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SHYRLENE MEIRY DA ROCHA ARAÚJO

AVALIAÇÃO DOS EFEITOS DO DERIVADO TIAZOLIDÍNICO LPSF/GQ-02 SOBRE AS VIAS DE SINALIZAÇÃO DO AMPK E SREBP-1c NO METABOLISMO LIPÍDICO HEPÁTICO DE CAMUNDONGOS LDLR-/-

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Dissertação apresentada dia 29/07/2015, no Aggeu Magalhães – FIOCRUZ/PE, às 9:00 horas.

Orientadora: Profª. Drª. Christina Alves Peixoto.

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A minha anjinha Anna Luiza.

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RESUMO

O sobrepeso e a obesidade têm sido identificados como os fatores de risco mais importantes para muitas doenças, incluindo doenças cardiovasculares, diabetes tipo 2 e distúrbios lipídicos como a doença do fígado gorduroso não-alcoólica (NAFLD). Atualmente, a NAFLD é considerada como a manifestação hepática da síndrome metabólica, sendo uma das doenças hepáticas mais prevalentes em todo o mundo. Evidências crescentes sugerem que o AMPK e SREBP são reguladores críticos do metabolismo de lipídios no fígado. As tiazolidinadionas (TZDs) são comumente utilizadas para o tratamento de diabetes tipo 2 e outras condições que ofereçam resistência a insulina como a NAFLD. No presente estudo, foi avaliada a atividade biológica do derivado tiazolidínico (LPSF/GQ-02) sobre a via metabólica de lipídios na patogênese da NAFLD. Foram utilizados camundongos deficientes do receptor de LDL (LDLr-/-) dividido em três grupos: 1- Dieta hipercalórica (HFD); 2- HFD + Pioglitazona (20 mg/kg/dia); 3- HFD+LPSF/GQ-02 (30mg/kg/dia). O experimento foi realizado por 12 semanas sendo que nas ultimas 4 semanas as drogas em estudo (PIO e LPSF/GQ-02) foram administradas via gavagem. Os resultados obtidos indicaram que a LPSF/GQ-02 foi eficaz em melhorar a arquitetura hepática diminuindo a acumulação de gordura no fígado, através da inibição da via da lipogênese (LXR/SREBP-1C/ACC/FAS), bem como através da ativação da via lipolítica (AMPK/FoxO1/ATGL). Estes resultados sugerem uma ação direta da LPSF/GQ-02 sobre o metabolismo lipídico e consequentemente na esteatose hepática, devido à diminuição de gordura nos hepatócitos por meio da inativação da via de síntese de lipídios e aumento da β-oxidação dos ácidos graxos e lipólise. Sendo assim, esses dados apoiam os resultados anteriormente publicado pelo Laboratório de Ultraestrutura do Aggeu Magalhães, que mostraram a propriedade hipolipemiante da LPSF/GQ-02, ao reduzir o acúmulo de triglicerídeo no fígado, bem como confirma o potencial desta TZD para o tratamento na NAFLD.

Palavras Chaves: Síndrome Metabólica, NAFLD, Tiazolidinadionas, LPSF/GQ-02.

ABSTRACT

Overweight and obesity have been identified as the more important risk factors for many diseases, including cardiovascular disease, type 2 diabetes and lipid disorders as the disease. Nonalcoholic fatty liver disease (NAFLD). Actually, NAFLD is considered as the hepatic manifestation of metabolic syndrome is one of the most prevalent liver disease worldwide. Growing evidences suggests that AMPK and SREBP are critical regulators of lipid metabolism in the liver. The thiazolidinediones (TZDs) are commonly used for the treatment of type 2 diabetes and other conditions that provide insulin resistance and NAFLD. In the present study, we evaluated the biological activity of LPSF / GQ-02 on the metabolic pathway of lipids in the pathogenesis of NAFLD. We used mice deficient in LDL receptor (LDLr - / -) divided into three groups: 1 hypercaloric diet (HFD); 2- HFD + pioglitazone (20 mg / kg / day); 3- HFD + LPSF / GQ-02 (30mg / kg / day). The experiment was conducted for 12 weeks and in the last four weeks the drugs were administered daily by gavage. The results indicated that LPSF / GQ-02 was effective in improving liver architecture by decreased the accumulation of fat in the liver, by inhibiting the lipogenic via (LXR / SREBP-1C / ACC / FAS), as well as activating the lipolytic pathway (AMPK / FoxO1 / ATGL). These results suggest a direct action of LPSF / GQ-02 on lipid metabolism in hepatic steatosis and, consequently, due to the decrease of fat in hepatocytes through the inactivation of lipid synthesis pathway and increase the β-oxidation of fatty acids and lipolysis. Thus, these data support the results previously published by Ultrastructure Laboratory Aggeu Magalhães, who showed lipid-lowering property of LPSF / QA-02 by reducing triglyceride accumulation in the liver, and confirms the potential of this TZD for treatment in NAFLD.

Key words: Metabolic syndrome, NAFLD, thiazolidinediones, LPSF / GQ-02, lipid-lowering.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

ACC	Acetil CoA carboxilase
ADP	Adenosina difosfato
ALT	Alanina transaminase
AMPK	Proteína quinase ativada por 5’AMP
AST	Aspartato aminotransferase
ATGL	Lipase de triglicerídeo do tecido adiposo
ATP	Adenosina trifosfato
CaMKK	Quinase Cálcio/calmodulina dependente da proteína quinase
COX-2	Ciclo-coxigenase-2
DM2	Diabetes mellitus tipo 2
eNOS	Sintase de óxido nítrico endontelial
FAS	Sintase de ácido graxo
FAs	Ácidos graxos livres
FFAs	Ácidos graxos livres
FoxO1	Proteína “forkhead box O1”
IL-6	Interleucina-6
iNOS	Sintase de óxido nítrico induzível
IRS-1	Substrato 1 do receptor de insulina
LDLr	Receptor de LDL
LKB1	Quinase B1 do fígado
LXR	Receptor X do fígado
MMP-9	Metaloproteinase de matriz-9
NAFLD	Doença do fígado gorduroso não alcoólico
NASH	Esteatohepatite não-alcoólica

NF-kB	Fator nuclear kapa B
PPARs	Receptores ativados por proliferadores de peroxissomos
RI	Resistência à insulina
RXR	Receptor X retinóide (RXR)
SCD1	Esteroil-CoA dessaturase
SM	Síndrome Metabólica
SREBP-1c	Proteína de Ligação ao Elemento Regulatório de Esterol
TG	Triglicerídeo
TNFα	Fator de necrose tumoral α
TZDs	Tiazolidinadionas
VLDL	Lipoproteína de muito baixa densidade

SUMÁRIO

1 INTRODUÇÃO

A Síndrome Metabólica (SM) é caracterizada pela combinação de várias condições metabólicas, como a obesidade, dislipidemia, aumento da pressão sanguínea, resistência à insulina e uma condição pró-inflamatória (REAVEN, 2002). A doença do fígado gorduroso não alcoólico (NAFLD) é considerada como a manifestação mais comum da síndrome metabólica e uma das doenças do fígado mais prevalente do mundo (KANURI & BERGHEIM, 2013), e tem se tornado um problema de saúde pública devido à incidência cada vez maior de obesidade em crianças e adultos (STANKOVIC et al., 2014).

A NAFLD é caracterizada pelo acúmulo de gordura em mais de 5% dos hepatócitos na forma de macro e/ou microvesículas lipídicas (STANKOVIC et al., 2014). Esse excesso de triglicerídeos nas células do fígado se dá por um desequilíbrio de sua aquisição e/ou remoção na célula. Essa disfunção pode ser resultado da interação entre diversos fatores tais como: dieta hipercalórica e/ou dieta desequilibrada, o aumento da lipogênese “de novo” ou maior lipólise no tecido adiposo, um menor gasto de energia devido ao estilo de vida sedentária, ou ainda uma susceptibilidade genética (KANURI & BERGHEIM, 2013; TILG & MOSCHEN, 2010).

A Proteína de Ligação ao Elemento Regulatório de Esterol (SREBP) é um fator de transcrição lipogênico que é regulado nutricionalmente por glicose e insulina. SREBP1 preferencialmente regula o processo lipogênico por atuar em genes envolvidos na síntese de triglicerídeos e ácidos graxos como a acetil CoA carboxilase (ACC) e a sintase de ácido graxo (FAS) (QUAN et al., 2013), e encontra-se ativado durante o processo patológico da NAFLD (TESSARI et al., 2009).

A Proteína quinase ativada por 5’AMP (AMPK) é uma serina/treonina quinase que medeia a adaptação celular aos fatores de estresse nutricional, metabolismo celular e/ou resistência à insulina. Quando ativado, o AMPK inibe vias biossintéticas de produção de ácidos graxos e esteróis, e ativa, simultaneamente, vias catabólicas produzindo ATP, como oxidação de ácidos graxos, através da regulação em curto prazo de enzimas específicas e expressão de genes (HARDIE, 2004). Evidências crescentes sugerem que o AMPK e SREBP-1c são reguladores críticos do metabolismo de lipídios no fígado (LEE et al., 2010). O AMPK inativa o SREBP-1c e

inibe a esteatose hepática induzida por dieta rica em gordura em modelos experimentais (KIM et al., 2009). Além disso, foi demonstrado que o AMPK regula a atividade transcricional da proteína “forkhead box O1” (FoxO1) apresentando papel crítico na regulação da gluconeogênese e metabolismo lipídico. A ativação do AMPK pode aumentar a expressão da enzima lipase do triglicerídeo do tecido adiposo (ATGL), a β-oxidação do ácido graxo, o transporte de glicose, gerando assim uma maior produção de ATP (CHEN et al., 2011).

Por sua vez, os receptores ativados por proliferadores de peroxissomos (PPARs) são receptores nucleares ativados por ligantes que estão envolvidos na regulação transcricional do metabolismo lipídico, balanço energético, inflamação e aterosclerose. Atualmente são conhecidos três isotipos PPAR-α, PPAR-δ/β e PPAR-γ (AHMED et al., 2007).

O PPAR-α, é predominantemente expresso no fígado e em menor intensidade no coração, musculo esquelético, intestino e rim onde exerce um papel importante no controle da oxidação dos ácidos graxos, metabolismos lipídicos e inflamação (LEFEBVRE et al., 2006; AHMED etal., 2007).O PPAR-δ/β possui expressão significativa em tecidos responsáveis em controlar o metabolismo lipídico, como os adipócitos, intestino delgado, coração, músculo esquelético e macrófagos (GROSS & STAELS, 2007). O PPAR-γ é o isotipo mais estudado e está relacionado com a adipogênese e efeitos de sensibilização à insulina através do desvio de ácidos graxos para o armazenamento de tecido adiposo, sendo presente em adipócitos, macrófagos, monócitos, hepatócitos, células musculares e endoteliais (SOUZA-MELLO, 2015).

As Tiazolidinadionas (TZDs) são agonistas sintéticos do PPAR-γ utilizadas clinicamente para tratar pacientes com diabetes tipo 2 (MIYAZAKI et al., 2002). São agentes antidiabéticos orais, os quais atuam principalmente através do aumento da sensibilidade à insulina do tecido adiposo. Estudos mostram que as TZDs (rosiglitazona e pioglitazona) diminuem os níveis séricos de glicose, a resistência à insulina, e a acumulação de ácidos graxos livres no tecido hepático (CHANG et al., 2013). Apesar de existirem diversos estudos mostrando os efeitos benéficos dos agonistas de PPAR-γ, os efeitos colaterais cardiovasculares das TZDS tornaram-se alvo de discussão. Desse modo, é necessária uma investigação de novos derivados tiazolidínicos que desenvolvam efeitos colaterais menores.

Recentes estudos demonstraram que o derivado tiazolidínico LPSF/GQ-02 (5-(4-Cloro-benzilideno)-3-(4-metil-benzil)-thiazolidina-2,4-diona) foi eficaz em diminuir a resistência à insulina e o acúmulo de gordura hepática, bem como reduzir a aterosclerose, além de apresentar efeitos anti-inflamatórios importantes (SOARES & SILVA et al., 2013, 2015). No presente estudo, as vias de sinalização AMPK / LXR / SREBP1 / ACC / FAS e AMPK / FoxO1 / ATGL envolvidas no desenvolvimento de esteatose hepática foram avaliadas em modelo de NAFLD tratados com LPSF/GQ-02.

2 JUSTIFICATIVA

A doença do fígado gorduroso não-alcoólica é a causa mais comum de lesões hepáticas crônicas em todo o mundo. Várias evidências sugerem que múltiplos mecanismos, incluindo a resistência à insulina, estresse oxidativo, inflamação e fatores genéticos interagem para iniciar o desenvolvimento da esteatose hepática. Entretanto, ainda não existe nenhuma terapia farmacológica aprovada para tratar a NAFLD e seus fatores de risco associados. Estudos recentes realizados no laboratório de Ultraestrutura do Aggeu Magalhães/FIOCRUZ-PE demonstram que o derivado tiazolidínico LPSF/GQ-02 exerce efeitos benéficos sobre a resistência à insulina, diminuição de aterosclerose e inflamação em animais LDLR-/- alimentados com uma dieta rica em gordura, além de reduzir o conteúdo lipídico hepático.

