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Curso de Pós-Graduação em Ciências Biológicas – Bioquímica

Modulação diferencial de fatores de transcrição adipogênicos, por indometacina e retinol, durante a conversão fenotípica de uma linhagem representativa de células estreladas hepáticas

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Sumário

PARTE I

RESUMO

A modulação fenotípica da célula estrelada hepática (CEH) é um dos eventos primários do processo de fibrose hepática. Durante este evento, a CEH modifica seu fenótipo lipocítico (quiescente) para miofibroblástico (ativado), fenômeno chamado de ativação. Estudos recentes sugerem que fatores de transcrição adipogênicos atuam no processo de ativação das CEH, e conseqüentemente teriam um papel chave na evolução do processo fibrótico. Neste estudo analisamos a expressão gênica de vários fatores de transcrição adipogênicos, como os receptores ativados por proliferadores de peroxissomos (RAPP), receptor X do fígadoα (RXFα), proteína ligante de CCAAT/*enhancer*α (PLCEα) e proteína ligante do elemento regulatório de esteróis-1 (PLERE-1), em uma linhagem representativa de CEH, a GRX. A linhagem GRX, é capaz de ser induzida *in vitro* a expressar o fenótipo quiescente através do tratamento com indometacina ou retinol. Após 24 horas ou 7 dias de tratamento, a expressão gênica foi analisada através de PCR em tempo real. Tratamento com indometacina aumentou a expressão de todos os genes avaliados, exceto PLCEα e RAPPβ. O mesmo resultado obtido com desmetil indometacina (LM 4511) sobre RAPPα e γ sugere que a ação da indometacina sobre a diferenciação fenotípica da GRX é independente de sua ação inibitória sobre ciclooxigenase, já que a LM 4511 não possui esta atividade. Tratamento com retinol levou à modulação de um número menor de genes, diminuindo a expressão de PLCEα e aumentando a de RAPPγ, e RAPPβ. Adipsina, gene característico de adipócitos, teve sua expressão induzida apenas nas células tratadas com indometacina. Pex16 e catalase, genes

modulados pelos RAPPs, foram diferencialmente expressos, dependendo do tratamento. Os resultados apresentados sugerem que a linhagem representativa de CEH, GRX, tem sua indução fenotípica modulada por fatores de transcrição adipogênicos, que são diferencialmente expressos dependendo do tratamento de conversão.

ABSTRACT

BACKGROUND: Hepatic stellate cell (HSC) phenotype modulation is a key event in the genesis of liver fibrosis. The phenotype transition and maintenance requires the integrated action of several transcriptional factors, and recent data suggests a role for those known to be involved in adipogenesis. AIM: Analyze gene expression modulation of the adipogenic transcription factors liver X receptorα (LXRα), CCAAT/enhancer binding proteinα (C/EBPα), sterol regulatory element binding protein-1 (SREBP-1) and peroxisome proliferator-activated receptors (PPARs) during quiescent conversion with retinol or indomethacin on a representative HSC cell line. METHODS: We used the HSC derived cell line GRX, that retains the capacity to express the quiescent phenotype when treated with retinol or indomethacin. After 24 hours or 7 days treatment, gene expression was quantified by real-time polymerase chain reaction. RESULTS: C/EBPα and PPARβ were the only transcription factors whose expression were not increased during quiescent conversion by indomethacin treatment. Furthermore, Indomethacin action was independent of ciclooxygenase inhibitory activity, since an analogue that lacks this activity exerted the same effect on PPARs expression. Retinol treatment modulated a fewer set of genes, only increasing PPARβ and PPARγ, and reducing C/EBPα expression along quiescent conversion. Catalase and Pex16, PPARs downstream regulated genes, had their expression increased during quiescent conversion. Adipsin, a marker of adipocyte differentiation, was augmented only by indomethacin treatment. CONCLUSION: These data support a role for adipogenic transcriptional regulation during quiescent conversion of a representative HSC cell

line, and differential modulation by indomethacin and retinol during phenotype transdifferentiation.

ABREVIATURAS

RAPP - receptor ativado por proliferador de peroxissomo
CEH - célula estrelada hepática
RAR - receptor de ácido retinóico
RXR - receptor X de retinóides
RXF - receptor X do fígado
PLCE - proteína ligante de CCAAT/*enhancer*
PLERE - proteína ligante do elemento regulatório de esteróis
Mio - miofibroblasto
ROH - retinol
Indo - indometacina
PCLR-I - proteína celular ligante de retinol-I

INTRODUÇÃO

Fibrose hepática é uma doença caracterizada pelo acúmulo excessivo de matriz extracelular e pode ser induzida por uma grande variedade de insultos, como a ingestão de álcool, infecção viral e erros inatos do metabolismo. Independente do insulto inicial, a fibrose hepática tem como fenômeno chave a ativação das células estreladas hepáticas (CEH) (Iredale, 2001). Esta célula apresenta-se na forma de lipócito no fígado saudável, e tem como função principal controlar o metabolismo hepático de vitamina A, através da captação, armazenamento e liberação de retinóides para o organismo (Geerts, 2001). Processos inflamatórios, derivados de danos ao fígado, induzem uma mudança de fenótipo das CEH, levando-as a expressar um fenótipo miofibroblastóide, característico de patologias hepáticas. Este processo é chamado de ativação e leva a um aumento na taxa de proliferação das CEHs, secreção de citocinas inflamatórias e componentes da matriz extracelular. Após a resolução do processo de fibrose, a CEH miofibroblástica sofre apoptose induzida por citocinas pró-apoptóticas e deprivação de fatores de sobrevivência (Iredale, 2001).

Dentre os fatores capazes de ativar a CEH temos componentes da matriz extracelular (Wells, 2005), espécies reativas de oxigênio (EROS) (Poli, 2000), citocinas inflamatórias (Marra, 2005), lipídios oxidados e aldeídos (Friedman,1999). Vários estudos demonstram a importância destes fatores na ativação e a inter-relação entre eles. Por exemplo, citocinas inflamatórias se ligam em receptores de membrana nas CEH, elevando a produção intracelular de EROS (Garcia-Trevijano, 1999). Este aumento leva a oxidação de lipídios de

membrana que atuam como sinalizadores para a expressão de proteínas de matriz extracelular características do processo fibrótico (Anania, 2001). Conseqüentemente, modificações quantitativas e qualitativas na expressão e secreção de componentes da matriz extracelular podem desencadear a ativação de CEH adjacentes (Schuppan, 2001). Atualmente, um grande número de trabalhos tenta identificar alvos moleculares, como proteínas e segundos mensageiros, com o objetivo final de gerar fármacos hepatoprotetores que interrompam a cadeia de eventos que culminam com a ativação das CEHs.

Embora seja de vital importância o reconhecimento de genes característicos da CEH miofibroblástica, o processo de ativação não é apenas induzido pela ativação destes genes, mas também pelo silenciamento de genes envolvidos na manutenção do fenótipo lipocítico. Por exemplo, o uso de inibidores de histonas deacetilases, enzimas envolvidas na supressão da expressão gênica, bloqueiam a ativação de CEHs, sugerindo que o silenciamento gênico pela perda de acetilação de histonas é parte do processo de ativação (Niki et al., 1999). Tendo em vista estes resultados, quais genes teriam sua expressão diminuída durante a ativação das CEHs? A resposta surgiu através do descobrimento de uma nova classe de compostos com atividade anti-fibrótica. Galli e colegas (2000) descobriram que tiazolidinedionas, drogas usadas na clínica contra diabetes, atenuam os efeitos fibróticos do fator de crescimento de transformação celularβ. A partir deste, outros estudos demonstraram que as tiazolidinedionas eram capazes de diminuir características das CEHs ativadas como a expressão de colágeno tipo I, proliferação e expressão de α-actina de músculo liso (Guo et al., 2005; Miyahara et al., 2000).

As tiazolidinedionas são ligantes específicos do receptor ativado por proliferadores de peroxissomo γ (RAPPγ). Este fator de transcrição pertence a grande família de receptores nucleares, e possui mais dois isotipos chamados RAPPα e RAPPβ. Os RAPPs são ativados por ligantes lipídicos e controlam a expressão de genes envolvidos no metabolismo de ácidos graxos, glicose e na resposta inflamatória (Desvergne e Wahli, 1999). Nas CEHs, a expressão das RAPPs é modulada conjuntamente com o processo de ativação (Miyahara et al., 2000), sendo que a expressão de RAPPγ diminui na transição do fenótipo lipocítico para o miofibroblástico enquanto que a expressão de RAPPβ, ao contrário, aumenta (Helemmans et al., 2003).

