice. *J. Agric. Food Chem.*, 52: 935-942.

Mermelstein CS, Guma FCR, Mello TG, Fortuna VA, Guaragna RM, Costa ML, Borojevic R, (2001). Induction of lipocyte phenotype in murine hepatic stellate cells : Reorganization of the action cytoskeleton. *Cell Tissue Res*, 306: 75-83.

Mgbonyebi OP, Russo J and Russo IH, (1998). Antiproliferative effect of synthetic resveratrol on human breast epithelial cells. *Int J Oncol*, 12: 865-869.

Monteiro ANA, Borojevic R, (1987). *In vitro* formation of fibrous septa by liver connective tissue cells. *In vitro Cellular Developmental Biology,* 23:10-14.

Moreira RK, (2007). Hepatic Stellate Cells and Liver Fibrosis. *Arch Pathol Lab Med*., 131:1728-34.

Muoio DM, MacLean PS, Lang DB, Li S, Houmard JA, Way JM, Winegar DA, Corton JC, Dohm GL, Kraus WE, (2002). Fatty acid homeostasis and induction of lipid regulatory genes in skeletal muscles of peroxisome proliferator-activated receptor (PPAR) alpha knock-out mice. Evidence for compensatory regulation by PPAR delta. *J. Biol. Chem*. 277: 26089–97.

Narayanan BA, Narayanan NK, Stoner GD and Bullock BP, (2002). Interactive gene expression pattern in prostate cancer cellsexposed to phenolic antioxidants. *Life Sci* 70: 1821-39.

Nigro P, Bloise E, Turco MC, Skhirtladze A, Montoro P, Pizza C, Piacente S, Belisario MA, (2007). Antiproliferative and pro-apoptotic activity of novel phenolic derivatives of resveratrol. *Life Sciences*, 81:873–883

Notas G, Nifli AP, Kampa M, Vercauteren J, Kouroumalis E, Castanas E, (2006). Resveratrol exerts itsantiproliferative effect on HepG2 hepatocellular carcinoma cells, by inducing cell cycle arrest, and NOS activation. *Biochim Biophys Acta*, 1760:11657-66.

Olaso E, Ikeda K, Eng, FJ, Xu L, Wang LH, Lin, C, Friedman, S. L. (2001). DDR2 receptor promotes MMP-2 mediated proliferation and invasion by hepatic stellate cells.*The Journal of clinical investigation,* 108:1369-78.

Olson ER, Naugle JE, Zhang X, Bomser JA and Meszaros JG, (2004). Inhibition of cardiac fibroblast proliferation and myofibroblast differentiation by resveratrol. *American Journal Physiology Heart Circulatory Physiology*, 288: 1131-38.

Passino M, Adams RA, Sikorski SL, Akassoglou K, (2007). Regulation of Hepatic Stellate Cell Differentiation by the Neurotrophin Receptor $p75^{NTR}$. *Science*, 315:1853-56.

Peet J, Janowski BA, Mangelsdorf DJ, (1998). The LXRs: a new class of oxysterol receptors. *Curr. Opin. Genet. Dev*., 8: 571-575.

Picard F, Kurtev M, Chung N, Topark-Ngarm A, Senawong T, Oliveira RM, Leid M, McBurney M, Guarente L, (2004). Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-γ. *Nature*, 429: 771-776.

Pozo-Guisado E, Alvarez-Barrientos A, Mulero-Navarro S, Santiago-Josefat B and Fernandez-Salguero PM, (2002). The antiproliferative activity of resveratrol results in apoptosis in MCF-7 but not in MDA-MB-231 human breast cancer cells: cell-specific alteration of the cell cycle. *Biochem Pharmacol*, 64: 1375-86.

Pozo-Guisado E, Lorenzo-Benazayas MJ, Fernadez-Salguero PM, (2004). Resveratrol modulates the phosphoinositide 3-kinase pathway through an estrogen receptor alpha-dependent mechanism : relevance in cell proliferation. *Int. J. Cancer*, 109: 167-173.

Qiang L, Wang H and Farmer SR, (2007). Adiponectin Secretion Is Regulated by SIRT1 and the Endoplasmic Reticulum Oxidoredutase Ero1-α. *Mollecular an Cellular Biology*, 27: 4698-4707.

Ragione FD, Cucciolla V, Borriello A, Pietra VD, Racioppi L, Soldati G, Manna C, Galletti P and Zappia V, (1998). Resveratrol arrests the cell division cycle at S/G2 phase transition. *Biochem. Biophys. Res. Commun*., *250:* 53–58.

Rayalam S, Della-Fera MA, Yang JY, Park HJ, Amabati S, Baile C, (2007). Resveratrol Potentiates Genistei's Antiadipogenic and Proapoptotic Effects in 3t3-L1 Adipocytes. *The journal of Nutrition*, 137: 2668-73.