Pesquisadores em todo o mundo têm investido na busca por novas moléculas com o intuito de se obter alternativas terapêuticas mais seletivas e com menos efeitos adversos contra a patogênese da NAFLD. Como resultados, esperamos caracterizar a ação da LPSF/GQ-02 para que possa ser utilizada como fármaco útil na terapêutica da NAFLD. Pretende-se ainda contribuir com as políticas de saúde, ciência e tecnologia brasileira através da identificação de moléculas com potencial farmacológico relevante para a saúde, atuando, portanto, dentro de um segmento estratégico para o desenvolvimento social e econômico.

3 OBJETIVOS

3.1 - OBJETIVO GERAL

Analisar o efeito do tratamento *in vivo* do derivado tiazolidínico LPSF/GQ-02, comparativamente com a Pioglitazona, sobre o metabolismo lipídico hepático em camundongos LDLr-/- induzidos com uma dieta rica em gordura.

3.2 - OBJETIVOS ESPECÍFICOS

a) Avaliar a ação da LPSF/GQ-02 sobre as modificações histopatológicas do fígado por coloração de hematoxilina-eosina;

b) Avaliar a ação da LPSF/GQ-02 sobre o acúmulo de lipídios no tecido hepático pela coloração de Oil Red O;

c) Caracterizar imunohistologicamente os marcadores envolvidos na lipogênese (SREBP-1c e ACC) e na lipólise (ATGL);

d) Avaliar a ação da LPSF/GQ-02 na expressão de marcadores lipogênicos (LXRα, SREBP-1c, ACC, p-ACC e FAS) e marcadores da lipólise (AMPK, p-AMPK, FoxO1 e ATGL) pela técnica de Western Blotting;

Capítulo I

4 REVISÃO BIBLIOGRÁFICA

4.1 FISIOPATOLOGIA DA NAFLD

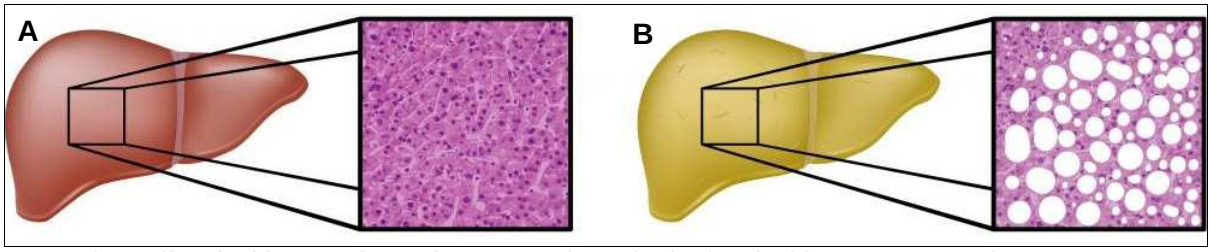
A obesidade é resultado de um desequilíbrio na relação da ingestão e gasto calórico, gerando um excesso de energia, que é armazenado sob a forma de gordura no tecido adiposo (CHUGH & SHARMA, 2012; MCKENNEY & SHORT, 2011). Essa condição aumenta os riscos de desenvolvimento das doenças cardiovasculares e diabetes mellitus tipo 2 (KOPELMAN, 2000). A SM é uma das principais causas de mortalidade e morbidade em países industrializados (SIMONS et al., 2011) e se caracteriza pela combinação de várias características metabólicas, incluindo a obesidade, dislipidemia, aumento da pressão sanguínea, resistência à insulina e um estado pró-inflamatório (REAVEN, 2002). A prevalência de síndrome metabólica se correlaciona com a epidemia global de obesidade e está crescendo a um ritmo alarmante, afetando mais de 20% da população mundial adulta (ONAT, 2011). A crescente epidemia de síndrome metabólica e suas complicações tais como doenças cardiovasculares, têm sido acompanhadas por um aumento em alterações hepáticas, incluindo a NAFLD. Essa patologia também se tornou uma doença hepática significativa, em crianças, devido ao aumento prevalência de obesidade infantil (TAKAHASHI & FUKUSATO, 2012).

A NAFLD é a manifestação patológica mais comum referente a doenças do fígado, sendo caracterizada por um acúmulo de Triglicerídeo (TG) macro e/ou micro vesicular nos hepatócitos (Figura 1), excedendo 5% do peso do fígado e com alterações persistentes nos níveis plasmáticos das enzimas hepáticas como aspartato aminotransferase (AST) e alanina aminotransferase (ALT) (TESSARI, 2008; SONSUZ et al., 2000). De acordo com Donnelly et al (2005), aproximadamente 60% dos TG no fígado é derivado de ácidos graxos livres oriundos da lipólise do tecido adiposo, 26% têm origem da lipogênese de novo e, 15% derivam da dieta. A maioria dos pacientes com NAFLD são assintomáticos, e para um possível diagnostico são utilizados testes para as enzimas hepáticas no sangue e exames de imagem, como o ultra-som (BERLANGA et al., 2014).

A prevalência da NAFLD na população de um modo geral está associada com a incidência da obesidade e de resistência a insulina (RI). Sua prevalência aumenta de 10-24% para 25-75% quando associados à obesidade e diabetes mellitus tipo 2

(CHATKIN et al., 2008). Essa patologia é compreendida em um amplo espectro de danos no fígado, variando de esteatose macrovesicular simples à esteatohepatite não alcóolica (NASH), fibrose avançada e cirrose (ÂNGULO, 2002).

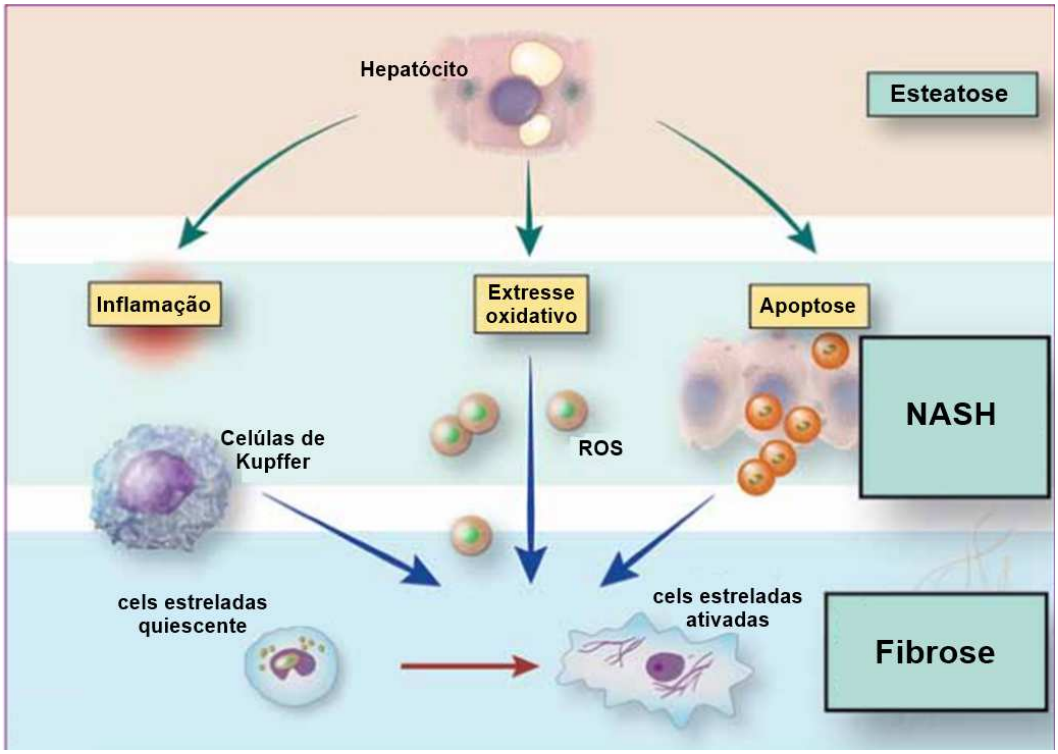
Figura 1: Aspectos Macroscópicos e Microscópicos (A) fígado normal, (B) Fígado com esteatose hepática.



Fonte: https://corticoides97unb.wordpress.com/2013/06/14/corticoides-e-esteatose/

A patogênese da NAFLD não é claramente bem descrita bem como os mecanismos envolvidos na progressão da esteatose para NASH. É sabido que a resistência à insulina, o estresse oxidativo e a inflamação tem um papel importante no desenvolvimento da NAFLD, bem como na sua progressão (ALKHOURI & MCULLOUGH, 2012). A teoria dos “dois hits” tem sido proposta para a fisiopatologia da NAFLD. O primeiro “hit” refere-se ao acúmulo de TG e ácidos graxos livres (FFAs) nos hepatócitos como uma consequência da RI. Em associação a RI promove a quebra de gordura do tecido adiposo liberando mais FFA na circulação que serão armazenados no fígado, resultando na esteatose. O segundo “hit” envolve a peroxidação lipídica, a disfunção mitocondrial, mediadores inflamatórios e uma série de interações complexas entre os hepatócitos, células de Kupffer e adipócitos resultando na NASH (THAN & NEWSOM, 2015; PAPANDREOU & ANDREOU, 2015) (Figura 2). A inflamação crônica contribui para a progressão da lesão hepática e, em longo prazo, para o desenvolvimento de fibrose e cirrose. Por outro lado, fatores dietéticos podem modular a esteatose hepática, de forma que uma dieta rica em gordura saturada promove uma maior elevação de lipídios no fígado e dos níveis de insulina no plasma, induzindo à resistência à insulina e por consequência afetando a função mitocondrial (TESSARI, 2008).

Figura 2: Esquema do desenvolvimento e progressão da NAFLD.



Fonte: Adaptada de ALKHOURIA & MCULLOUGH, 2012.

4.2 METABOLISMO LIPÍDICO E NAFLD

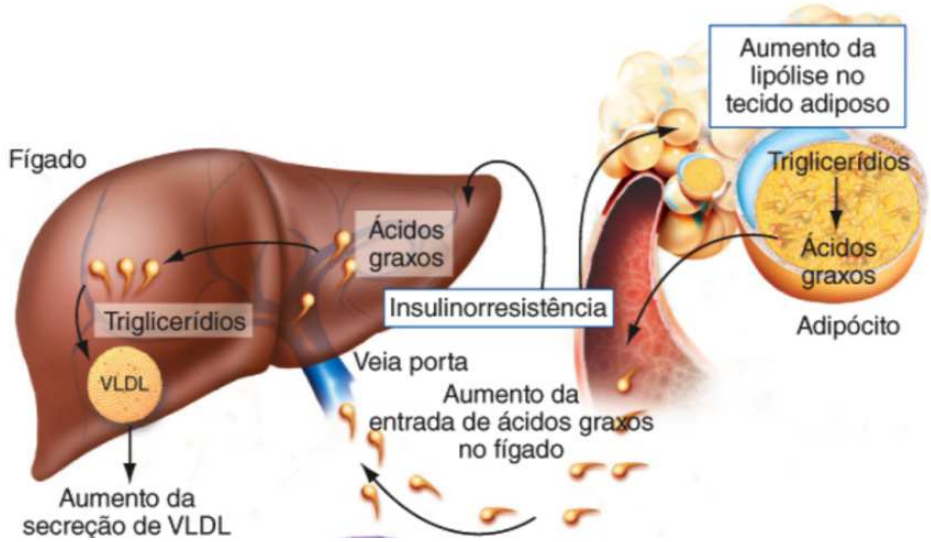
O fígado desempenha um papel importante no metabolismo lipídico, como na importação e síntese de FFAs, armazenamento e exportação de lipídios; um desequilíbrio em algum desses processos pode levar ao desenvolvimento da NAFLD (MUSSO et al., 2009). O aumento crônico de FFAs pode perturbar diversas vias metabólicas e induzir a RI em vários órgãos (PETTA et al., 2009). Por outro lado, FFAs abundantes causam lipotoxicidade por meio da produção de ROS, causando inflamação, apoptose, induzindo a progressão para a NASH e fibrogênese (MALHI et al., 2008).

O TG é a forma de armazenamento do excesso de FFAs em condições fisiológicas. Os TG podem ser armazenados na forma de gotículas de lipídios dentro dos hepatócitos ou distribuídos para o sangue na forma de Lipoproteína de muito baixa densidade (VLDL). O acúmulo excessivo de TGs hepáticos está associado com um aumento do fornecimento de FFAs do tecido adiposo periférico para o fígado e da lipogênese de novo através da via lipogênica. Por outro lado, a eliminação de lipídios hepáticos, que ocorrem pela β-oxidação e exportação de

VLDL, são moderadamente afetados (LEWIS et al., 2002). Os TG e o colesterol quando acumulados na mitocôndria, são considerados agressivos, por conduzirem o dano no fígado mediada por Fator de necrose tumoral α (TNF-α) e por meio da formação de espécies reativas de oxigênio (MARI et al., 2006).