Os RAPPs heterodimerizam obrigatoriamente com o receptor X de retinóides (RXR) para modular a expressão gênica (Desvergne e Wahli, 1999). Este fator de transcrição pertence a mesma família dos RAPPs, de receptores nucleares, e tem como ligante específico o *9-cis*-ácido retinóico (Bastien and Rochette-Egly, 2004). A formação do heterodímero RXR-RAPP pode acontecer pela ativação de qualquer um dos parceiros, RXR ou RAPPs. Uma vez que ativadores de RAPPγ atuam como anti-fibróticos, estudos recentes avaliaram se retinóides, ligantes de RXR, teriam semelhante característica anti-fibrótica. Embora alguns estudos demonstrem a possibilidade de retinóides atuarem como agentes inibidores do processo de ativação das CEHs *in vitro* (Milliano and Luxon, 2005; Wang et al., 2004), resultados controversos obtidos *in vivo* indicam que o papel dos retinóides durante a fibrose hepática depende de vários fatores, como: disponibilidade plasmática, expressão de proteínas carregadoras e de receptores responsivos (Vollmar et al., 2002; Chen et al., 2004).

Outros estudos sugeriram a analogia entre a modulação fenotípica das CEHs e a diferenciação de fibroblastos em adipócitos, processo chamado adipogênese. Este fenômeno é controlado por uma série de fatores de transcrição que atuam cooperativa e seqüencialmente levando a expressão de genes secundários, que definem a fisiologia característica de células adipocíticas. Dentre os fatores de transcrição envolvidos, temos RAPPγ, RAPPβ, receptor X do fígadoα (RXFα), proteína ligante de CCAAT/*enhancer*α (PLCEα) e proteína ligante do elemento regulatório de esteróis-1 (PLERE-1) (Seo et al., 2004; Fajas et al., 1999 e Mandrup e Lane 1997). Estes fatores de transcrição expressos nas CEHs têm sua expressão diminuída nas CEHs miofibroblásticas, sendo que vários estudos indicam um possível papel para eles na manutenção do fenótipo lipocítico: (I) A expressão ectópica de RAPPγ aumenta a expressão de RXFα e PLCEα ao mesmo tempo que são atenuadas características de ativação, (II) o tratamento com indutores adipocíticos restauram a expressão destes fatores de transcrição (She et al., 2005), (III) as CEHs expressam genes marcadores de adipócitos controlados por estes fatores de transcrição, como a adipsina (She et al., 2005) e (IV) a expressão ectópica de PLCEα atenua características do fenótipo miofibroblástico e aumenta a de lipocítico (Huang et al., 2004).

Culturas primárias de CEHs sofrem conversão espontânea para o fenótipo ativado; o processo de ativação inicia após 24 horas de cultura (Hellemans et al., 2003) dificultando o estudo *in vitro* do fenótipo lipocítico. O estudo do fenótipo lipocítico foi facilitado pelo isolamento de linhagens celulares capazes de expressar este fenótipo. Em 1985, Borojevic e colaboradores isolaram a linhagem celular GRX, a partir de granulomas fibróticos hepáticos resultantes da infestação

experimental de camundongos com *Schistossoma mansoni.* Em condições basais de cultivo as células GRX são miofibroblastos, representando o fenótipo ativado das CEHs. Estudos posteriores mostraram que as células GRX apresentam vários marcadores de CEHs, como α-actina de músculo liso, desmina, proteína ácida fibrilar glial e proteína celular ligante de retinol (Guma et al., 2001; Vicente et al, 1998). A principal característica da linhagem GRX é a capacidade de poder ser induzida a expressar o fenótipo lipocítico quando tratada com retinóides, indometacina ou β-caroteno por cerca de 7 dias (Margis and Borojevic, 1989; Borojevic et al., 1990; Martucci et al., 2004). Nestas condições a taxa de proliferação e a secreção de colágeno diminuem (Chiarini et al., 1994; Margis et al., 1992). Como acontece nas CEHs, recentemente foi mostrada a relação entre o estado de ativação da célula GRX e o grau de estresse oxidativo da célula (Guimarães, comunicação pessoal, 2006). A importância da linhagem GRX foi reconhecida em uma ampla revisão sobre as CEHs, onde ela é citada como sendo a primeira linhagem celular permanente reconhecida como representativa das CEHs (Geerts, 2001).

Nossa hipótese de trabalho se baseia na proposta de que a caracterização, nos dois fenótipos das CEHs, da expressão dos principais genes adipocíticos e de alguns genes que sabidamente são controlados pelos mesmos, é o passo principal para o controle do processo fibrótico e para o desenvolvimento de terapias que controlem essa patologia. Nesse contesto, a linhagem GRX por sua capacidade de conversão de miofibroblasto (fenótipo ativado) para lipócito (fenótipo quiescente) das CEHs, se torna uma ferramenta bastante apropriada para o estudo proposto.

Os objetivos específicos foram:

- Analisar, durante a conversão fenotípica, a expressão de 3 grupos de genes: (I) Os 3 isotipos de RAPPs α, β, γ. (II) Fatores de transcrição envolvidos no processo de adipogênese, tendo sido estudados o receptor X do fígadoα (RXFα), proteína ligante de CCAAT/*enhancer*α (PLCEα) e proteína ligante do elemento regulatório de esteróis-1 (PLERE-1). (III) Genes regulados por RAPPs, como catalase e Pex16, e o marcador de conversão adipocítica, adipsina.
- Avaliar a expressão destes 3 grupos de genes durante a conversão lipocítica da GRX induzida por retinol e indometacina, diferenciando a ação destes compostos sobre os genes estudados.
- Determinar se o efeito de indometacina sobre os genes estudados se deve a sua ação inibitória sobre ciclooxigenase, ou de ligante de RAPPα e γ, através do uso de um análogo que não possui ação sobre ciclooxigenase.

PARTE II

Artigo a ser submetido ao periódico Gut

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The study should be presented in sections, namely:

Abstract
No more than 250 words, summarising the problem being considered, how the study was performed, the salient results, and the principal conclusions under subheadings Background, Aims, Patients (or Subjects), Methods, Results, and Conclusions. Please follow the Statistical Advice for Contributors to Gut (PDF).

Keywords
Authors should provide up to five keywords or phrases for the index. It is recommended to use terms included in the Medical Subject Heading vocabulary thesaurus (MeSH).

Introduction
Brief description of the background that led to the study (current results and conclusions should not be included).

Methods
Details relevant to the conduct of the study. Wherever possible give numbers of subjects studied (not percentages alone). Statistical methods should be clearly explained at the end of this section. Please see the Statistical Advice for Contributors to Gut (PDF).

Results
Work should be reported in SI units apart from blood pressure measurements which should be given in mm Hg, and drugs in metric units. Undue repetition in text and tables should be avoided. Comment on validity and significance of results is appropriate but broader discussion of their implication is restricted to the next section. Subheadings that aid clarity of presentation within this and the previous section are encouraged.

Discussion
The nature and findings of the study are placed in context of other relevant published data. Caveats to the study should be discussed. Avoid undue extrapolation from the study topic.

Acknowledgements and affiliations
Individuals with direct involvement in the study but not included in authorship may be acknowledged. Financial support and industrial affiliations (and their nature) of all those involved must be stated. Click here for more information on acknowledgements.

References

The number of references should be limited to those critical and relevant to the current manuscript.
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Tables/Figures

Images submitted should be those which uniquely display the data and not repetition of information available either in the text or as a table. Figures take up considerable space and need to be thoroughly justified.
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Differential modulation of adipogenic transcription factors during phenotypic conversion of a hepatic stellate cell line

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BACKGROUND: Hepatic stellate cell (HSC) phenotype modulation is a key event in the genesis of liver fibrosis. The phenotype transition and maintenance requires the integrated action of several transcriptional factors, and recent data suggests a role for those known to be involved in adipogenesis. AIM: Analyze gene expression modulation of the adipogenic transcription factors liver X receptorα (LXRα), CCAAT/enhancer binding proteinα (C/EBPα), sterol regulatory element binding protein-1 (SREBP-1) and peroxisome proliferator-activated receptors (PPARs) during quiescent conversion with retinol or indomethacin on a representative HSC cell line. METHODS: We used the HSC derived cell line GRX, that express a myofibroblast phenotype in basal medium and retains the capacity to express quiescent phenotype when treated with retinol or indomethacin. After 24 hours or 7 days treatment, gene expression was quantified by real-time polymerase chain reaction. RESULTS: C/EBPα and PPARβ were the only transcription factors analyzed whose expression were not increased during quiescent conversion by indomethacin treatment. Furthermore, indomethacin action was independent of ciclooxygenase inhibitory activity, since an analogue that lacks this activity exerted similar effect on PPARs expression. Retinol treatment modulated a fewer set of genes, increasing PPARβ and PPARγ, and reducing C/EBPα expression along quiescent conversion. Catalase and Pex16, PPARs downstream regulated genes, had their expression increased during conversion. The expression of adipsin, marker of adipocyte differentiation, increased only with indomethacin treatment. CONCLUSION: These data support a role for adipogenic transcriptional regulation during quiescent conversion of a representative HSC cell line, and differential modulation by indomethacin and retinol during phenotype transdifferentiation.