Reddy JK, Hashimoto T, (2001). Peroxisomal β-oxidation and peroxisome proliferators-activated receptor α: an adaptive metabolic system. *Annu. Rev. Nutr*., 21:193-230.

Repa JJ, Turley JA, Lobaccaro JA, Medina J, Li L, Lustig K, Shan B, Heyman RA, Dietschy JM, Mangelsdorf DJ, (2000). Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. Science, 289: 1524-29.

Rodgers JT, Lerin C, Hass W, Gygi SP, Spiegelman BM, Puigserver P, (2005). Nutrient control of glucose homeostasis through a complex of PGC1-alpha and SIRT1. *Nature*, 434: 113-118.

Roman V, Billard C, Kern C, Ferry-Dumazet H, Izard JC, Mohammad R, Mossalayi DM, Kolb JP,(2002). Analysis of resveratrol-induced apoptosis in human B-cell chronic leukemia. *British Journal of Haematology*, 117:842-851.

Roosen A, Schober A, Strelau J, Bottner M, Faulhaber J, Bendner G, McIlwrath SL, Seller H, Ehmke H, Lewin GR, Unsicker K., (2001). Lack of neurotrophin-4 causes selective structural and chemical deficits in

sympathetic ganglia and their preganglionic innervation. *J Neurosci*., 21: 3073-84.

Sgambato A, Ardito R, Faraglia B, Boninsegna A, Wolf FI, Cittadinini A, (2001). Resveratrol, a natural phenolic compound, inhibits cell proliferation and prevents oxidative DNA damage. *Mutation Research*, 496:171-180.

Shih A, Zhang S, Cao HJ, Boswell S, Wu YH, Tang HY, Lennartz MR, Davis FB, Davis PJ, Lin HY, (2004). Inhibitory effect of epidermal growth factor on resveratrol-induced apoptosis in prostate cancer cells is mediated by protein kinase C-α. *Molecular Cancer Therapeutics*, 11:1355-63.

Schultz JR, Tu H, Luk A, Repa JJ, . Medina JC, Li L, Schwendner S, Wang S, Thoolen M, Mangelsdorf DJ,. Lustig KD, Shan B, (2000). Role of LXRs in control of lipogenesis. *Genes Dev*., 14:2831-38.

Signorelli P, Ghidoni R, (2005). Resveratrol as an anticancer nutrient: molecular basis open questions and promises. *Journal of Nutritional Biochemistry*, 16: 449-466.

Soleas GJ, Diamandis EP, and Goldberg DM, (1997). Resveratrol: a molecule whose time has come? And gone? *Clin. Biochem*., *30:* 91–113.

Stervbo U, Vang O, Bonnesen C, (2006). Time- and concentrationdependent effects of resveratrol in HL-60 and HepG2 cells. *Cell Prolif*., 39:479.

Stewart JK, Artime MC, O'Brian CA, (2003). Resveratrol: A candidate Nutritional Substance for Prostate Cancer Prevention. *J. Nutr*., 133:2440-43.

Song C, Kokontis JM, Hiipakka RA, Liao S, (1994). Ubiquitous receptor: a receptor that modulates gene activation by retinoic acid and thyroid hormone receptors. *Proc. Natl. Acad. Sci. USA*, 91: 10809-813.

Stivala LA, Savio M, Carafoli F, Perucca P, Bianchi L, Magas G, Forti L, Pagnoni UM, Albini A, Prosperi E, Vannini V, (2001). Specific Structural Determinants Are Responble for the Antioxidant Activity and the Cell Cycle Effects of Resveratrol. *The journal of Biological Chemistry*, 276: 22586-594.

Subbaramaiah K, Michaluart P, Chung WJ, Tanabe T, Telang Nand Dannenberg AJ, (1999). Resveratrol inhibits cyclooxygenase-2 transcription in human mammary epithelial cells. *Ann NY Acad Sci* , 889: 214-223.

Sun NJ, Woo SH, Cassady JM, and Snapka RM,(1998). DNA polymerase and topoisomerase II inhibitors from *Psoralea corylifolia*. *J. Nat. Prod. (Lloydia)*, *61:* 362–366.

Surh YJ, Hurh YJ, Kang JY, Lee E, Kong G and Lee SJ, (1999). Resveratrol, an antioxidant present in red wine, induces apoptosis in human promyelocytic leukemia (HL-60) cells. *Cancer Lett* 140: 1-10.

Taub R, (2004). Liver regeneration: from myth to mechanism. *Nature Reviews Molecular Cell Biology*, 5:836-47.

Tavares H, Hirata MH, Hirata RDC, (2007). Receptor Ativado por Proliferadores de Peroxissoma Gama (PPARγ): Estudo Molecular na Homeostase da Glicose, Metabolismo de Lipídeos e Abordagem Terapêutica. Arq Brás Endocrinol Metabol., 51/4: 526-533.