Em suma, a acumulação de TG nos hepatócitos é resultado de um desequilíbrio entre as vias de aquisição de lipídios, a absorção de ácidos graxos e a lipogênese *de novo*, e os mecanismos de remoção, a oxidação mitocondrial e exportação de partículas de VLDL. A fim de controlar a progressão da esteatose hepática, é importante compreender os mecanismos de regulação da acumulação dos lipídios no fígado (BERLANGA et al., 2014) (Figura 3).

Figura 3: Mecanismo de regulação do acumulo de TG hepático.



Fonte: http://www.asaudeebemestar.com/2013/02/sindrome-metabolica.html

4.3 LIPOGÊNESE "*DE NOVO*"

Compreende o processo de síntese endógena de ácidos graxos (FAs) no fígado, e consiste na conversão da glicose em acetil-CoA, seguida da sua conversão a malonil-CoA pela enzima ACC. Finalmente, a enzima FAS catalisa a formação de ácido palmítico a partir do malonil-CoA. Dependendo do estado metabólico, FAs são então processados em TG e armazenados ou rapidamente metabolizados (BECHMANN et al., 2012; KAWANO et al., 2013).

Em um estado de jejum, uma diminuição dos níveis de insulina estimula a hidrolase de TG nos adipócitos, liberando os FFAs que são transportados para o fígado. No fígado os FFAs recém chegados podem ser utilizados para a produção de energia mitocondrial através da β-oxidação, ou podem ser esterificados e armazenados como gotículas lipídicas, ou empacotadas com a apoliproteina B em VLDL, que é secretada para a circulação (KAWANO et al., 2013; FUCHS,2012) . Na NAFLD a aquisição de FAs através da absorção e da lipogênese de novo são aumentados e compensados pela oxidação de FAs ou pela produção de partículas de VLDL (BERLANGA et al, 2014).

A regulação da via lipogênica ocorre no nível da transcrição de vários fatores nucleares, como o Receptor X do fígado (LXR), proteína 1c ligadora do elemento regulado por esteróis (SREBP-1c), e das enzimas (ACC, FAS, Esteroil-CoA dessaturase-1 [SCD1]). A esteatose hepática nos seres humanos tem sido ultimamente associada com o aumento da expressão de vários genes envolvidos na lipogênese de novo (MITSUYOSHI et al., 2009).

4.3.1 LXR

São fatores de transcrição ativados por ligantes que pertencem à superfamília de receptores nucleares (BARANOWSKI, 2008). Apresentam-se em duas isoformas α e β. LXRα tem sua expressão predominantemente no fígado, no tecido adiposo e no intestino. LXRβ está distribuído de forma homogênea nos tecidos (FAULDS et al., 2010). A sua ativação está associada com o aumento da lipogênese, hipertrigliceremia e acumulação de lipídios no fígado por meio da lipogênese *de novo* e na progressão da esteatose (KIM et al., 2009). Esse acúmulo de gordura no fígado por meio dessa ativação do LXR é devido à indução dos genes envolvidos na síntese de ácidos graxos, como a ACC, FAS, SCD-1 e o fator de transcrição nuclear SREBP-1c (HAN et al., 2014).

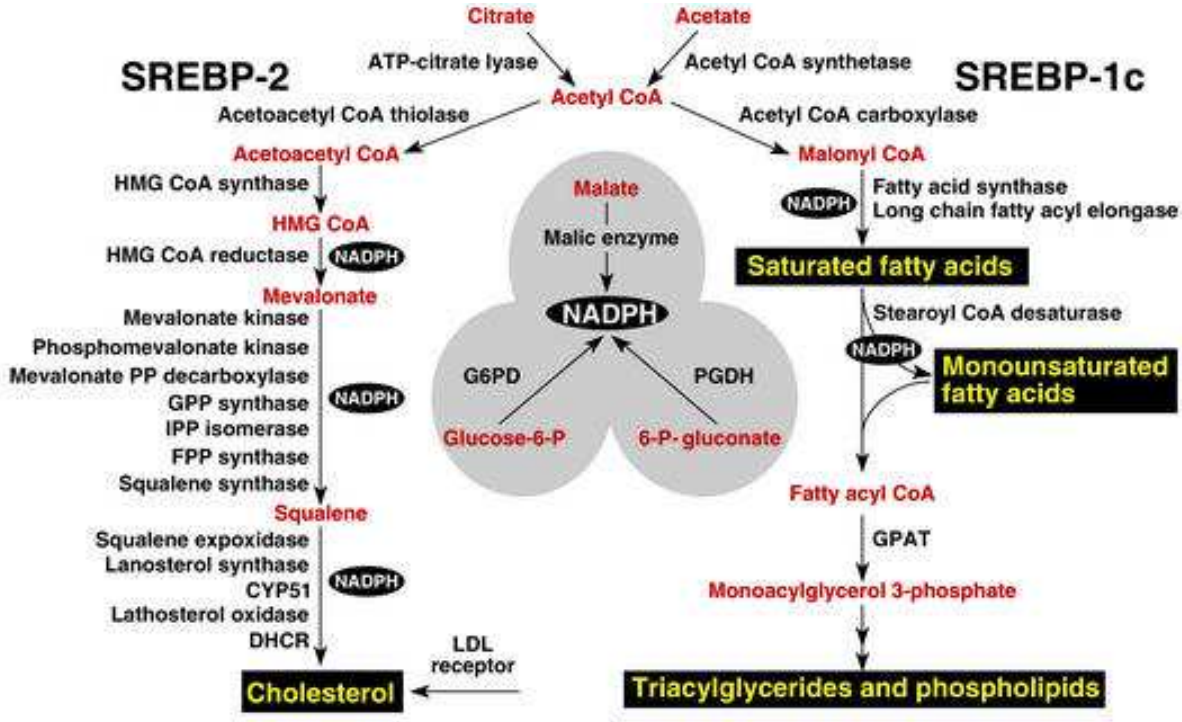
4.3.2 SREBP-1c

SREBPs são uma família de fatores de transcrição ligados à membrana. SREBPs, sintetizadas como precursores inativos ligados às membranas do retículo

endoplasmático e, portanto, devem ser submetidos à clivagem proteolítica para liberação do seu domínio N-terminal, que constitui o fator de transcrição maduro (FERRÉ & FOUFELLE, 2010)

O SREBP apresenta-se sob diferentes isoformas: SREBP-1c e SREBP-2 e SREBP-1a, esta última expressa em níveis muito baixos no fígado (HORTON et al., 2002). O SREBP-1c, a isoforma predominante no fígado, é responsável preferencialmente pela transcrição de genes envolvidos na regulação da lipogênese de novo, enquanto a isoforma SREBP-2 regula os genes envolvidos na biossíntese de colesterol e no metabolismo (Figura 4). A isoforma SREBP-1a, apesar de seus níveis muito baixos no fígado, ativa os genes das vias lipogênicas e colesterol (BERLANGA et al., 2014).

Figura 4: Esquema dos genes regulados por SREBPs. In vivo o SREBP-2 ativa preferencialmente os genes do metabolismo do colesterol, enquanto que SREBP-1c tem preferencia em ativar os genes do metabolismo do ácido graxo e triglicerídeos.



Fonte: Horton et al., 2002.

A regulação do SREBP-1c ocorre principalmente no nível transcricional (FERRÉ & FOUFELLE, 2010). Essa transcrição é induzida por dois estímulos muito diferentes: a insulina, um hormônio liberado em resposta à ingestão de hidrato de

carbono e que conduz a um aumento paralelo tanto do precursor de SREBP-1c ligado à membrana do retículo endoplasmático, quanto da sua translocação para o núcleo; e o LXRα que também tem um papel importante na transcrição de genes relacionados á lipogênese. Em resposta à alimentação, SREBP-1c liga aos seus genes lipogênicos, tais como ACC1, FAS, e de SCD1, e para o seu próprio gene, estimulando a lipogênese hepática (BERLANGA et al., 2014).

4.3.3 ACC e FAS

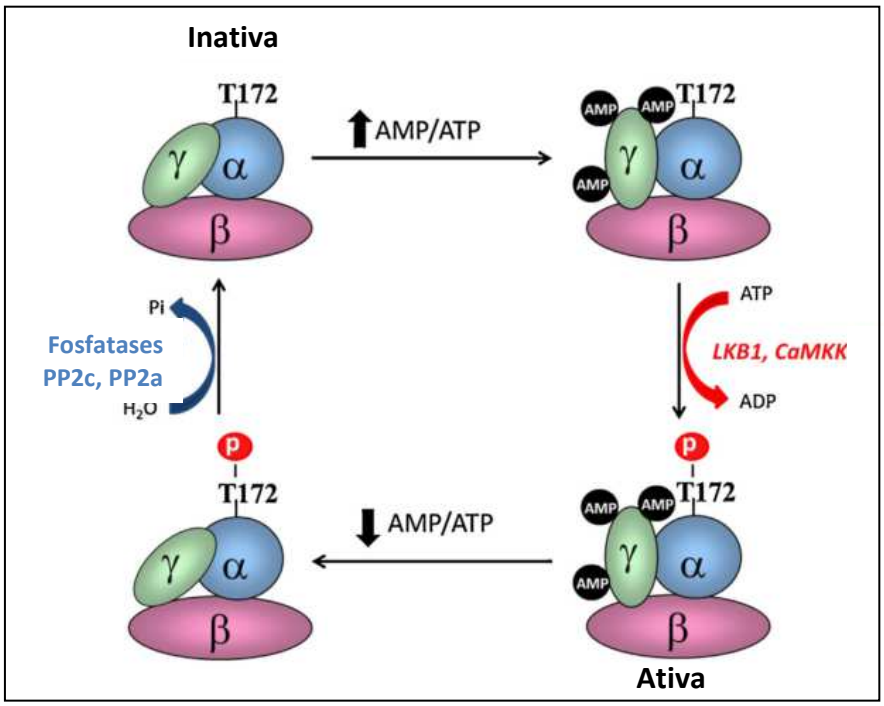
No processo de síntese dos ácidos graxos, a enzima ACC converte o acetil-CoA à malonil-CoA. Em seguida, a enzima FAS, utiliza tanto a acetil-CoA e o malonil-CoA para formar o ácido palmítico. Ambas as enzimas são reguladas por SREBP-1c, e passam a desempenhar um papel importante no metabolismo energético dos ácidos graxos livres. Atualmente essas enzimas são consideradas como possíveis alvos para a regulação da obesidade, diabetes, câncer e complicações cardiovasculares e apresentaram uma maior expressão na NAFLD, sugerindo suas ações como possíveis marcadores de diagnostico ou alvo terapêutico para NAFLD (DORN et al., 2010; BERLANGA et al., 2014).

4.4 PROTEÍNA QUINASE ATIVADA POR AMP – AMPK

A AMPK é uma enzima que induz uma cascata de eventos intracelulares em resposta a mudança da carga energética celular e possuem o papel de manutenção da homeostasia energética no metabolismo celular (HARDIE et al., 2003; CARLING et al., 2004). Todas as células para sobreviver devem diariamente manter a relação entre adenosina trifosfato (ATP) e adenosina difosfato (ADP) alta. Isso é obtido por intermédio do catabolismo que aumenta a energia celular convertendo ADP e fosfato em ATP, enquanto o anabolismo diminui o componente energético celular, por converter ATP em ADP e fosfato. Convém ressaltar o fato de que a relação ATP–ADP nas células geralmente permanece quase constante, indicando que o mecanismo que regula esse processo é muito eficiente. A AMPK é um componente-chave para a manutenção desse equilíbrio fisiológico (CARLING et al., 2004; SCHIMMACK et al., 2006).

AMPK é ativado por meio da fosforilação no resíduo de treonina 172, localizado no domínio catalítico da subunidade α. A ativação do APMK ocorre por meio da quinase B1 do fígado (LKB1), pela quinase cálcio/calmodulina dependente da proteína quinase (CaMKK) (SEO et al., 2014) (Figura 5), ou, por qualquer estresse que cause aumento na relação intracelular AMP–ATP, tanto aqueles que interferem com a produção de ATP quanto também aqueles que aumentam o consumo de ATP (HARDIE et al., 2003) e através de ativadores químicos como a Metformina e Tiazolidinedionas (TZDs) (ZHOU et al., 2001).

Figura 5: Esquema representativo das subunidades do AMPK e sua regulação. O AMPK torna-se ativado em condições de alta relação AMP/ATP. Nessas circunstâncias, o AMP liga-se ao AMPK, facilitando a sua ativação por meio da fosforilação na Thr172, catalisada por LKB1 e CaMKK.



Fonte: SID et al., 2013.

Uma vez ativada, AMPK exerce efeitos sobre o metabolismo da glicose e dos lipídios, sobre expressão gênica e sobre síntese protéica. Essa enzima atua em diversos órgãos, incluindo fígado, músculo esquelético, coração, tecido adiposo e pâncreas (ZHOU et al., 2001). No fígado, a ativação do AMPK por fosforilação inativa as enzimas associadas à lipogênese, tais como ACC (SEO et al., 2014). Com a inativação da ACC não há concentrações de malonil CoA suficientes para inibir a

carnitina acil transferase e, portanto, há uma predominância da β-oxidação sobre a síntese de ácidos graxos, permitindo que a produção de energia prevaleça sobre o gasto (ZHOU et al., 2001). Mais ainda, de acordo com estudo publicado por Li et al (2011), a ativação do AMPK inibe a clivagem e consequentemente a ativação da transcrição do SREBP-1c por meio da fosforilação direta. SREBP-1c quando inativado por consequência irá impedir a transcrição dos genes responsáveis da lipogênese.