Keywords: Peroxisome proliferator-activated receptor, hepatic stellate cell, adipogenesis

INTRODUCTION

Hepatic stellate cells (HSCs) are vitamin A-storing cells that reside in the Disse's space and play a key role during intrahepatic uptake, storage and release of retinoids.[1] During liver injury they transdifferentiate into myofibroblast-like cells, which proliferates, alters the extracellular matrix (ECM) of the liver qualitatively and quantitatively and produces inflammatory cytokines.[2] [3] As the major source of ECM in the fibrotic liver; the activation process of HSCs, a phenotypic switch from vitamin A-storing cell to myofibroblast-like cell, has been studied in order to understand this phenomenon and desenvolve methodologies to prevent and attenuate liver fibrosis.

A growing number of studies begin to stablish peroxisome proliferator-activated receptor γ (PPARγ) as a potent target for the treatment of liver fibrosis.[4] This transcription factor belongs to the nuclear receptors superfamily, forming a subfamily along with PPARα, PPARβ, functioning as lipid-activated receptors that control a variety of genes involved on lipid metabolism, adipogenesis and inflammation.[5][6][7][8] PPARγ has its expression diminished during HSC activation, and ectopic expression ameliorate fibrotic markers such as collagen type I production, smooth muscle α-actin and transforming growth factor β expression.[9] Furthermore, thiazolidinediones, synthetic ligands of PPARγ, suppress proliferation, expression of collagen type I and smooth muscle α-actin on activated HSCs and inhibits liver fibrosis on a model of bile duct ligation.[10] [11]

As PPARγ acts as obligatory heterodimer with retinoid X receptor (RXR) and HSC activation is accompanied by the lost of retinoid stores, many studies focused on the effect of retinoids on HSC function and liver fibrosis.[12][13][14] Although several studies demonstrate retinoids as possible inhibitors of HSC activation *in vitro*, controversial *in vivo* results has been obtained.[15] [16] Apparently, the antifibrotic or fibrotic effect of retinol shows to be dependent of the concentration treatment and the availability of liver RXR.[17] [18]

The differentiation of fibroblast to adipocytes is tightly coordinated by a cascade of transcription factors that acts inducing other downstream transcription factors and genes that typify the adipocyte phenotype.[19] In addition to PPARγ, other transcription factors that play key roles during this process are CCAAT/enhancer-binding protein (C/EBP) α, β and δ, liver X receptor (LXR) and sterol regulatory element-binding protein (SREBP).[19] Evidences has raised to support the notion of an adipogenic characteristic of HSC quiescent phenotype. The adipocyte differentiation mixture is able to increase the expression of several key regulators of adipogenesis on HSC, including PPARγ.[20] C/EBPα, SREBP-1c and PPARγ are also expressed in the quiescent HSC, and its ectopic expression inhibit markers of activation as proliferation, ECM production and smooth muscle α-actin.[9] [20] [21] These growing data indicating a role for adipogenesis regulators on the development and maintenance of the quiescent HSC and made us wonder if all-trans-retinol or indomethacin, an anti-inflammatory used to induce adipogenesis,[22] [23] would be able to modulate gene expression of adipogenic transcription factors on HSC.

As primary culture of HSC are activated during culture time course, the study of a possible adipogenesis regulation of quiescent cells can be facilitated by the induction *in vitro* of cell lines representative of HSCs. The GRX cell line was the first permanent cell line derived of HSC.[24] This cell line express various markers of HSC as smooth muscle α-actin, desmin, glial fibrillary acidic protein and cellular retinol binding protein.[1] [25] GRX express a miofibroblast-like phenotype on basal medium, and can be induced to fully express the quiescent phenotype by treatment with retinoids, β-carotene or indomethacin, with an overall increase of lipid storage.[26][27][28] Under such conditions, proliferation and extracellular matrix production decreases.[29] [30] In the present study we evaluated the expression of several adipogenesis related transcription factors on GRX cells induced to express the quiescent phenotype by retinol or indomethacin. We also evaluated the effect of a desmethyl indomethacin, named LM 4511, that lacks ciclooxigenase inhibitor activity, on the expression of PPARs.

MATERIALS AND METHODS

Cell cultures

GRX cells were obtained from the Rio de Janeiro Cell Bank (PABCAM, Federal University, Rio de Janeiro, Brazil). For gene expression experiments, 10^5 cells/well or $3x10^5$ cell/well were seeded for 7 days or 24 hours respectively into 6-well culture plates (Nunc, Roskilde, Denmark). Cells were maintained in Dulbecco's culture medium (DMEM) with 2 g / L HEPES buffer (both from Sigma Chemical Company, St Louis, MO, USA), supplemented with 5% fetal bovine serum (Cultilab, Campinas, SP, Brazil), pH 7.4, in a humidified atmosphere with 5% CO_2. In the experimental series, cells were treated with 5 µM of *all-trans*-retinol or 130 µM of indomethacin or LM 4511 for the indicated times. 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 $cm^{-1}M^{-1}$.

RNA extraction, cDNA synthesis and Real-time PCR

RNA was isolated from GRX cells using the TRIzol Reagent (Invitrogen). Approximately 2 µg of total RNA was added to each cDNA synthesis reaction using the SuperScript-II RT preamplication system (Invitrogen). Reactions were performed at 42ºC for 1 h using the primer T23V (5' TTT TTT TTT TTT TTT TTT TTT TTV). Real-time PCR amplification was carried out using specific primer pairs designed with Oligo Calculator version 3.02 (http://www.basic.nwu.edu/biotools/oligocalc.html) and synthesized by RW-Genes.

The sequences of the primers used are listed in table I. Real-time PCRs were carried out in an Applied-Biosystem 7500 real-time cycler and done in quadruplicates. 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, and 15 s at 72ºC; samples kept for 2 min at 40ºC for reannealing and then heated from 55 to 99ºC with a ramp of 1ºC/sec to acquire data to produce the denaturing curve of the amplified products. Real-time PCRs were made in 10 µl final volume composed of 5 µl of each reverse transcription sample diluted 50 times, 1 µl of Platinum Taq 10 times PCR buffer, 0.6 µl of 50 mM $MgCl_2$, 0.2 µl of 5 mM dNTPs, 0.2 µl of 10 µM primer pairs, 2.45 µl of water, 0.5 µl of SYBR (1:100,000 Molecular Probe), and 0.05 µl of Platinum Taq DNA polymerase (5 U/µl) (Invitrogen). All results were analyzed with the $2^{-\Delta\Delta C}$T methodusing β-actin as internal control gene.[31]

Table I

Primers used for real time PCR

	foward primer	reverse primer
PPARγ	5'-TGGAATTAGATGACAGTGACTTGG	5'-CTCTGTGACGATCTGCCTGAG
PPARα	5'-CAAGGCTATCCCAGGCTTTG	5'-CGGACTCGGTCTTCTTGATG
PPARβ	5'-AGCACCCTGTGAATGACTCC	5'-GGGTGTCAGCCTGTATGGTT
C/EBPα	5'-CCGACTTCTACGAGGTGGAG	5'-GTCGATGTAGGCGCTGATGT
LXRα	5'-TGTTGACTTTGCCAAACAGC	5'-GGGTTGATGAACTCCACCTG
SREBP-1	5'-GTACCTGCGGGACAGCTTAG	5'-TCAGGTCATGTTGGAAACCA
Adipsin	5'-CGTACCATGACGGGGTAGTC	5'-ATCCGGTAGGATGACACTCG
Catalase	5'-ACATGGTCTGGGACTTCTGG	5'-CAAGTTTTTGATGCCCTGGT
Pex16	5'-GTGGACACCCTGGCTACTGT	5'-GTCTGTAAGCAGCTGGAGCA
β-actin	5'-TATGCCAACACAGTGCTGTCTGG	5'-TACTCCTGCTTGCTGATCCACAT

Statistics

All experiments were done in quadruplicates. Data represents mean ± standard deviation (SD). Differences were assessed by using ANOVA followed by Student-Newman-Keuls. Treatment was considered statistically significant when $p<0.05$.