Teboul M, Enmark E, Li Q, Wikstrom AC, Pelto-Huikko M, Gustafsson JA, (1995) . OR-1, a member of the nuclear receptor superfamily that interacts with 9-*cis*-retinoic acid receptor. *Proc. Natl. Acad. Sci. USA*, 92: 2096-2100.

Tessitore L, Davit A, Sarotto I, and Caderni G (2000). Resveratrol depresses the growth of colorectal aberrant crypt foci by affecting *bax* and *p21CIP* expression. *Carcinogenesis,* 21**:**1619–1622.

Thompson KC, Trowern A, Fowell A, Marathe M, Haycock C, Arthur MJP, Sheron N, (1998). Primary rat and mouse hepatic stellate cells express the macrophage inhibitor cytokine interleukin-10 during the course of activation in vitro. *Hepatology***,** 28:1518–24

Tontonoz P, Hu E, Spiegelman BM, (1994). Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. Cell, 79: 1147-76.

Ulrich S, Loitssch SM, Rau O, Von Knethen A, Brune B, Schubert-Zsilavecz M, Stein JM, (2006). Eroxisome Proliferator-Activated Receptor γ as a Molecular Target of Resveratrol-Induced Modulation of Polyamine metabolism. *Cancer Res*., 66: 7348-54.

Wake K, (1971). ‘‘Sternzellen’’ in the liver: perisinusoidal cells with special reference to storage of vitamin, *A. Am J Anat.*, 132:429–462.

Walle T, Hsieh F, DeLegge MH, Oatis JE, Jr. and Walle UK, (2004). *The American Society for Pharmacology and Experimental Therapeutics*. 32: 1377-82.

Wang SC, Tsukamoto H, Rippe R A, Schrum L, Ohata M, (1998). Expression of interleukin-10 by in vitro and in vivo activated hepatic stellate cells. *J. Biol. Chem.*, 273: 302–308

Wang Z, Huang Y, Zou J, Cao K, Wu JM, (2002). Effects of red wine and wine polyphenol resveratrol on platelet aggregation in vivo and in vitro. *Int. J. Mol. Med*., 9(1):77-9.

Waterhouse AL, (2002). Wine phenolics. *Ann. N.Y. Acad. Sci*., 957: 21-36.

Wheeler MD, Kono H, Yin M, (2001). The role of Kupffer cell oxidant production in early ethanol-induced liver disease. *Free Radic Biol Med.* 31:1544–49.

Wenzel E, Somoza V, (2005). Metabolism and biovailability of *trans*-resveratrol. *Molecular nutrition & Food research*, 49:472-81.

Werman A, Hollenberg A, Solanes G, Bjorbaek C, Vidal-Puig AJ, Flier JS, (1997). Ligand-independent activation domain in the N terminus of peroxisome proliferator-activated receptor gamma (PPARgamma). Differential activity of PPARgamma1 and -2 isoforms and influence of insulin. *J Biol Chem*., 272: 20230-5. 230.

Wood JG, Rogina B, Lavu S, Howitz K, Helfand SL, Tatar M, Sinclair D, (2004). Sirtuin activators mimic caloric restriction and delay ageing in metazoans. *Nature*, 430/6: 686-689.

Yang JY, Fera AD, Rayalan S, Ambati S, Hartzell D, Park HJ, Baile CA, (2008). Enhanced inhibition of adipogenesis and induction of apoptosis in 3T3-L1 adipocytes with combinations of resveratrol and quercetin. *Life Sciences*, 82:1032-39.

Yeung F, Hoberg J, Ramsey CS, Keller MD, Jones DR, Frye RA, Mayo MW, (2004). Modulation of NF-κB-dependent transcription and cell survival by the SIRT1 deacetylase. *EMBO J*., 23: 2369-80.

Xiuyun H, Xu S, Maitland-Toolan KA, Sato K, Jiang B, Ido Y, Lan F, Walsh K, Wierzbicki M, Verbeuren TJ, Cohen RA, Zang M, (2008). SIRT1 Regulates Hepatocyte Lipid Metabolism through Activating AMP-Activated Protein Kinase. *Journal of Biological Chemistry*, 283:20015-026.

Zhang P, Li H, Wu ML, Chen XY, Kong QY, Wang XW, Sun Y, Wen S, Liu J, (2006). c-Myc downregulation: a critical molecular event in resveratrol-induced cell cycle arrest and apoptosis of human medulloblastoma cells. *J Neurooncol*., 80: 123-31.

Zhang W, Fei Z, Zhen Hn, Zhang Jn, Zhang X, (2006). Resveratrol inhibits cell growth and induces apoptosis of rat C6 glioma cells. *J Neurooncol*., 81: 231-40.

ANEXOS

LISTA DE FIGURAS

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ARTIGO 2

RESULTADOS COMPLEMENTARES

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