Em contrapartida, a ativação do AMPK regula positivamente a enzima lipase ATGL, que é responsável pelo passo inicial do catabolismo em TG, e a sua deficiência provoca uma acumulação de TG em diversos órgãos, incluindo o fígado (FUCHS et al., 2012). Por outro lado, a proteína ATGL também tem sua expressão regulada por FoxO1 que é uma classe de proteínas “forkhead”. A falta de nutrientes pode estimular a translocação do FoxO1 do citoplasma para o núcleo, podendo se ligar a uma região promotora do gene ATGL aumentando a sua transcrição (CHEN et al., 2012). Em resumo, a AMPK atua no fígado diminuindo a síntese de lipídios e estimulando a queima de gordura, além de bloquear a produção hepática de glicose (ZANG et al., 2004).

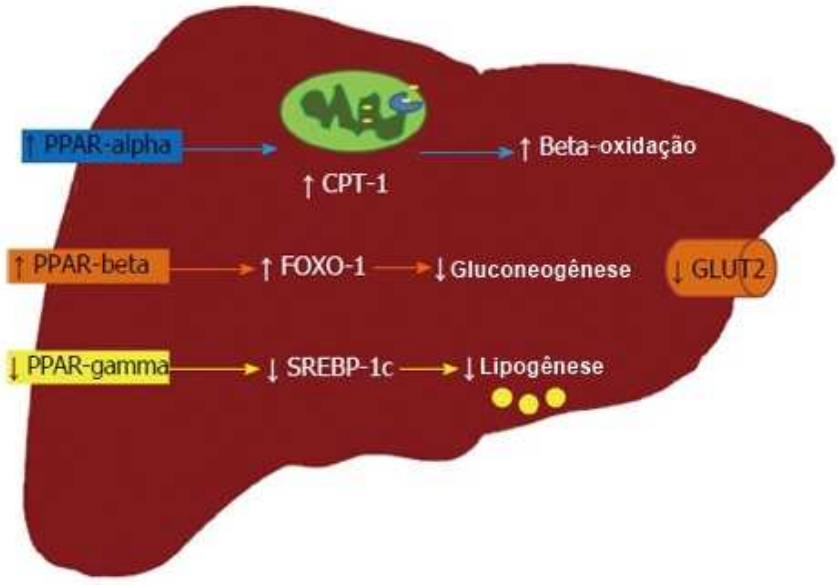
Os efeitos da ativação da AMPK no metabolismo em geral são benéficos para pacientes com NAFLD, sendo um potencial alvo para futuros agentes terapêuticos no tratamento da síndrome metabólica.

4.5 RECEPTORES ATIVADOS POR PROLIFERADORES DE PEROXISSOMOS (PPARS) E NAFLD

Os PPARs possuem uma organização estrutural semelhante a outros membros da superfamília de receptores nucleares, como o receptor do ácido retinóico e vitamina D. De modo geral, contém um domínio C que se liga ao DNA, uma região N-terminal que apresenta uma região de ativação do PPAR e uma região C-terminal a qual apresenta uma região de ligação ao ligante TZD (FEIGE et al., 2006). Há três isótopos em mamíferos de PPARs: PPARα, PPARδ e PPARγ, e desempenham um papel chave na modulação da acumulação de TGs hepáticos. PPARα regula a β-oxidação do ácido graxo, enquanto que o PPARγ aumenta a

sensibilidade à insulina bem como a regulação do armazenamento de TG no tecido adiposo (KALLWITZ et al., 2008) (Figura 6).

Figura 6: Ação de cada isoforma do receptores PPARs na NAFLD.



Fonte: SOUZA-MELLO, 2015.

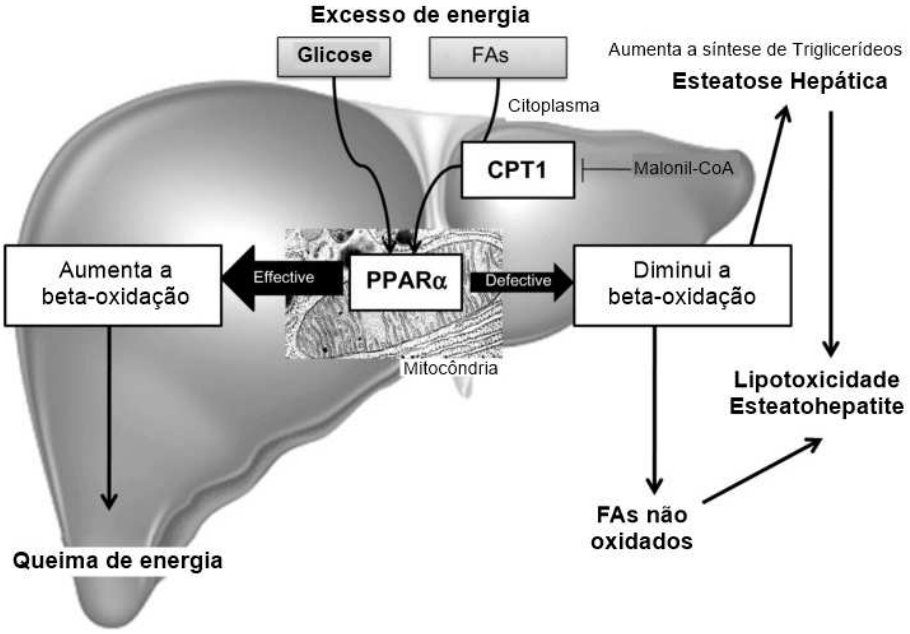
4.5.1 PPARα

É expresso no fígado e em outros tecidos metabolicamente ativos, incluindo músculo estriado, rim e pâncreas (KALLWITZ et al., 2008). Sua ativação está relacionada com a transcrição de aproximadamente 80-100 genes, atuando sobre a oxidação dos ácidos graxos, metabolismo lipídico e inflamação (AHMED et al., 2007). São responsáveis pelo transporte de ácido graxos e β-oxidação, diminuindo o acúmulo de lipídios. Estudos mostraram que quando os camundongos tem o gene do PPARα silenciados e expostos a condições de aumento do metabolismo de ácidos graxos no fígado, resultam na esteatose hepática do animal (KALLWITZ et al., 2008). Em contra partida, animais que receberam uma dieta deficiente de colina e metionina associado com a administração de uma agonista de PPAR, apresentou uma diminuição na esteatose hepática (NAGASAWA et al., 2006).

Outros fatores podem interagir com o PPARα regulando o conteúdo de triglicerídeos hepáticos. Estes incluem a adiponectina, que é um hormônio de origem peptídica produzido em adipócitos que limita a acumulação de gordura no fígado por

diversos mecanismos, incluindo a ativação de PPARα para aumentar a oxidação dos ácidos graxos hepáticos (YAMAUCHI et al., 2003). Em modelos de cultura celular, o tratamento com adiponectina resultou em maior atividade de PPARα, e consequentemente os genes envolvidos na oxidação dos ácidos graxos (YOON et al., 2006). Por outro lado, a adiponectina é regulada positivamente também pelo PPARy, fornecendo uma ligação entre os dois isotipos (NESCHEN et al., 2006) (Figura 7). Estudos observaram que uma ativação parcial do PPARy juntamente com uma ativação seletiva do PPARα no fígado por Telmisarfan, mostrou efeitos positivos na arquitetura dos hepatócitos e na ultraestrutura em camundongos alimentados com dietas com alto nível de gordura, apresentaram uma redução da expressão do SREBP-1c e nos níveis de insulina, e uma maior densidade numérica mitocondrial (SOUZA-MELLO et al., 2010; KUDO et al., 2009).

Figura 7: Ação regulatória do PPARα na oxidação dos ácidos graxos.



Fonte: Adaptada de BERLANGA et al., 2014.

4.5.2 PPAR-δ/β

É o menos estudado entre os isotipos de PPARs, embora possua expressão significativa em tecidos responsáveis por controlar o metabolismo lipídico, como os adipócitos, intestino delgado, coração, musculo esquelético e macrófagos (GROSS & STALES, 2007). Essa isoforma é importante não só para a regulação metabólica

como para o desenvolvimento do organismo (GRIMALDI, 2007). Encontra-se envolvido em muitos mecanismos biológicos, principalmente nas vias envolvidas na gliconeogênese e do metabolismo lipídico, sugerindo que ações farmacológicas dessa isoforma podem ser benéficas sobre a síndrome metabólica (GRAHAM et al., 2005).

4.5.3 PPAR-γ

É expresso em níveis elevados no tecido adiposo e desempenha um papel importante no aumento da sensibilidade à insulina, bem como na promoção da absorção de ácidos graxos em adipócitos e diferenciação de adipócitos. Pettinelli et al (2011) mostraram que pacientes com NASH exibiram uma elevada expressão PPAR-γ no fígado, e que coincidia com uma super-expressão de SREBP-1c, e por consequência aumento da lipogênese hepática. Em modelos murinos de NAFLD, a ativação total do PPAR-γ por Rosiglitazona não foi eficiente em reduzir a esteatose hepática. Portanto, a ativação completa do PPAR-γ favorece a transcrição de fatores envolvidos na lipogênese, aumentando o acúmulo de TG hepático garantindo a manutenção da NAFLD (FRAULOB et al., 2012; FERNANDES-SANTOS et al., 2009; SOUZA-MELLO, 2015).

Em contrapartida, outros estudos mostraram que camundongos deficientes para o gene PPAR-γ são protegidos contra o desenvolvimento da esteatose hepática, sugerindo um papel benéfico, evitando o acúmulo de TG no fígado (MATSUSUE et al., 2003; GAVRILOVA et al., 2003). Devido à existência de resultados controversos a respeito do mecanismo de ação dessas moléculas, são necessários estudos mais detalhados para um melhor esclarecimento.

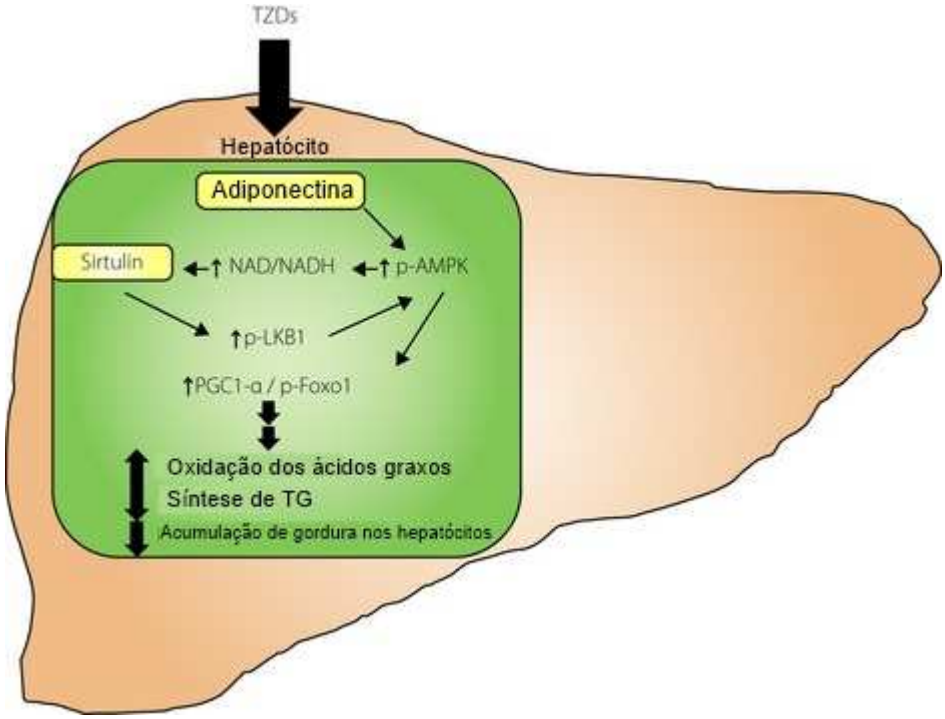
4.6 TZDS E NAFLD

TZDs são agonistas de PPAR-γ e melhoram o controle glicêmico em pacientes com diabetes mellitus tipo 2, aumentando a sensibilidade à insulina (MIYAZAKI et al., 2002). As TZDs promovem aumento na sensibilidade à insulina no tecido adiposo, fígado e musculo esquelético (PROMRAT et al., 2004).

Essas moléculas possuem como características principais a presença de um anel tiazolidinico. A Ciglitazona, o primeiro agonista do PPAR-γ apresentou bons resultados em melhorar a glicemia em modelo de resistência à insulina, entretanto não foi comercializada por causar hepatotoxicidade. Troglitazona foi o primeiro agonista de PPAR-γ aprovada pela *US food and drugAdministration* (FDA) para o tratamento da diabetes tipo 2 (WATKINS & WHITCOMB, 1998), porém quando registrado graves hepatotoxicidades e morte foi retirada do mercado (ISLEY, 2003). Rosiglitazona e Pioglitazona, aprovadas para uso pela FDA em 1999, são consideradas a segunda geração de agonistas do PPAR-γ.