RESULTS

Isolated hepatic stellate cells present PPARα, β and γ with different pattern of expression, which is modificated during *in vitro* activation.[32] Previous studies from our laboratory demonstrated that GRX cells express an activated phenotype on basal medium, showing activation markers as smooth muscle α-actin.[25] We aimed to analyze the expression pattern of PPARs in GRX cultured in basal medium (Fig.1). All three isoforms of PPARs were expressed, with PPARβ being massively more expressed than PPARα and PPARγ, rising up to 5000 and 50 times more, respectively. In the first day of culture, HSC freshly isolated from rats shown high levels of PPARγ and low levels of PPARβ. After 7 days in culture following activation, this is inverted, being PPARβ the predominant PPAR expressed.[20] Therefore, GRX cells cultured in basal medium exhibit similar PPARs expression pattern as in vitro activated HSCs.

GRX cells are known to convert to quiescent cells when treated with indomethacin or retinol for 7 days, accumulating neutral lipids and retinol on perinuclear lipids droplets and reducing activation markers (Andrade et al., 2003; Guaragna et al., 1991, Margis et al., 1989). [26] [33] [34] Hence, we look forward to determine the expression of the different PPAR subtypes during the GRX quiescent conversion with indomethacin or retinol.

PPAR expression was investigated in the first 24 hours and in the seventh day of conversion. Indomethacin and retinol did not modulated PPARγ or PPARβ expression in the first 24 hours (Fig.2A and E). PPARα expression was

responsiveness to indomethacin in the two times examined, with a overall increase of 2 times (Fig.2C and D). After 7 days, with the appearance of lipid droplets, the expression of PPARγ had already increased 4 times with retinol treatment and 3 times with indomethacin in comparison with control cells (Fig.2B). Surprisingly, retinol treatment slightly increased PPARβ expression (Fig.2F), although previously reported to have diminished expression in quiescent HSC.[20]

Following the observation that both treatments increased PPARs isoforms during the quiescent conversion of the GRX cells, we wondered if downstream PPARs related genes could also be modulated during time course of treatments. We evaluated the expression of the enzyme catalase, the peroxisomal protein Pex16 and the adipogenic marker adipsin (Fig.3). Indomethacin lead to an increased catalase gene expression after 7 days treatment, while retinol showed no effect (Fig.3B). Adipsin expression has been used as a marker of adipocyte differentiation on many models of adipogenesis *in vitro*.[23] [35] [36] Therefore, we sought to evaluate if adipsin had its expression modulated after 7 day treatment with retinol or indomethacin. Similarly to catalase, only indomethacin increased adipsin expression (Fig.3C). Peroxisomes in rodents can be induced by peroxisome proliferators, compounds that have PPARs ligand activity and can augment the expression of peroxisins (Pex), family involved in peroxisomes biogenesis and proliferation.[37] [38] Indomethacin and retinol differentially increased the expression of the peroxin Pex16, with the former being more potent than the last (Fig.3A).

This different effect of retinol and indomethacin on PPAR related genes expression points to the possibility that these treatments could be also differentially

modulating adipogenic genes expression. Hence, we evaluated the expression of LXRα, C/EBPα and SREBP-1, all of them involved on adipogenesis (Fig.4).[39] [40] LXRα is a nuclear hormone receptors with a major role as cholesterol sensor,[41] and a recently report showed a role on adipogenesis by modulating PPARγ.[42] Among these three genes evaluated, LXRα was the only that had its expression modulated by indomethacin in the first 24 hours and after 7 days indomethacin treatment stayed with an increased expression compared to control myofibroblast (fig.4A and B). C/EBPα, a transcription factor that modulates and is modulated by PPARγ,[19] [43] had its expression not affect by indomethacin, but after 7 days of retinol treatment (fig.4C and D) its expression decreased compared to control myofibroblasts and indomethacin treated cells (fig.4D). Next, we evaluated the expression of SREBP-1, whose activation has been related with PPARγ expression.[36] [44] Although PPARγ expression increased with retinol and indomethacin treatment after 7 days (fig.2B), only indomethacin induced SREBP-1 expression in the same treatment period (fig.4E). Neither treatments modulated this gene in the first 24 hours (fig.4F).

Indomethacin has been extensively studied in several liver diseases because of its COX inhibitory activity.[45] [46] However, with the concentration used in this study (130 μM), it is reported that indomethacin also exert PPARγ and PPARα ligand activity.[47] As we observed an augment in the gene expression of PPARα and PPARγ on GRX cells treated with indomethacin, we aimed to evaluate the participation of its COX inhibitory activity by using a modified indomethacin lacking the 2'-methyl group, named LM-4511, that poorly inhibit COX but retains the capacity to activates PPARγ (Marnett LJ, personal communication, 2005).[48]

When we evaluated the effect of LM-4511 on PPARα and PPARγ expression, we observed a result similar to the obtained with indomethacin (Fig.5A and C). Furthermore, this compound also induces the expression of adipsin (fig.5B).

DISCUSSION

The discovery that PPARγ expression is modulated during HSC activation and that PPARγ ligands exerts anti-fibrotic effects highlighted a possible anti-fibrotic effect of this transcription factor during liver fibrosis.[49] More importantly, the demonstration that ectopic expression of PPARγ and C/EBP-α were enough to restore several markers of the quiescent phenotype and reduce fibrotic characteristics of HSCs suggested a analogy between the maintenance of the quiescent and the adipocyte phenotype.[9] [21]

GRX cells cultured in basal medium presents myofibroblast phenotype with several characteristics of activated HSCs.[25] [27] Furthermore, this cell line has a pattern of PPARs expression similar to the observed in HSCs activated *in vitro*: a higher expression of PPARβ compared to the others PPARs isotypes with PPARα having the lowest expression (Fig.1).[20] The expression of PPARβ has been inversely related to PPARγ during HSC transdifferentiation.[20] Although this study correlates PPARβ function with characteristics of activated phenotype, a recent work showed that genes involved in retinol uptake and sterification are regulated by PPARβ on activated cells.[32] This discovery suggests a possible analogy between the role of PPARβ on activated HSCs and preadipocytes. PPARβ participates in the proliferation rate of both cells and on the expression of proteins involved in the uptake of substrate for the formation of lipid droplets, as CD36 and fatty acid binding proteins. Likewise, long chain fatty acids are able to increase PPARβ expression on both cells.[32] [43] Furthermore, as occurs on the commitment of preadipocytes to mature adipocytes, PPARγ and PPARβ

expression are inversely correlated during HSC phenotype switch.[20] These observations points to the possibility that the pattern of PPARs expression seen on activated HSCs and GRX maintained on basal medium is analogue to the observed in preadipocytes.

In spite of the fact that retinol and indomethacin induce the GRX quiescent phenotype,[26] [50] we show a different expression of adipogenic transcription factors along this process. These distinct action could be related to the ligand activity and specificity that they have. Indomethacin is able to activate PPARγ and PPARα in the concentrations used in this work (>100μM), with 2 times more activity on PPARα.[47] *All-trans*-retinol metabolism can lead to the formation of RXR or RAR ligands. By process of oxidation and isomerization, cells are able to convert *all-trans*-retinol to 9-*cis* retinoic acid, that binds to RXR and RAR, and *all-trans* retinoic acid that can bind RAR only.[51] [52]

Among the three PPARs isotypes, PPARα was the only one to augment on the first 24 hours of quiescent conversion (Fig.2C). *In vitro* experiments showed that this PPAR expression is low in comparison to the other PPAR isotypes along the conversion induction of adipocytes.[43] On HSCs, the synthetic PPARα ligand Wy-14,643 diminish activation markers, as DNA and collagen synthesis, at concentrations known to activate both PPARγ and PPARα but not at those that only activate PPARα or by a synthetic PPARα-selective agonist.[53] An *in vivo* study showed that administration of Wy-14,643 to rats ameliorates steatohepatitis induced by a methionine and choline deficient diet on rodents.[54] Although this could indicate a direct effect of PPARα on the inhibition of HSC activation and a possible induction of the quiescent phenotype, it is likely that treatment with a

PPARα ligand on the rodent liver reverses fibrosis indirectly by reducing stimuli, such as lipid peroxides and macrophages migration, known to activate HSC.[55] [56] Therefore, our observation that indomethacin treatment increases PPARα on 24 hours and 7 days after GRX treatment could be related to the high affinity ligand characteristic of this compound on PPARα[47] and not to a direct role during the quiescent conversion of GRX cells.

Interestingly, 7 days treatment with retinol increased PPARβ expression (Fig.2F). Retinoic acid, a metabolic product of retinol, is a high affinity ligand of PPARβ, activating this transcription factor at nanomolar concentrations.[57] As we treated GRX cells with 5 μM of retinol, we could be leading to a forced activation and expression of this gene along 7 days treatment. The indomethacin inability to exert same effect suggests this possibility (Fig.2F).