A terapia com TZD aumenta os níveis de adiponectina, que estão associados com o aumento da sensibilidade à insulina (MAEDA et al., 2001). Da mesma forma, o aumento da adiponectina estimula a oxidação de ácidos graxos por meio da ativação do AMPK, inibindo o acúmulo de lipídios no tecido hepático (CHANG et al., 2013) (Figura 8).

Figura 8: Mecanismos pelos quais o tratamento com TZD melhora a esteatose hepática. No fígado, TZDs regulam positivamente a adiponectina e/ou sirtulin, que consequentemente, alteram reguladores hepáticos, levando ao aumento da oxidação dos ácidos graxos e diminuindo a acumulação de gordura.



Fonte: Adaptada de CHANG et al., 2013.

As TZDs também aumentam a expressão da proteína AMPK (SAHA et al., 2004). O AMPK como dito anteriormente atua na regulação da oxidação dos ácidos graxos, bem como diminui a lipogênese (HARDIE, 2003). A redução na lipogênese é mediada através de fosforilação e inibição da ACC, o que diminui a formação de malonil-CoA, inativando o SREBP (BROWNING & HORTON, 2004). Finalmente, TZDs têm propriedades anti-inflamatórias e anti-fibróticas que podem ser benéficas na NASH.

Apesar de existirem diversos estudos mostrando os efeitos vasculares favoráveis dos agonistas de PPAR-γ, os efeitos cardiovasculares das TZDs tornam-se alvo de intensa discussão. Em um estudo de metanálise, o tratamento com Rosiglitazona foi associado a um aumento dos riscos de infarto do miocárdio e mortalidade por causas cardiovasculares (NISSEN & WOLSKI, 2007). De forma controversa, o tratamento com Pioglitazona foi associado à redução do risco combinado de infarto agudo do miocárdio, acidente vascular cerebral e mortalidade em 16%, entre pacientes diabéticos de alto risco cardiovascular (DORMANDY et al., 2005). É possível que os efeitos danosos apresentados pela Rosiglitazona sejam específicos dessa molécula, não representando um efeito de classe. Entretanto, os mecanismos determinantes do aumento de risco cardiovascular associado às TZDs ainda não são conhecidos.

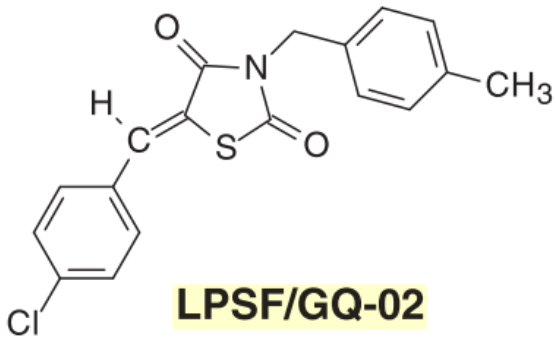
4.7 NOVO DERIVADO DO PPAR-Γ (LPSF/GQ-02)

O envolvimento do PPAR-γ em diversos processos biológicos, principalmente na modulação do metabolismo lipídico e resposta inflamatória torna esse receptor nuclear um importante alvo para o desenvolvimento de novas moléculas. As TZDs utilizadas clinicamente para tratar pacientes com DM2, são moléculas que possuem interação especifica com o PPAR-γ e por essa razão são consideradas potentes agonistas desse receptor nuclear. Entretanto, a utilização das TZDs está relacionada com diversos efeitos colaterais, como ganho de peso, retenção de fluido e hepatotoxicidade (YKI-JARVINEN, 2004), os quais podem ser responsáveis pelos riscos cardíacos associados com a utilização desses fármacos (NISSEN & WOLSKI, 2007). Dessa forma, diversas pesquisas estão sendo realizadas para encontrar bons

candidatos a fármacos, que possibilite o tratamento dessas patologias sem causar efeitos colaterais tão severos.

O novo derivado tiazolidínico LPSF/GQ-02 (5-(4-Cloro-benzilideno)-3-(4-metil-benzil)-thiazolidina-2,4-diona) (Figura 9) sintetizado pelo Departamento de Antibióticos da Universidade Federal de Pernambuco foi eficaz em melhorar a resistência à insulina, reduzir a área de lesões ateroscleróticas oferecendo um efeito protetor ao endotélio, além de exercer efeitos anti-inflamatórios aumentando a expressão do óxido nítrico sintase endotelial (eNOS) e reduzindo a expressão da matriz metaloproteinase 9 (MMP-9) (SOARES & SILVA et al., 2013). Recentemente, resultados obtidos com camundongos deficiente do receptor de LDL (LDLr-/-) em modelo para NAFLD mostraram que a LPSF/GQ-02 foi eficaz em reduzir o acumulo de gordura e colágeno, bem como diminuiu os efeitos dos marcadores inflamatórios como interleucina 6 (IL-6), de óxido nítrico sintase (iNOS), a ciclo-coxigenase -2 (COX-2) e F4/80 e aumentou a expressão da proteína IκBα, Fator nuclear kapa B citoplasmática (NFkB-65), sintase de óxido nítrico endotelial (eNOS) e do substrato 1 do receptor de insulina (IRS-1), sugerindo uma ação direta desse novo derivado tiazolidinico sobre os fatores que afetam a inflamação, resistência a insulina e acúmulo de gordura no fígado destes animais (SOARES & SILVA et al., 2015).

Figura 9: Estrutura química do novo derivado tiazolidinico.



Fonte: Soares & Silva et al., 2013.

Visto os resultados promissores da LPSF/GQ-02 sobre a aterosclerose e a inflamação hepática, o presente estudo propõe avaliar a ação da LPSF/GQ-02 sobre a as vias lipogênicas e lipolíticas hepáticas em modelo de NAFLD.

5 REFERÊNCIAS

AHMED, W.; ZIOUZENKOVA, O.; BROWN, J. et al. PPARs and their metabolic modulation: new mechanisms for transcriptional regulation? **Journal of Internal Medicine.** v. 269, p.184-198, 2007.

ALKHOURI, N; MCCULLOUGH, A. Noninvasive Diagnosis of NASH and Liver Fibrosis Within the Spectrum of NAFLD. **Gastroenterology & Hepatology.** v.8, 2012.

ANGULO P. Nonalcoholic fatty liver disease. **N Engl J Med.** v.346, p.1221-1231, 2002.

BARANOWSKI M. Biological role of liver X receptors. **J Physiol Pharmacol**. v. 59, 2008.

BECHMANN, LP; HANNIVOORT, RA; GERKEN, G, et al. The interaction of hepatic lipid and glucose metabolism in liver diseases. **J Hepatol**. v.56(4), p.952–964, 2012.

BERLANGA, A; GUIU-JURADO, E; PORRAS, JA; AUGUET, T. Molecular pathways in non-alcoholic fatty liver disease. **Clinical and Experimental Gastroenterology.** v. 7,p.221–239,2014.

BROWNING, JD; HORTON, JD. Molecular mediators of hepatic steatosis and liver injury. **J Clin Invest**. v.114, p.147-152, 2004.

CARLING, D. The AMP-activated protein kinase cascade – a unifying system for energy control. **Trends Biochem**. **Sci**. v.29, p.18-24, 2004.

CHANG. E, PARK. CY, PARK. SW. Role of thiazolidinediones, insulin sensitizers, in non-alcoholic fatty liver disease. **J Diabetes Investig**. v.4(6), p.517-24, 2013.

CHATKIN, J; MORETTO, M; MAGGIONI, L. et al. Doença hepática não-alcoólica gordurosa e risco de cirrose. **Scientia Medica.** v.18, n. 4, p.172-176, 2008.

CHEN, WL; CHEN, YL; CHIANG, YM; et al. Fenofibrate lowers lipid accumulation in myotubes by modulating the PPARα/AMPK/FoxO1/ATGL pathway. Biochem Pharmacol. v.84, p.522-31, 2012.

CHEN. WL,WEI. HW, CHIU. WZ, et al. Metformin regulates hepatic lipid metabolismo through activating AMP-Activated protein kinase and inducing ATGL in laying hens. Eur J Pharmacol. 671(1-3), 107-12, 2011.

CHUGH, PK.; SHARMA, S. Recent advances in the pathophysiology and pharmacological treatment of obesity. **J. Clin. Pharm**. **Ther.** v.37, p.525–535, 2012

DONNELLY, KL; SMITH, CI; SCHWARZENBERG, SJ; et al. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. **J Clin Invest**. v.115, p.343-51, 2005.

DORMANDY, JA; CHARBONNEL, B; ECKLAND, DJ; et al. Secondary prevention of macrovascular events in patients with type 2 diabetes in the PROactive Study (PROspective pioglitAzone Clinical Trial In macroVascular Events): a randomised controlled trial. **Lancet.** v. 366, p. 1279-1289, 2005.

DORN, C; RIENER, MO; KIROVSKI, G, et al. Expression of fatty acid synthase in nonalcoholic fatty liver disease. **Int J Clin Exp Pathol**. v.3(5), p.505–514, 2010.

FAULDS, MH; ZHAO, C; DAHLMAN-WRIGHT, K. Molecular biology and functional genomics of liver X receptors (LXR) in relationship to metabolic diseases. **Curr Opin Pharmacol**. v.10(6), p.692–697, 2010.

FEIGE, JN; GELMAN, L; MICHALIN, L. et al. From molecular action to physiological outputs: peroxisome proliferator-activated receptors are nuclear receptors at the crossroads of key cellular functions. **Prog Lipid Res**. v.45 (2), p.120-159, 2006.

FERNANDES-SANTOS, C; CARNEIRO, RE; DE SOUZA MENDONCA, L; et al. Pan-PPAR agonist beneficial effects in overweight mice fed a high-fat high-sucrose diet. **Nutrition**. v.25, p.818-827, 2009.

FERRÉ, P; FOUFELLE, F. Hepatic steatosis: a role for de novo lipogenesis and the transcription factor SREBP-1c. **Diabetes Obes Metab**. v.12, 2010.

FRAULOB, JC; SOUZA-MELLO, V; AGUILA, MB; et al. Beneficial effects of rosuvastatin on insulin resistance, adiposity, inflammatory markers and non-alcoholic fatty liver disease in mice fed on a high-fat diet. **Clin Sci (Lond).** v.123: p.259-270, 2012.

FUCHS M. Non-alcoholic fatty liver disease: the bile acid-activated farnesoid x receptor as an emerging treatment target. **J Lipids**, 201; 2012:934396.

GAVRILOVA, O; HALUZIK, M; MATSUSUE, K et al. Liver peroxisome proliferator-activated receptor gamma contributes to hepatic steatosis, triglyceride clearance, and regulation of body fat mass, **J. Biol. Chem.** v. 278, p.34268–34276, 2003.

GRAHAM, TL; MOOKHERJEE, C; SUCKLING, KE; et al. The PPARdelta agonist GW0742X reduces atherosclerosis in LDLR(−/−) mice. **Atherosclerosis.** v.181. p.29–37, 2005.

GRIMALDI, P. A. Regulatory functions of PPARβ in metabolism: implications for the treatment of metabolic syndrome. **Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids** v. 1771, p. 983-990, 2007.

GROSS, B.; STAELS, B. PPAR agonists: multimodal drugs for the treatment of type-2 diabetes. **Best Practice & Research Clinical Endocrinology & Metabolism** v. 21 (4), p. 687–710, 2007

GUIU-JURADO, E; PORRAS, J; AUGUET, T. Molecular pathways in non-alcoholic fatty liver disease. **Clin Exp Gastroenterol**. v.7: p.221–239, 2014.

HAN, JY; PARK, SH; YANG, JH; et al. Licochalcone suppresses LXRα-induced hepatic lipogenic gene espression through AMPK/Sirt1 pathway activation. **Toxicological Research**. v. 30, p19-25, 2014.

HARDIE, DG. Minireview: the AMP-activated protein kinase cascade: the key sensor of energy status . **Endocrinology**. v.144, p.5179-83, 2003.

HARDIE, DG. The AMP-activated protein kinase pathway new players upstream and downstream. Journal of cell Science. v. 117, p. 5479-87, 2004.

HORTON, JD; GOLDSTEIN, JL; BROWN, MS. Critical review SREBPs: activa- tors of the complete program of cholesterol and fatty acid synthesis in the liver. **J Clin Invest**. v.109(9), p.1125–1131, 2002.

ISLEY, WL., Hepatotoxicity of thiazolidinediones, **Expert. Opin. Drug Saf**. v. 2, p. 581–586, 2003.

KALLWITZ, ER; MCLACHLAN, A; COTLER, SJ. Role of peroxisome proliferators-activated receptors in the pathogenesis and treatment of nonalcoholic fatty liver disease. **Diabetes, obesity & metabolismo**. v.14. P.22-28, 2008.

KANURI, G; BERGHEIM, I. In Vitro and in Vivo Models of Non-Alcoholic Fatty Liver Disease (NAFLD). **International Journal of Molecular Sciences**. v.14, p. 11963-80, 2013.

KAWANO, Y; COHEN, DE. Mechanisms of hepatic triglyceride accumula- tion in non-alcoholic fatty liver disease. **J Gastroenterol**. v.48(4), p.434–441, 2013.

KOPELMAN, PG. Obesity as a medical problem. **Nature**. v. 6;404(6778), p.635-43. 2000.

KUDO, H; YATA, Y; TAKAHARA, T; et al. Telmisartan attenuates progression of hepatic macrophage infiltration and effects on adipose tissue. **Liver Int**. v.29, p.988-996, 2009.

LEE, MS; KIM, D; JO, K; HWANG, JK. Nordihydroguaiaretic acid protects against high-fat diet-induced fatty liverby activating AMP-activated protein kinase in obese mice. **Biochemical and Biophysical Research Communications**. v. 401, p.92–97, 2010.

LEFEBVRE, P; CHINETTI, G; FRUCHART, JC; STAELS, B. Sorting out the roles of PPAR alpha in energy metabolism and vascular homeostasis, **J. Clin. Invest.** v. 116, p. 571–580, 2006.

LEWIS, GF; CARPENTIER, A; ADELI, K; GIACCA, A. Disordered fat storage and mobilization in the pathogenesis of insulin resistance and type 2 diabetes. **Endocr Rev.** v. 23(2), p. 201–229, 2002.

LI, Y; XU, S; MIHAYLOVA, M; et al. AMPK phosphorylates and inhibits SREBP activity to attenuate hepatic steatosis and atherosclerosis in diet-induced insulin-resistant mice. **Cell Metabolism**. v. 13, p. 376-388, 2011.

MAEDA, N, TAKAHASHI, M, FUNAHASHI, T; et al. PPARgamma ligands increase expression and plasma concentrations of adiponectin, an adipose-derived protein. **Diabetes.** v.50, p. 2094-2099, 2001.

MALHI, H; GORES GJ. Molecular mechanisms of lipotoxicity in nonalcoholic fatty liver disease. **Semin Liver Dis**. v. 28(4), p. 360–369, 2008.

MARÍ, M; CABALLERO, F; COLELL, A. et al. Mitochondrial free cholesterol loading sensitizes to TNF- and Fas-mediated steatohepatitis. **Cell Metab.** v.4(3), p.185–198, 2006.

MATSUSUE, K; HALUZIK, M; LAMBERT, G. et al. Liver-specific disruption of PPARgamma in leptin-deficient mice improves fatty liver but aggravates diabetic phenotypes. **J. Clin. Invest**.v.111, p. 737–747, 2003.

MCKENNEY, RL.;SHORT, DK. Tipping the balance: the pathophysiology of obesity and type 2 diabetes mellitus. **Surg. Clin. North Am**. v.91, p.1139–1148, 2011.

MITSUYOSHI, H; YASUI, K; HARANO, Y ; et al. Analysis of hepatic genes involved in the metabolism of fatty acids and iron in nonalcoholic fatty liver disease. **Hepatol Res**. v.39(4), p.366–373, 2009.

MIYAZAKI, Y; MAHANKALI, A; MATSUDA, M; et al. Effect of pioglitazone on abdominal fat distribution and insulin sensitivity in type 2 diabetic patients. **J Clin Endocrinol Metab**. v.87, p. 2784-2791, 2002.

MUSSO, G; GAMBINO, R; DURAZZO, M. et al. Adipokines in NASH. postprandial lipid metabolism as a link between adiponectin and liver disease. **Hepatology**. v.42, p.1175–1183, 2005.

NAGASAWA, T; INADA, Y; NAKANO, S. et al. Effects of bezafibrate, PPAR pan-agonist, and GW501516, PPARdelta agonist, on development of steatohepatitis in mice fed a methionine- and choline-defi cient diet. **Eur J Pharmacol**. v.536, p.182-191, 2006.

NESCHEN, S; MORINO, K; ROSSBACHER, JC. et al. Fish oil regulates adiponectin secretion by a peroxisome proliferator-activated receptor- gamma-dependent mechanism in mice. **Diabetes**. v.55, p.924-928, 2006.

NISSEN, SE; WOLSKI, K. Effect of Rosiglitazone on the Risk of Myocardial Infarction and Death from Cardiovascular Causes. **N. Engl. J. Med**. v. 356 (24), p. 2457-2471, 2007.

ONAT, A. Metabolic syndrome: nature, therapeutic solutions and options. **Expert Opin. Pharmacother**. v.12, p.1887–1900. 2011.

PAPANDREOU, D; ANDREOU, E. Role of diet on non-alcoholic fatty liver disease: An updated narrative review. World journal of hepatology. v. 7, p. 575-82, 2015.

PETTA, S; MURATORE, C; CRAXÌ, A. Non-alcoholic fatty liver disease pathogenesis: the present and the future. **Dig Liver Dis**. v. 41(9), p. 615–625, 2009.

PETTINELLI, P; DEL POZO, T; ARAYA, J; et al. Enhancement in liver SREBP-1c/PPAR-alpha ratio and steatosis in obese patients: correlations with insulin resistance and n-3 long-chain polyunsaturated fatty acid depletion. **Biochim Biophys Acta.** v.1792, p.1080-1086, 2009.

PROMRAT, K.; LUTCHMAN, G.; UWAIFO, G. I. et al. A pilot study of pioglitazone treatment for non-alcoholic steatohepatitis. **Hepatology.** v. 39, p. 188-96, 2004.

REAVEN, G. Metabolic syndrome: pathophysiology and implications for management of cardiovascular disease. **Circulation**. v. 106, p.286–288, 2002.

SAHA, AK; AVILUCEA, PR; YE, JM; et al. Pioglitazone treatment activates AMP-activated protein kinase in rat liver and adipose tissue in vivo. **Biochem Biophys Res Commun**. v.314, p. 580-585, 2004.

SCHIMMACK, G; DeFRONZO, RA; MUSI, N. AMP-activated protein kinase: role in metabolism and therapeutic implications. **Diabetes Obes Metab.** v.8,p.591-602, 2006.

SEO, SM; KIM, JH; KIM, HJ. Honokiol activates the LKB1-AMPK signaling pathaway and attenuates the lipid accumulation in hepatocytes. **Toxicology and Applied Pharmacology**. 2015. (*article in press*)

SID, B; VERRAX, J; CALDERON, P. Role of AMPK activation in oxidative cell damage: Implications for alcohol-induced liver disease. **Biochemical pharmacology**. v. 86, p.200-9, 2013.

SIMONS, LA; SIMONS, J; FRIEDLANDER, Y; MCCALLUM, J. Is prediction of cardiovascular disease and all-cause mortality genuinely driven by the metabolic

syndrome, and independently from its component variables? The Dubbo study. **Heart Lung Circ**. v. 20, p. 214–219. 2011.

SOARES E SILVA, A. et al. LPSF/GQ-02 inhibits the development of hepatic steatosis and inflammation in a mouse model of non-alcoholic fatty liver disease (NAFLD). **PloS one.** v. 10, p. e0123787, 2015.

SOARES E SILVA, A. et al. Effect of new thiazolidine derivatives LPSF/GQ-02 and LPSF/GQ-16 on atherosclerotic lesions in LDL receptor-deficient mice (LDLR-/-). Cardiovascular Pathology. v.22, P. 81-90, 2013.

SONSUZ, A; BASARANOGLU, M; OZBAY, G. Relationship between aminotransferase levels and histopathological findings in patients with nonalcoholic steatohepatitis. **Am J Gastroenterol**. v.95: p.1370-1371, 2000.

SOUZA-MELLO, V. Peroxisome proliferator-activated receptors as targets to treat non-alcoholic fatty liver disease. **World journal of hepatology**. v. 7, p. 1012-9, 2015.

STANKOVIC, M.N et al., Time-dependent changes and association between liver free fatty acids, serum lipid profile and histological features in mice model of nonalcoholic fatty liver disease. **Arch. Med. Res**. v.45 (2), p. 116-124, 2014.

TAKAHASHI, Y.; SOEJIMA, Y.; FUKUSATO, T. Animal models of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. **World J. Gastroenterol**. v.18, p.2300–2308, 2012.

TESSARI, P. et al. Hepatic lipid matabolismand non alcoholic fatty liver disease. **Nutr Metab Cardiovasc Dis**. v.19(4), p.291-302, 2009.

THAN, N; NEWSOME, P; A concise review of non-alcoholic fatty liver disease. Atherosclerosis. v 239, p.192-202, 2015.

TILG, H & MOSCHEN, A. R. evolutions os inflammation in nonalcoholic fatty liver disease: the multiple hits hypothesis**. Hepatology**. v.52(5), p.1836-46, 2010.

WATKINS, P.B.; WHITCOMB, R.W. Hepatic dysfunction associated with troglitazone. **N. Engl. J. Med**. v. 338, p. 916–917, 1998.

Y. KIM, Y. KIM, Y. YANG et al. Inhibition of SREBP-1c-mediated hepatic steatosis and oxidative stress by sauchinone, an AMPK-activating lignan in Saururus chinensis. **Free Radical Biology and Medicine**. v.48(4), p.567-578, 2009.

YAMAUCHI, T; KAMON, J; ITO, Y. et al. Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. **Nature**. v.423, p.762-769, 2003.

YKI-JARVINEN, H. Thiazolidinediones. **N Engl J Med**. v.351, p.1106–1118, 2004.

YOON, MJ; LEE, GY; CHUNG, JJ. et al. Adiponectin increases fatty acid oxidation in skeletal muscle cells by sequential activation of AMP-activated protein kinase, p38 mitogen-activated protein kinase, and peroxisome proliferator-activated receptor alpha. **Diabetes**. v.55, p.2562-2570, 2006.

ZANG, M; ZUCCULLO, A; HOU, X; et al. AMP-activated protein kinase is required for the lipid-lowering effect of metformin in insulin-resistant human HepG2 cells. **J Biol Chem** v.279, p.47898–47905, 2004.

ZHOU, G; MYERS, R; LI, Y; et al. Role of AMP-activated protein kinase in mechanism of metformin action. **J Clin Invest**. v.108, p.1167-74, 2001.

Capítulo II

Effects of LPSF / GQ-02 on hepatic lipid metabolism pathways in non-alcoholic fatty liver disease (NAFLD)

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is considered the more common manifestation of metabolic syndrome. One of its most important features is the accumulation of triglycerides in the hepatocyte cells. Thiazolidinediones (TZDs) act as insulin sensitizers and are used to treat patients with type 2 diabetes and other conditions that are resistant to insulin, such as hepatic steatosis. Controversially, TZDs are also associated with the development of cardiovascular events and liver problems. For this reason, new therapeutic strategies are necessary to improve liver function in patients with chronic liver diseases. The aim of the present study was to evaluate the effects of LPSF/GQ-02 on the liver lipid metabolism in a murine model of NAFLD. Eighty male LDLR-/- mice were divided into 3 groups: 1–fed with a high-fat diet (HFD); 2–HFD+pioglitazone (20 mg/kg/day); 3–HFD+LPSF/GQ-02 (30mg/kg/day). The experiments lasted 12 weeks and drugs were administered daily by gavage in the final four weeks. The liver was processed for optical microscopy, Oil Red O, immunohistochemistry and western blot analysis. LPSF/GQ-02 effectively decreased fat accumulation, increased the hepatic levels of p-AMPK, FoxO1, ATGL and p-ACC, and reduced the expression of LXRα, SREBP-1c and ACC. These results suggest that LPSF/GQ-02 acts directly on the hepatic lipid metabolism through the activation of the PPAR-α/AMPK/FoxO1/ATGL lipolytic pathway, and the inhibition of the AMPK/LXR/SREBP-1C/ACC/FAS lipogenic pathway.

INTRODUCTION

Overweight and obesity have been identified as the most important risk factors of many diseases, including cardiovascular disease, type 2 diabetes and lipid disorders such as metabolic syndrome. Non-alcoholic fatty liver disease (NAFLD) is considered the most common manifestation of metabolic syndrome, is one of the most prevalent liver diseases in the world (Kanuri & Bergheim, 2013) and has become a major public health problem due to the impact of increasing obesity among children and adults (StankovI et al., 2014).

NAFLD is characterized by the accumulation of fat in more than 5% of hepatocytes in the form of macro and/or micro lipid vesicles in the cytoplasm (Stankovi et al., 2014). This excess of triglycerides in the liver cells occurs through an imbalance in the acquisition and/or removal of the cell. This dysfunction may be the result of interaction between various factors such as a high calorie and/or unbalanced diet, increased lipogenesis or greater lipolysis in the adipose tissue, inadequate energy expenditure due to a sedentary lifestyle, or a genetic susceptibility (Kanuri & Bergheim, 2013; Tilg & Moschen, 2010).

Sterol regulatory element-binding protein 1 (SREBP-1c) is a lipogenic transcription factor regulated by insulin and glucose, responsible for the regulation of key genes involved in hepatic lipogenesis, such as fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC), and lipoprotein cholesterol carriers (Quan et al., 2013; Kim et al., 2009). SREBP-1c is upregulated by the Liver X receptor (LXR) (Higuchi et al., 2008), and is activated during the pathological process of NAFLD (Tessari et al., 2009).