Indomethacin, a non-steroidal anti-inflammatory drug, is largely used as COX inhibitor.[58] Previous reports demonstrated that this compound is also able to act as PPARγ and PPARα ligands and activators.[47] [59] We asked whether this compound could be increasing PPARα and PPARγ gene expression by its COX inhibitory or PPAR ligand activity. In order to test this, we used LM-4511, an indomethacin with the 2'-methyl group removed that reduces dramatically the COX inhibitory activity.[48] Importantly, LM-4511 retains the capacity to activate PPARγ at the micromolar range (Marnett LJ, personal communication, 2005). When we evaluated the effect of LM-4511 on PPARα and PPARγ expression we observed a result similar to the obtained with indomethacin (Fig.5A and C, respectively). Furthermore, this compound also induces the expression of adipsin, but opposite to the observed with PPARα and PPARγ, LM-4511 increased adipsin expression

in a lesser degree when compared to indomethacin (Fig.5B). These results suggests that indomethacin is acting by its PPAR ligand activity rather than its COX inhibitory activity.

PPARγ and PPARα are known to induce gene expression of catalase. This antioxidant enzyme contains on the promoter a functional PPRE and appears to be a highly PPAR regulated gene.[60] *In vitro* studies showed that PPARγ ligands can induces the expression and activity of catalase.[60] Furthermore, the administration of Wy-14,643 have an antifibrotic action in the rat thioacetamide model of liver cirrhosis, probably due to an antioxidant effect of enhanced catalase expression and activity in the liver.[61] The peroxin family protein regulates the biogenesis and proliferation of peroxisomes and members can be induced by activators of PPARα and PPARγ.[62] [63] Catalase and Pex16, a member of the peroxin family, were modulated during quiescent conversion of GRX cells (Fig.3A and B). Hence, an augment on expression of genes known to be regulated in a positive fashion by PPARs suggests that not only PPARs expression was increased during quiescent conversion of GRX cells, but also their transcriptional activity.

Retinoids inhibits the process of adipocyte differentiation. They impede adipogenesis by blocking C/EBPβ-mediating induction of downstream genes.[64] PPARγ and C/EBPα are included on the set of downstream genes not inducted during retinoids adipogenesis inhibition, and it was shown that this molecule acts in the early events of adipogenesis.[65] On GRX cells, 7 days treatment with retinol decreased C/EBPα gene expression (Fig.4D), while LXRα and SREBP-1, transcription factors also involved in adipogenesis,[36] [42] remained with the same level of gene expression as the untreated myofibroblast (Fig.4B and F). In the other

hand, indomethacin treatment increased gene expression of LXRα and SREBP-1 (Fig.4B and F). Interestingly, as with indomethacin, PPARγ expression augment on GRX cells treated by 7 days with retinol (Fig.2B). The activation of HSCs is accompanied by loss of retinoid stores and reduced RXR expression,[66] [67] then PPARγ activity and expression may be limited by the reduced expression of dimerization partner and ligands. Although retinol is not a PPARγ ligand, the formation of a retinol pool during the time course of the treatment could be leading to the induction of RXR-PPARγ dimerization by the ligand activation of RXR and the following PPARγ expression. In fact, a study demonstrated that retinoid treatment on myeloid cells potentiate the expression and activity of PPARγ.[68]

These results points to a different mode of action of retinol and indomethacin during quiescent conversion. Although both compounds mediates the accumulation of lipid droplets,[26] [27] previous studies showed other differences between these two treatments on GRX cells. A recent work reported that retinol treatment increase cellular retinol binding protein-I (CRBP-I) expression during quiescent conversion, whereas indomethacin treated GRX cells remains with the same level of CRBP-I as the untreated myofibroblasts.[50] Moreover, the same study showed that myofibroblasts that were previously treated during 8 days with retinol, followed by 48 hours in basal medium, had a greater capacity to uptake retinol and synthesizes retinyl esters than indomethacin treatment.

These previous results on GRX retinol metabolism, together with our observations on this study, suggests that retinol induces a lipogenic phenotype on GRX that do not seems to be accompanied by adipogenic characteristics. In another hand, indomethacin appears to be inducing an adipogenic pathway that is

poorly followed by lipogenesis during conversion to quiescent cells. Lipogenesis encompasses the processes of fatty acid and triglyceride synthesis by inducing proteins that transport and accumulates fatty acids. Although lipogenesis can follow adipogenesis, it should not be mistaken for the former, which refers to the differentiation of cells by expressing adipocytes-specific genes.[69] This hypothesis is also suggested in view of adipsin gene expression along quiescent conversion, where only indomethacin treatment increased the expression of this adipogenic marker (Fig.3C). Detailed elucidation of retinol and indomethacin role on the induction of GRX quiescent phenotype and their interaction with transcription factors that modulates this phenotype transdifferentiation will help to differentiate the role of both processes on HSC physiology during liver fibrosis.

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LEGENDS OF FIGURES:

Fig 1. PPARα, γ and β relative expression on GRX cells maintained on basal medium. After 24 hours on six-well plates total RNA was extracted and gene expression was analyzed as described in materials and methods. Briefly, total RNA was reverse transcribed and PPARs expression quantified by real-time PCR using SYBR green as detection method. Relative quantification was normalized with β-actin expression and calculated by the 2-ΔΔ CT method.

Fig 2. PPARs gene expression during quiescent conversion of GRX. Cells were treated with indomethacin (130 μM) or retinol (5 μM) for 24 hours (A,C,E) or 7 days (B,D,F) and gene expression was measured as described in materials and methods. A, no change in PPARγ gene expression and B, increased expression of PPARγ during quiescent conversion either with indomethacin or retinol on the seventh day. C and D, increased PPARα expression on indomethacin treated cells on both times analyzed. E, unaltered expression of PPARβ on the first 24 hours of quiescent conversion and F, augment of PPARβ expression on cells treated with retinol during 7 days. Data represents mean ± SD. *, $p<0,05$.

Fig 3. Gene expression of PPARs related genes and an adipogenic marker during quiescent conversion. Cells were treated with indomethacin or retinol during 7 days and gene expression was measured as indicated in materials and methods. A,

Pex16, a peroxisome proliferation gene, expression increases with retinol or indomethacin treatment. B, catalase gene expression increases only with indomethacin treatment. C, Adipsin, an adipogenic marker, has its expression increased with indomethacin treatment. Treatment concentrations correspond to the same as in figure 2. Data represents mean ± SD. *, p<0,05.

Fig 4. Adipogenic transcriptional factors expression during quiescent conversion of GRX. Cells were treated with indomethacin (130 μM) or retinol (5 μM) for 24 hours (A,C,E) or 7 days (B,D,F) and gene expression was measured as described in materials and methods. A and B, LXRα shows increased expression when GRX cells were treated with indomethacin on both times analyzed. C, C/EBPα expression is unaltered with both treatments. D, decreased C/EBPα expression on 7 days retinol treated cells. E, unaltered expression of SREBP-1 on the first 24 hours of quiescent conversion. F, augment of SREBP-1 expression on cells treated with indomethacin during 7 days. Data represents mean ± SD. *, p<0,05.

Fig 5. PPARα, PPARγ and adipsin expression on LM4511 treated cells. GRX cells were treated with 130 μM LM4511 or indomethacin for 7 days followed by gene expression analysis as described in materials and methods. A, PPARα shows the same expression pattern on indomethacin or LM4511 treated cells. B, Adipsin induced expression by LM4511 shows half induction compared to indomethacin treated cells. C, Indomethacin and LM4511 induce PPARγ in the same extend. Data represents mean ± SD. *, p<0,05.