The 5'-AMP-activated protein kinase (AMPK) is a serine/threonine kinase that mediates cell adaptation to nutritional stress factors, cell metabolism and/or insulin resistance. When activated, AMPK inhibits the biosynthetic pathways from producing fatty acids and sterols, and simultaneously activates the catabolic pathways that produce ATP, such as the oxidation of fatty acids, by regulating specific short-term enzymes and gene expression (Hardie, 2004). Mounting evidence suggests that AMPK and SREBP-1c are critical regulators of lipid metabolism in the liver (Lee et al., 2010). The AMPK inactivates the SREBP-1c and inhibits hepatic steatosis induced by a high fat diet in experimental models (Kim et al., 2009). Therefore, AMPK and SREBP-1c are promising therapeutic targets for the prevention of liver diseases related to lipid accumulation. Furthermore, it has been shown that the AMPK regulates the transcriptional activity of the forkhead box protein O1 (FoxO1), playing a critical role in the regulation of gluconeogenesis and lipid metabolism. The activation of AMPK can increase expression of adipose triglyceride lipase (ATGL), the β-oxidation of fatty acids, and glucose transport, thereby generating greater ATP production (Chen et al., 2011). Therefore, the AMPK/FoxO1/ATGL pathway is a possible therapeutic target for dyslipidemia and fat in the liver (Chen et al., 2012).

Thiazolidinediones (TZDs) are oral antidiabetic agents, which act primarily by increasing the insulin sensitivity of adipose tissue. Studies have shown that thiazolidinediones (rosiglitazone and pioglitazone) decrease serum glucose levels, insulin resistance, and the accumulation of free fatty acids in liver tissue (Chang et al., 2013). Recently, results obtained with a murine model of NAFLD indicated that a new thiazolidione derivative LPSF/GQ-02 was effective in decreasing fat accumulation and the amount of collagen, as well as decreasing inflammation by reducing IL-6, iNOS, COX-2 and F4/80, and increasing the protein expression of

IκBα, cytoplasmic NFκB-65, eNOS and IRS-1 in LDLr -/- mice. These results suggest a direct action by LPSF/GQ-02 on the factors that affect inflammation, insulin resistance and fat accumulation in the liver of these animals (Soares and Silva et al., 2015)

In the present study, mice with NAFLD induced by a high-fat diet, were treated with LPSF/GQ-02 and the signaling pathways PPRAα/AMPK/LXR/SREBP-1c/ACC/FAS and PPARα/AMPK/FoxO1/ATGL were assessed for the development of hepatic steatosis.

MATERIALS AND METHODS

Synthesis of Thiazolidine Derivative LPSF/GQ-02

LPSF/GQ-02 representing the compounds 5-(4-chloro-benzylidene)-3-(4-methyl-benzyl)-thiazolidine-2,4-dione was synthesized at the Department of Antibiotics of the Universidade Federal de Pernambuco (Brazil) in accordance with the methodology described by Mourão et al (2005).

Study Design

Eighty mice were divided into 3 groups, all of which were homozygous for the absence of the LDL receptor gene (LDLr-/-), generated from C57BL6/J genetic background mice, obtained from Jackson Laboratories (USA) and bred in the vivarium of the Centro de Pesquisas Aggeu Magalhães. The state of health of the mice was determined and they were acclimated in a laboratory environment with a temperature of 22°C (±1°) and artificial light from fluorescent lamps for a light/dark period of 12/12 hours. After weaning, the animals were submitted to a standard diet

for eight days of adaptation. After this period, they received an fat diet for 12 weeks (Gupte et al., 2001) and the drugs were administered during the last four weeks of the experimental diet, totaling 30 days of treatment with LPSF/GQ-02.

HFD – This group received an fat diet (HFD) consisting of 21% milk fat and 1.25% cholesterol.

PIO – This group received HFD and was treated with 20 mg/kg/day of pioglitazone for 30 days.

LPSF/GQ-02- This group received HFD and was treated with 30 mg/kg/day of LPSF/GQ-02 for 30 days.

The fat diet was acquired commercially (PragSoluções Biociências). The animals had free access to water and were kept in a controlled light cycle of 12 hours light/darkness. At the end of the treatment, the animals were anesthetized (Ketamine/Xylazine) before blood collection by cardiac puncture (without anticoagulant). The serum was separated and stored at -20°C for biochemical analyses. The livers were dissected and fixed for posterior processing (morphological analysis) and frozen at - 80°C for posterior western blotting analysis.

Histopathology

Liver fragments were fixed in 10% formalin for 24 hours, before being processed and embedded in paraffin. Sections of 4-5 µm were cut and mounted on glass slides. The sections were stained with hematoxylin-eosin (HE) and assessed with an inverted microscope (Observer Z1, Zeiss MicroImaging GmbH), equipped with a camera and 4.7.4 image analysis software (AxionCam MRm Zeiss), at a magnification of 400 x.

Oil Red O Staining

In order to specifically detect lipids, samples of hepatic tissue were fixed in paraformaldehyde at 4% for 2 hours and embedded in O.C.T (Tissue-Tek, Zoeterwoude, Netherlands) in the presence of liquid nitrogen. Afterwards, frozen cuts (8µm thickness) were made on a cryostat and the samples were fixed with pure formaldehyde solution for 15 minutes. Next, the slides were stained with hematoxylin for 30 seconds to identify the nuclei of the cells. The cells were then washed in distilled water and stained with Oil Red O for 15 minutes. Five images of the same magnification were quantitatively analyzed using Gimp 2.6 software (GNU Image Manipulation Program, UNIX platforms).

Immunohistochemical Assays

Five sections (5 µm in thickness) of each group were cut and adhered to slides treated with 3-amino-propyl-trietoxi-silane (APES [Sigma, USA]). The sections were deparaffinized with xylene and rehydrated in graded ethanol (100 to 70%). To increase epitope exposure, the sections were heated for 30 minutes in a sodium citrate buffer (0.01 M, pH 6.0). To minimize endogenous peroxidase activity, the slides were treated with 0.3% (v/v) H_2O_2 in water for five minutes. The sections were washed with 0.01M PBS (pH 7.2) and then blocked with 1% BSA, 0.2% Tween 20 in PBS for 1 hours at room temperature. The sections were then incubated for 12 hours at 4ºC with antibodies against SREBP-1c (1:50 Santa Cruz Biotechnology, CA), ATGL (1:50 Abcam, Cambridge, UK) and ACC (1:50 Cell signaling, Danvers, MA). The antigen-antibody reaction was visualized with avidin-biotin peroxidase (Dako Universal LSAB ® + Kit, Peroxidase), using 3.3-diaminobenzidine as the chromogen.

The slides were counterstained in hematoxylin. Positive staining resulted in a brown reaction product. Negative controls were treated as above, but with the omission of the first antibody. Five pictures at the same magnification were quantitatively analyzed using Gimp 2.6 software (GNU Image Manipulation Program, UNIX platforms).

Cytosolic and Nuclear Protein Extraction

Cytosolic and nuclear proteins from the liver were isolated using Cayman's Nuclear Extraction kit (Item No. 10009277, Cayman chemical company, Ann Arbor, Michigan, USA). The liver fragments were homogenized in a hypotonic buffer supplemented with DTT and Nonidet P-40 per gram of tissue. The livers were centrifuged and re-suspended by adding specified assay reagents, following the manufacturer's instructions. The cytosolic and nuclear fractions were stored in pre-chilled vials at -80˚C until further analysis. Liver cytosols were used to determine the quantity of AMPK, p-AMPK, ACC, p-ACC, FAS, ATGL, FoxO1, SREBP-1c and β-actin in immunoblotting, whereas nuclear fractions were used for SREBP-1c, FoxO1, LXR and Lamin immunoblotting.

Measurement of Protein Levels

The total, cytosolic and nuclear extraction protein levels were determined using the Bradford method, with bovine serum albumin as standard (Bradford, 1970). The samples were read in a spectrophotometer at 660 nm. All samples were run in duplicate and the mean of the two absorbance levels was used to determine the

protein quantity. The protein concentration per sample amount was determined using the equation from a calibration curve. The curve was generated using the same method as the samples, with the substitution of bovine serum albumin at five concentration levels.

Western Blot Analysis

The proteins (40 µg) were separated to 10% (p-AMPK, AMPK, ATGL, LXR, SREBP-1c and FoxO1) and to 8% (ACC, p-ACC and FAS) sodium dodecyl sulfate–polyacrylamide by gel electrophoresis under reduced conditions and were electrophoretically transferred onto nitrocellulose membranes (Bio Rad, CA, USA, Ref. 162-0115). After overnight blocking at 4ºC with 5% non-fat milk in TBS-T (Tris-buffered saline 0.1% plus 0.05% Tween 20, pH 7.4), the membranes were incubated at room temperature for 3 hours with antibodies against the following: p-AMPK (1:1000, Danvers, MA); AMPK (1:1000, Abcam Cambridge, UK); ATGL (1:1000, Abcam Cambridge, UK); LXR (1:100, Santa Cruz Biotechnology, CA); SREBP-1c (1:200, Santa Cruz Biotechnology, CA); FoxO1 (1:200, Santa Cruz Biotechnology, CA); ACC (1:1000, Abcam Cambridge, UK); p-ACC (1:1000, Abcam Cambridge, UK); and FAS (1:100, Santa Cruz Biotechnology, CA), diluted in TBS-T buffer solution containing 3% non-fat milk. After washing (six times, 10 min each) in TBS-T, the membranes were further reacted with horseradish peroxidase-conjugated anti-rabbit antibody (1:3000, Sigma, USA) and anti-mouse antibody (1:8000, Sigma, USA) diluted in TBS-T with 1% nonfat milk, for 1 hours 30 min at room temperature. An enhanced chemiluminescence reagent (Super Signal, Pierce, Ref. 34080) was used to visualize the labeled protein bands and the blots were developed on X-ray film

(Fuji Medical, Kodak, Ref. Z358487-50EA). For quantification, the density of pixels of each band was determined by Image J 1.38 software (available at http://rsbweb.nih.gov/ij/download.html; developed by Wayne Rasband, NIH, Bethesda, MD). The results were confirmed in three sets of experiments for each protein investigated. Immunoblotting for β-actin was performed as a control for the above protein blots. After protein blot visualization with enhanced chemiluminescence, the protein antibodies were stripped from the membranes, which were reprobed with monoclonal anti-β-actin antibody (1:2000, Sigma, USA), and protein densitometry was performed.

Statistical Analysis

GraphPad Prism software (version 5) was used for statistical analysis. Data was expressed as mean ± standard deviation. Differences between the control and treated groups were analyzed using analysis of variance (ANOVA), prior to the performance of Tukey's post hoc test or the Student's t-test. Probability values below 0.05 were considered significant.

RESULTS

LPSF/GQ-02 action in liver histopathology

Histopathological analysis revealed a structural disorganization of hepatocytes in the HFD group, with the hexagonal shape being replaced with a ballooning form, as well as the presence of steatosis and micro and macro vesicular and inflammatory infiltrates (Figure 1A). The PIO group displayed even more severe cell structure

disorganization with a significant presence of lipid inclusions and infiltrated inflammatory foci (Figure 1B). Contrastingly, the structural organization of hepatocytes was preserved in the group treated with LPSF/GQ-02, and there was a reduced presence of vesicles in the liver fat and fewer inflammatory cells (Figure 1C).

LPSF/GQ-02 decreased hepatocyte lipids in the NAFDL model

Oil Red staining was used to quantify lipids in the liver tissue. The HFD group showed many lipid inclusions distributed in the liver parenchyma (Figure 1D). Treatment with pioglitazone, meanwhile, did not reduce the accumulation of hepatic lipids in comparison with the HFD group (Figure 1D). Treatment with LPSF/GQ-02, however, promoted a significant reduction in lipid droplets in the tissue (Figure 1F). Quantitative analysis was performed using ANOVA and Tukey's post hoc tests (Figure 1G).

Immunolocalization markers of lipogenic pathways (SREBP1/ACC/) and lipolysis pathway (ATGL) acted on by LPSF/GQ-02

SREBP-1c is an important transcription factor that regulates the metabolism of fatty acids and cholesterol in the liver (Nakatani, 2005). Immunohistochemical analysis of tissue specimens from the HFD group and the PIO group showed cytoplasmic labeling characteristic of SREBP-1c, mainly around the lipid inclusions (Figure 2A-B). On the other hand, the group treated with LPSF/GQ-02 revealed a significant decrease in SREBP-1c expression when compared to the HFD and PIO groups (Figure 2C).

The ACC enzyme has an important role in the metabolism of fatty acid synthesis in most living organisms (Tong et al., 2005). The PIO group revealed intense staining around the lipid inclusions, in comparison to the HFD and LPSF /GQ-02 groups. LPSF/GQ-02 did not reduce the immunoreactivity of ACC compared to the HFD group, however, it was able to significantly reduce ACC expression when compared to the PIO group (Figure 2E-G)

The ATGL is responsible for TG hydrolysis, turning them into free fatty acids and glycerol (Chen et al., 2011). The LPSF/GQ-02 group revealed an increase in ATGL immunostaining compared to the HFD and PIO groups (Figure 2I-K), therefore suggesting the possible lipolytic activity of this drug.