Figures

Figure 1

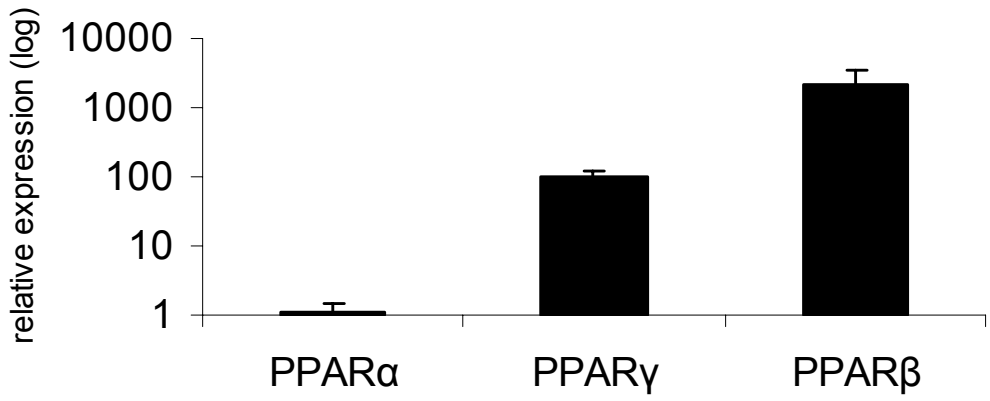
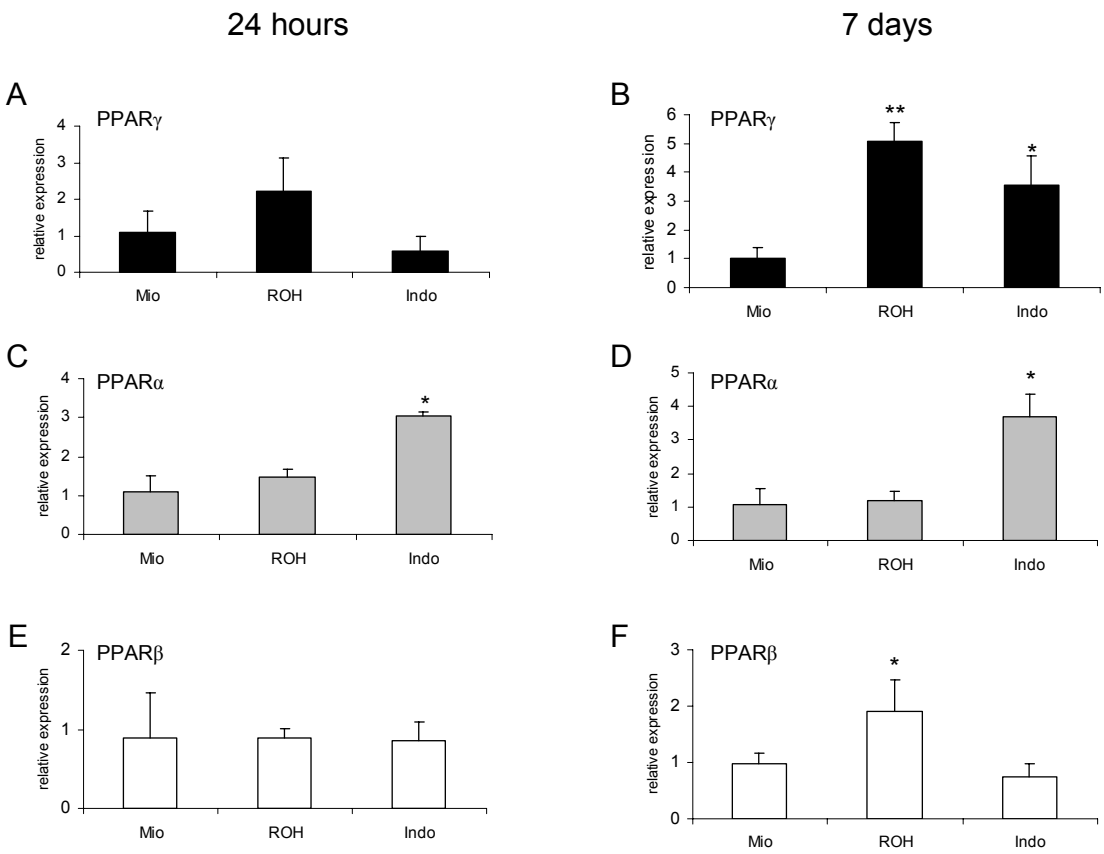


Figure 2


24 hours
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*

Figure 3

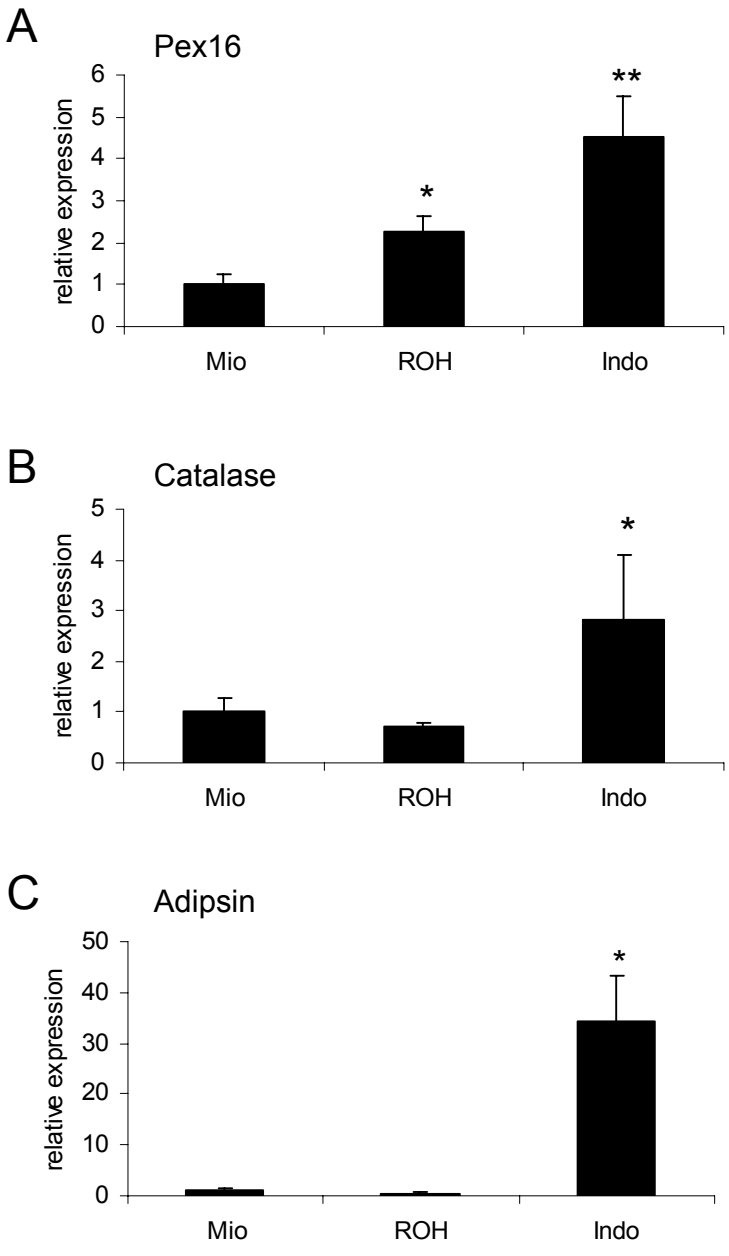

A
Pex16
relative expression
0
1
2
3
4
5
6
*
**
Mio
ROH
Indo
B
Catalase
relative expression
0
1
2
3
4
5
*
Mio
ROH
Indo
C
Adipsin
relative expression
0
10
20
30
40
50
*
Mio
ROH
Indo

Figure 4

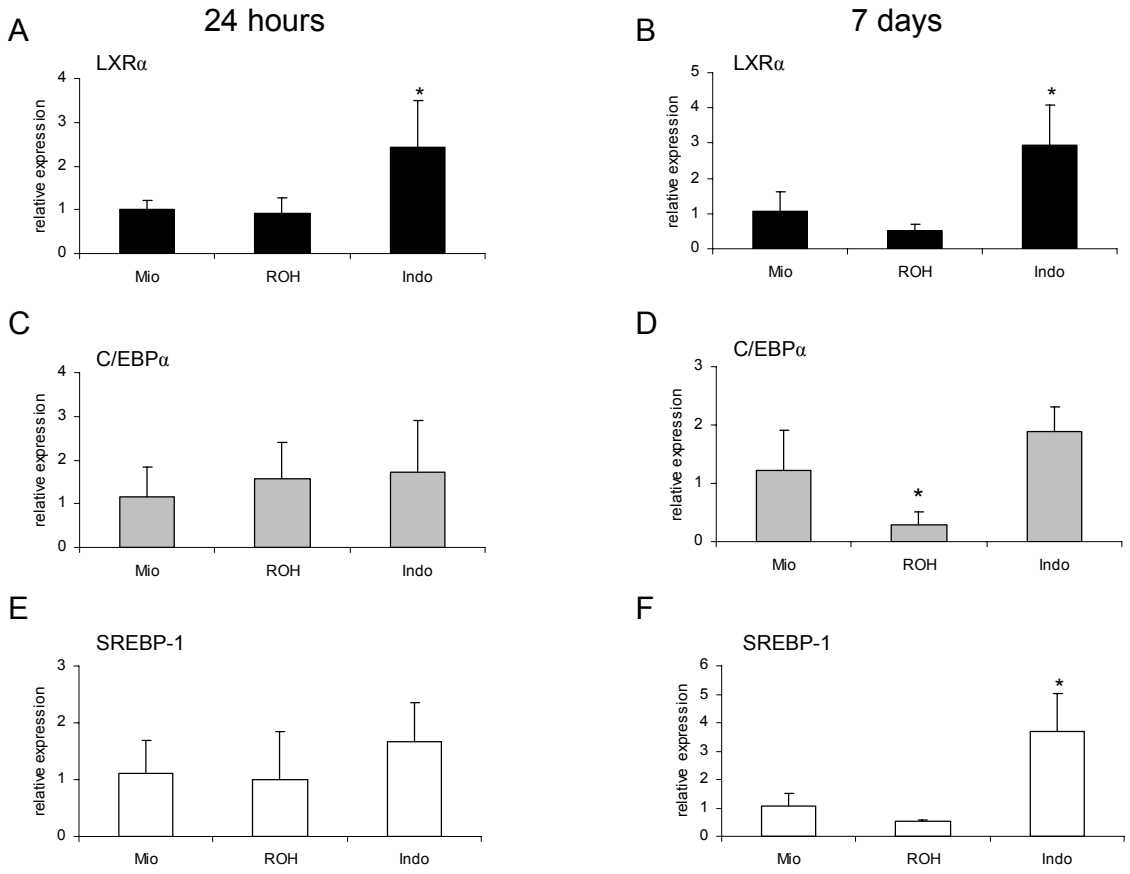
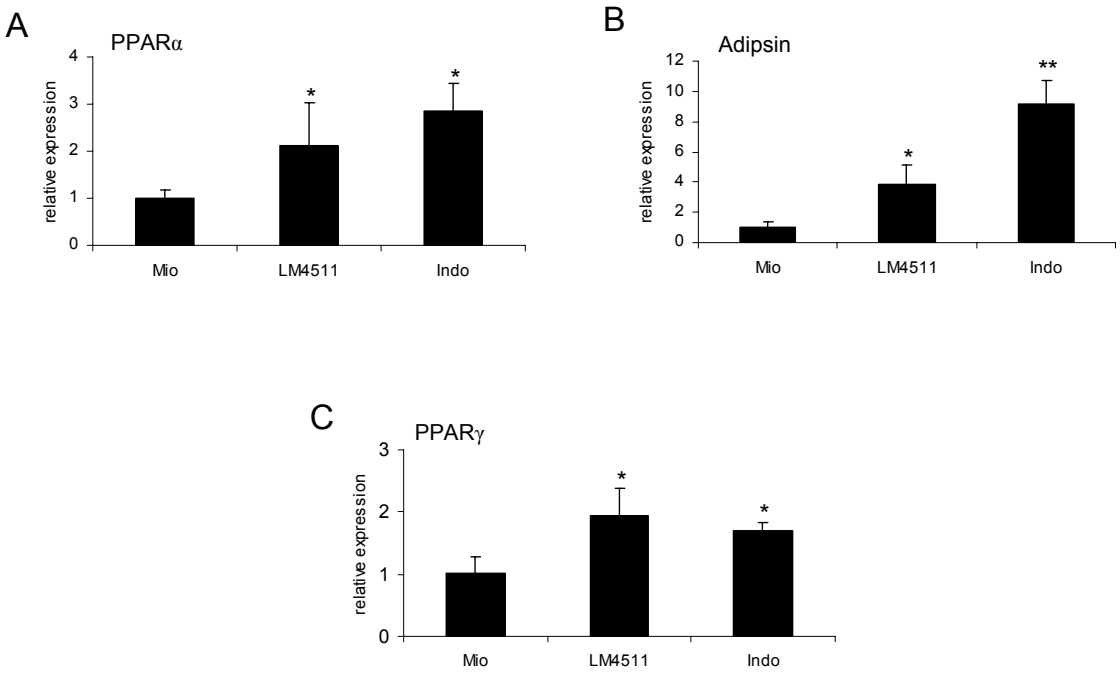

A
24 hours
LXRα
relative expression
Mio
ROH
Indo
B
7 days
LXRα
C
C/EBPα
D
C/EBPα
E
SREBP-1
F
SREBP-1

Figure 5


A
PPARα
relative expression
0
1
2
3
4
*
*
Mio
LM4511
Indo
B
Adipsin
relative expression
0
2
4
6
8
10
12
*
**
Mio
LM4511
Indo
C
PPARγ
relative expression
0
1
2
3
*
*
Mio
LM4511
Indo

PARTE III

RESULTADOS E DISCUSSÃO

A descoberta de que RAPPγ é modulado durante a ativação das CEHs, e que ligantes específicos deste fator de transcrição possuem efeitos anti-fibróticos (Galli et al., 2000) abriu a possibilidade de que RAPPs, e outros genes da mesma família, pudessem ter um papel importante no desenvolvimento da fibrose hepática. Além desses fatos, a demonstração de que a expressão ectópica de RAPPγ ou PLCEα é suficiente para restaurar várias características do fenótipo lipocítico das CEHs levantou a possibilidade de analogia entre a transdiferenciação das CEHs e adipócitos (She et al., 2005). Pré-adipócitos mantidos *in vitro* são capazes de se diferenciarem para adipócitos apenas com a expressão ectópica de RAPPγ ou PLCEα (Darlington et al., 1998; Tontonoz et al., 1994).

Células da linhagem GRX mantidas em meio basal de cultura apresentam as três isoformas de RAPPs (Fig.1). Nestas condições de cultivo, as células GRX apresentam o fenótipo miofibroblástico, e possuem características semelhantes a CEHs ativadas *in vitro*, como a produção de matriz extracelular, alta taxa proliferativa e a expressão de α-actina de músculo liso (Borojevic et al., 1990; Guma et al., 2001). O padrão de expressão dos RAPPs também é semelhante ao encontrado nas CEHs ativadas: uma alta expressão de RAPPβ comparada com os outros RAPPs, sendo RAPPα o de menor expressão (She et al., 2005). A expressão de RAPPβ tem sido inversamente relacionada com a expressão de

RAPPγ durante a conversão fenotípica das CEHs (She et al., 2005). Embora estes estudos relacionem a expressão de RAPPβ com características clássicas de ativação das CEHs. No entanto, Hellemans e colegas (2003) demonstraram que este fator de transcrição está envolvido com a expressão de genes relacionados com a captação e metabolismo de retinol no fenótipo miofibroblástico, genes que até então se pressupunham serem expressos apenas em CEHs lipocíticas. Esta descoberta demonstra que a CEH ativada mantém a capacidade de captar e metabolisar seu principal substrato de acúmulo, semelhante ao observado no início de indução adipogênica de fibroblastos pré-adipocíticos, onde ocorre aumento da expressão de genes relacionados com a captação e metabolismo de ácidos graxos (Grimaldi, 2001). Várias características semelhantes aos dois tipos celulares indicam uma possível analogia entre eles. Por exemplo, em ambos tipos celulares, o RAPPβ está envolvido no aumento da taxa proliferativa (Grimaldi, 2001; Hellemans et al., 2003), a expressão de RAPPγ e RAPPβ parece estar inversamente relacionada com a adipogênese e o tratamento com seus respectivos substratos de acúmulo induz à expressão das proteínas carregadoras e translocadoras de ácidos graxos.

Apesar do fato de que retinol e indometacina são capazes de induzir o fenótipo lipocítico na linhagem GRX (Vicente et al., 1998; Margis et al., 1989), nossos resultados mostram uma expressão diferencial de fatores de transcrição adipogênicos em respostas aos dois agentes indutores de fenótipo. Estas diferenças podem estar relacionadas a atividade de ligante e a especificidade de cada um dos compostos. Indometacina possui a capacidade de se ligar a RAPPγ e RAPPα, ativando ambos, mas com potência 2 vezes maior sobre RAPPα

(Lehmann et al., 1997). O metabolismo de *all-trans* retinol pode levar a produção de ligantes de RXR e de receptores de ácido retinóico (RAR). Através de processos de isomerização e oxidação, células são capazes de converter *all-trans* retinol em 9-*cis* ácido retinóico, ligante de RXR e RAR, e de *all-trans* ácido retinóico que é capaz de se ligar apenas em RAR (Bastien e Rochette-Egly, 2004; Paik et al., 2000).

Entre as três isoformas de RAPPs, RAPPα foi o único que teve a expressão aumentada nas primeiras 24 horas de conversão (Fig.2C). RAPPα não é modulado durante a diferenciação de adipócitos *in vitro*, e sua expressão é baixa relativamente a dos outros PPARs durante este processo (Grimaldi, 2001). O tratamento de CEHs, com Wy-14,643, ligante sintético de RAPPα, diminui marcadores de ativação, como a síntese de DNA e colágeno. Este efeito pode ser devido a ação do Wy-14,643 sobre o RAPPγ, já que um ligante específico de RAPPα não consegue reproduzir este resultado (Myiahara et al., 2000). Um estudo *in vivo* realizado por Ip e colegas (2004) mostrou que a administração de Wy-14,643 em ratos atenua a esteato-hepatite induzida por uma dieta deficiente em metionina e colina. Apesar desses resultados poderem indicar um efeito direto de RAPPα na inibição da ativação das CEHs, é possível que o tratamento com Wy-14,643 atenue a fibrose hepática indiretamente através da redução de estímulos, como a migração de macrófagos e a produção de lipídios oxidados, fenômenos reconhecidos como estimuladores de fibrose hepática (Olynyk et al., 2002; Imamura et al., 2005). Portanto, nossa observação de que indometacina leva a um aumento na expressão de RAPPα após 24 horas e 7 dias de tratamento pode estar relacionado a alta afinidade de ligação e ativação que este composto possui

sobre RAPPα e não diretamente com um papel sobre a conversão das células GRX.

Ao final de 7 dias de tratamento com retinol, quando as células apresentam o fenótipo lipocítico, observamos um aumento na expressão de RAPPβ (Fig.2F). Em culturas primárias de CEHs, a expressão de RAPPβ diminui durante a ativação das CEHs (She et al., 2005), então esperaríamos uma diminuição na expressão deste fator de transcrição durante a conversão das células GRX para lipócitos. No entanto sabe-se que o ácido retinóico, produto do metabolismo do retinol, é um ligante com alta afinidade por RAPPβ, ativando este fator de transcrição a partir de concentrações de nanomolar (Shaw et al., 2003). Uma vez que as células foram tratadas com retinol na concentração de 5μM, existe a possibilidade que esta concentração, cerca de mil vezes maior do que o necessário para ativar RAPPβ, esteja forçando a expressão deste gene. A inabilidade do tratamento de conversão com indometacina de reproduzir este resultado também sugere um efeito específico do retinol.

Indometacina, uma droga anti-inflamatória não esteroidal, é amplamente usada como inibidora de ciclooxigenase, enzima responsável pela produção de prostaglandinas (Mitchell et al., 1993). Estudos anteriores demonstraram que além desta atividade, indometacina também pode atuar como ligante de RAPPα e RAPPγ em concentrações acima de 100 μM (Lehmann et al., 1997; Jaradt et al., 2001). Como nas células GRX, o tratamento com indometacina aumentou a expressão de RAPPα após 24 horas e 7 dias (Fig.2C e D) e a de RAPPγ após 7 dias (Fig.2B), nos perguntamos se estes resultados eram derivados da atividade inibidora de COX ou de sua ação como ligante de RAPPα e RAPPγ. Para tentar

responder esta pergunta tratamos as células com LM 4511, uma molécula análoga a indometacina onde a única diferença estrutural é a ausência de um grupo metil. Esta modificação leva a uma drástica perda da atividade inibitória sobre ciclooxigenase, não alterando a capacidade de ligação e ativação de RAPPγ (Prusakiewicz et al., 2004; Marnett J, comunicação pessoal). Nas células tratadas com o análogo a expressão de RAPPα e RAPPγ foi semelhante a encontrada com indometacina (Fig.5A e C, respectivamente). Também avaliamos se LM-4511 é capaz de induzir a expressão de adipsina, um marcador de conversão adipocítica. Como mostrado na figura 5B, LM4511 aumentou a expressão de adipsina, mas com menor intensidade do que indometacina. Estes resultados sugerem que a indometacina induza o fenótipo lipocítico nas células GRX através de sua atividade como ligante de RAPPs.

Sabe-se que os RAPPγ e RAPPα são capazes de induzir a expressão gênica de catalase (Girnun et al., 2002). Esta enzima antioxidante contém no seu promotor um elemento responsivo a proliferadores de peroxissomos. Estudos *in vitro* demonstraram que ligantes específicos de RAPPγ são capazes de aumentar a expressão e a atividade desta enzima (Girnun et al., 2002). Em ratos, a administração de Wy-14,643 diminui a fibrose hepática foi induzida por tioacetamida, provavelmente devido a maior expressão e atividade antioxidante de catalase no fígado (Toyama et al., 2004). As peroxinas (Pex), são uma família de proteínas conhecidas por regularem a biogênese e proliferação de peroxissomos, e a expressão de seus membros pode ser induzida por ativadores de RAPPα e RAPPγ (Eckert and Erdmann, 2003; Shimizu et al., 2004). Os resultados apresentados nas figuras 3A e B mostram que nas células GRX a

expressão de catalase e Pex16, foi modulada durante a indução lipocítica. Portanto, um aumento na expressão de genes que são regulados positivamente por RAPPs sugere que não apenas a expressão gênica dos RAPPs esteja aumentada durante a conversão lipocítica das células GRX, mas também a atividade transcricional destas proteínas.

Retinóides inibem o processo de adipogênese. Eles impedem a diferenciação adipocítica bloqueando a indução de genes controlados por PLCEβ, fator de transcrição que atua inicialmente neste processo (Schawrz et al., 1997). RAPPγ e PLCEα estão incluídos no grupo de genes que têm a sua expressão inibida durante o bloqueio de adipogênese por retinóides (Xue et al., 1996). Nas células GRX, 7 dias de tratamento com retinol diminui a expressão de PLCEα (Fig.4D), enquanto que RXFα e PLERE-1, fatores de transcrição também envolvidos na adipogênese (Kim et al., 1998; Seo et al., 2004), continuaram com o nível de expressão gênica de miofibroblastos não tratados (Fig.4B and F). Já o tratamento com indometacina aumentou a expressão destes fatores de transcrição (Fig.4B and F). Apesar de estar demonstrado que em adipócitos, retinóides inibem a expressão de RAPPγ, nós observamos um aumento de expressão deste fator de transcrição com 7 dias de tratamento com retinol (Fig.2B). A ativação das CEHs é acompanhada da perda das reservas de retinol e redução da expressão de RXR (Ohata M et al., 1997; Yamane et al., 1993), então talvez a expressão e atividade de RAPPγ seja limitada pelo pequeno número de parceiro de dimerização e ligante deste parceiro. Apesar de retinol não ser um ligante de RAPPγ, um aumento de concentração intracelular de retinol durante o tratamento de conversão pode estar levando a formação do heterodímero RXR-RAPPγ.

Szanto e Nagy (2005) demonstraram que o tratamento com retinol em células mielóides potencia a expressão e atividade de RAPPγ.

Os resultados mostrados neste trabalham indicam que retinol e indometacina agem de modo diferente durante a conversão lipocítica das células GRX. Embora ambos tratamentos induzam o aparecimento de gotas lipídicas (Borojevic et al., 1990; Margis et al., 1989), estudos prévios mostram que há outras diferenças além das observadas aqui. Vicente e colegas (1998) demonstraram que a indução do fenótipo lipocítico com retinol aumenta a expressão da proteína celular ligante de retinol-I (PCLR-I), enquanto que a indução com indometacina mantém a expressão de PCLR-I nos mesmos níveis encontrados no fenótipo miofibroblástico. O mesmo estudo mostrou que após o tratamento durante 8 dias com retinol, seguido por 48 horas em meio basal, as células tinham uma maior capacidade de captar retinol e sintetizar ésteres de retinila do que células previamente tratadas com indometacina.

Estes prévios resultados, conjuntamente com os nossos, sugerem que o retinol induz um fenótipo lipogênico enquanto que indometacina induz um fenótipo adipogênico. Lipogênese é o processo de síntese de ácidos graxos e triacilglicerídeos através da indução de proteínas que transportam e acumulam ácidos graxos. Apesar de a lipogênese poder ser acompanhada de adipogênese, os dois processos não devem ser confundidos, uma vez que a adipogênese é caracterizada pela expressão de genes específicos de adipócitos, como a adipsina e fatores de transcrição envolvidos neste processo (Kersten, 2001). Esta hipótese pode ser reforçada pelo resultado da análise da expressão de adipsina ao longo do processo conversão fenotípica das GRX, onde verificou-se que apenas o

tratamento com indometacina aumentou a expressão deste marcador de adipócitos (Fig.3C).

A elucidação detalhada do papel da indometacina e do retinol ao longo da conversão fenotípica da linhagem GRX e sua interação com fatores de transcrição que modulam esta diferenciação poderá ajudar na descoberta de vias de manutenção do fenótipo lipocítico das CEHs e conseqüentes tratamentos para a fibrose hepática.

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