Quantitative analysis was performed using ANOVA and Tukey's post hoc tests (Figures 2D, H, L).

LPSF/GQ-02 action on the lipogenic (LXR/SREBP-1c/ACC/FAS) and lipolysis pathway (AMPK/FoxO1/ATGL).

The activation of the LXR receptor is associated with increased lipogenesis, and with the accumulation of fat by lipid synthesis, whereas LXR induces increased expression of the lipogenic genes, such as SREBP-1c, FAS and ACC (Han et al., 2014). Treatment with LPSF/GQ-02 significantly decreased LXR expression in comparison with the group that received Pioglitazone (Figure 3A, C).

For analysis of SREBP-1c expression nuclear (active) and cytosolic (inactive) fractions were prepared. In the cytoplasmic fraction there was significantly greater expression in the LPSF/GQ-02 group when compared to the HFD and PIO groups

(Figure 3B, C), while there was a significant decrease of SREBP-1c expression in the nuclear fraction in the LPSF/GQ-02 groups compared to the HFD and PIO groups (Figure 3A, C).

The ACC enzyme is responsible for the synthesis of malonyl-CoA, which is the substrate for the biosynthesis of fatty acids. For analysis of the expression of this enzyme its active (ACC) and inactive (p-ACC) forms were used. ACC expression was higher in the PIO group than in the HFD and LPSF/GQ-02 groups. On the other hand, expression in the LPSF/GQ-02 group was significantly lower than in the PIO group (Figure 3B, C). In turn, the LPSF/GQ-02 group revealed an increase in p -ACC expression compared to the HFD group (Figure 3B, C).

Fatty acid synthase (FAS) is another enzyme involved in lipid synthesis in the lipogenic pathway. The PIO group had a greater expression of FAS than the HFD and LPSF/GQ-02 groups. The LPSF/GQ-02 group had FAS levels similar to the control group (Figure 3B, C).

AMPK inactivates enzymes involved in lipogenesis, such as the acetyl-CoA carboxylase and activators of the nuclear transcriber SREBP-1c, preventing the development of hepatic steatosis (Seo et al., 2015). AMPK positively regulates FoxO1 and ATGL, which play an important role in the activation of lipolysis. For analysis of the expression of this enzyme its inactive (AMPK), and active form (p-AMPK) were used. The present study found that treatment with PIO and LPSF/GQ-02 induced increased expression of AMPK when compared to the HFD group (Figure 4A, B). Moreover, only the LPSF/GQ-02 group showed a significant increase in p-AMPK expression when compared to the HFD group (Figure 4A, B).

FoxO1 is a transcription factor that plays important roles in the regulation of the genes responsible for lipid and glucose metabolism by insulin and AMPK signaling. The phosphorylation and translocation of FoxO1 from the cytoplasm to the nucleus is the primary regulatory mechanism of the action of insulin (Deng et al, 2012). For this analysis the nuclear and cytosolic forms of FoxO1 were assessed. The LPSF/GQ-02 groups revealed a higher expression of the cytosolic FoxO1 protein compared to the HFD groups and the Pioglitazone group (Figure 4A, B). Furthermore, mices treated with pioglitazone and LPSF/GQ-02 displayed a higher nuclear expression of FoxO1 than the HFD group (Figure 4A, B).

Adipose triglyceride lipase is an enzyme that catalyzes the rate-limiting hydrolysis step of triglycerides in the triacylglycerol lipolysis cascade. Treatment with LPSF/GQ-02 induced a higher expression of ATGL than the HFD and pioglitazone groups. Therefore, the new thiazolidine derivative LPSF/GQ-02 displayed possible lipase activity in hepatocytes, confirmed by histopathological and immunohistochemical analysis (Figure 4A, B).

DISCUSSION

Thiazolidinediones (TZDs) are a class of oral anti-diabetic medication that improves insulin resistance by acting as a selective agonist of the peroxisome proliferator activated receptor (PPARs) (Girard, 2001; Yki-jarvinen, 2004). In the present study, the effects of the thiazolidine derivative LPSF/GQ-02 on hepatic lipid metabolism among LDLr -/- mice was assessed and compared with the effects of pioglitazone, a sensitizer of insulin action that has been used to treat patients with type 2 diabetes and other insulin resistant conditions, including NAFLD.

NAFLD is the most common manifestation of chronic liver disease and is closely linked to the diabetes mellitus type 2, obesity and insulin resistance (Ferré and Foufelle, 2010). The main characteristic of NAFLD is the accumulation of triglycerides (TG) in the liver due to the influx of FFA from the adipose tissue, new lipogenesis and ingested through the diet (Seo et al., 2015). From our group showed that mice with an HFD diet displayed an increase in serum cholesterol, LDL and TG. Treatment with LPSF/GQ-02 for 30 days, while not altering serum levels of cholesterol, LDL and TG, did increase the serum level of HDL, a fraction considered important as it is responsible for the reverse transport of cholesterol (Soares and Silva et al., 2015).

Histopathological examination has described the accumulation of TG as hepatic steatosis, in the form of macro and/or micro bubbles in the majority of hepatocytes (Kanuri and Bergheim, 2013). In the present study, Oil red O staining was used to confirm the presence and distribution of lipids in the liver. Mice with a high-calorie diet had only macro and microvesicular hepatocyte steatosis. Moreover, after administration of LPSF/GQ-02 there was a significant reduction in hepatic lipid content, indicating that LPSF/GQ-02 was effective in decreasing hepatic steatosis. It is well known that PPARα can modulate physiopathological mechanisms implicated in NAFLD and atherosclerosis (Larter et al., 2012). Huang et al (2013) showed that PPARα directly regulates the genes necessary for the oxidation of fatty acids and the enzymes that act in ketogenesis, thereby promoting the use of fat as an energy source. The reduction of lipid content after treatment with LPSF/GQ-02 could be explained by a possible PPARα agonist action. Yet although this Thiazolidine derivative did not affect the expression of PPARα in a mice model of NAFLD by western blot analysis (Soares e Silva et al., 2015), further studies on gene

expression are necessary to confirm this data. In contrast, the group that received pioglitazone, a drug considered an agonist of PPARγ, did not present a reduction in hepatic lipid content, but exhibited a large accumulation of lipids, similar to the HFD group. The accumulation of lipids is strongly associated with heightened levels of PPARγ (Varas-Lorenzo et al., 2014). Therefore, pioglitazone may promote the accumulation of fat through the activation of genes of the lipogenic pathway by PPARγ, contributing to the development of steatosis (Huang et al, 2013). The data of the present study in relation to the action of LPSF/GQ-02 on lipidic content in a murine model of NAFLD confirmed the results observed by Soares and Silva et al (2015).

AMP-activated kinase (AMPK) is an energetic sensor of increased levels of AMP and ADP originating from ATP depletion, and is a crucial requirement of cellular homeostasis. The activation of AMPK has a wide variety of metabolic effects, including glucose uptake by the skeletal muscles and increased fatty acid oxidation, and may play an important role not only in the prevention of diabetes mellitus type 2 but also in the context of the metabolic syndrome.

According to Chen et al (2012), fenofibrate, a possible PPAR-α agonist, increases the phosphorylation of AMPK with a subsequent increase in β-oxidation of fatty acids. On the other hand, the GW9662, an inhibitor of PPARα, inhibited the oxidation of fatty acids through AMPK regulation. Treatment with LPSF/GQ-02 promoted increased expression of p-AMPK, suggesting possible regulation via PPARα. In contrast, such effects were not observed in treatment with Pioglitazone.

FoxO1 is a transcription factor with an important action for the metabolic pathways, as it regulates the expression of genes involved in glycogenolysis,

gluconeogenesis and in lipid metabolism. Its regulation occurs through deacetylation allowing its translocation to the nucleus, and thus inducing the transcription of enzymes involved in lipolysis. AMPK signaling is intimately involved in the regulation of FoxO1 by decreasing its acetylation and increasing transcriptional activity (Wang et al., 2011; Yang et al., 2014). In the present study it was observed that the action of LPSF/GQ-02 increased the expression of cytosolic and nuclear forms of FoxO1. These results confirm the data obtained by Chen et al (2012), who demonstrated that Fenofibrate stimulated lipolysis through the AMPK/FoxO1 pathway. Similarly, LPSF/GQ-02 may exert an inhibitory effect on hepatic steatosis.

The ATGL enzyme transforms TG into free fatty acids and glycerol. Yang et al (2014) showed that the lipid-lowering action of Alpha-lipoic acid was mediated via the SIRT1/LKB1/AMPK pathway activating the FoxO1/ATGL signaling pathway. The activation of AMPK results in the enhancement the expression of ATGL through FoxO1, causing a decrease in intracellular triglycerides and ATP generation (Chen et al., 2011). Similarly, LPSF/GQ-02 significantly increased the expression of ATGL in liver tissue, indicating a possible lipid-lowering action through the AMPK/ATGL pathway.

LXRs are important regulators of cholesterol and lipid metabolism (Huang et al., 2013). In the liver function, LXRs stimulate new lipogenesis, positively inducing SREBP-1c, which is a transcription factor responsible for activating genes of the lipogenic enzymes ACC, FAS and SCD1 (Berlanga et al, 2014). Previous studies have shown an inverse correlation between the activity of AMPK and LXR/SREBP-1c in hepatocytes, leading to the suppression of the genes of the lipogenic pathway (Quan et al., 2013, YANG et al., 2009). Similarly, Kim et al (2009) observed that

animals with a hypercaloric diet, when treated with the bioactive compound Sauchinone, displayed reduced lipogenesis due to the inhibition of phosphorylation of LXR by AMPK. Treatment with LPSF/GQ-02 promoted the increase of phosphorylated AMPK well as reducing expression of LXRα and nuclear levels of SREBP-1c, confirming that LPSF/GQ-02 acts on the AMPK/LXR/ SREBP-1c pathway.

Besides the inhibition of transcription of the lipogenic pathway genes, AMPK phosphorylates and inactivates the ACC enzyme, resulting in decreased production of malonyl-CoA and increased carnitine palmitoyltransferase activity, stimulating the β-oxidation of the fatty acids (Zhang et al., 2015). Similar results were obtained by the group treated with LPSF/GQ-02, which had higher levels of phosphorylated ACC. Pioglitazone, however, increased the levels of both the ACC and FAS enzymes, confirming its lipogenic action.

Chen et al (2012) demonstrated that the lipid-lowering effects of fenofibrate, a potential agonist of PPAR-α, were due to the activation of PPAR-α/AMPK/FoxO1/ATGL signaling, resulting in an increase of lipolysis and β-oxidation of fatty acids. Similarly, the new thiazolidine derivative LPSF-GQ02, reduced the hepatic lipid content, possibly through the activation of the PPAR-α /AMPK/FoxO1/ATGL lipolytic pathway, and inhibition of the AMPK/LXR/SREBP-1C/ACC/FAS lipogenic pathway. However further studies are needed to further clarify the beneficial action of LPSF/GQ-02 in reducing hepatic steatosis.

REFERENCES

KANURI, G; BERGHEIM, I. In Vitro and in Vivo Models of Non-Alcoholic Fatty Liver Disease (NAFLD). **International Journal of Molecular Sciences**. v.14, p. 11963-80, 2013.

STANKOVIC, M.N et al., Time-dependent changes and association between liver free fatty acids, serum lipid profile and histological features in mice model of nonalcoholic fatty liver disease. **Arch. Med. Res**. v.45 (2), p. 116-124, 2014.

TILG, H & MOSCHEN, A. R. evolutions os inflammation in nonalcoholic fatty liver disease: the multiple hits hypothesis**. Hepatology**. v.52(5), p.1836-46, 2010.

H. QUAN, D. KIM, S. KIM et al. Betulinic acid alleviates non-alcoholic fatty liver by inhibiting SREBP1 activity via the AMPK-mTOR-SREBP signaling pathway. **Biochemical Pharmacology**. v.85(9), p.1330-1340, 2013.

Y. KIM, Y. KIM, Y. YANG et al. Inhibition of SREBP-1c-mediated hepatic steatosis and oxidative stress by sauchinone, an AMPK-activating lignan in Saururus chinensis. **Free Radical Biology and Medicine**. v.48(4), p.567-578, 2009.

HIGUCHI, N; KATO, M; SHUNDO, Y; et al. Liver X receptor in coop- eration with SREBP-1c is a major lipid synthesis regulator in nonalcoholic fatty liver disease. **Hepatol. Res**. v.38, p.1122–1129, 2008.

TESSARI, P. et al. Hepatic lipid matabolismand non alcoholic fatty liver disease. **Nutr Metab Cardiovasc Dis**. v.19(4), p.291-302, 2009.

HARDIE, DG. The AMP-activated protein kinase pathway new players upstream and downstream. **Journal of cell Science**. v. 117, p. 5479-87, 2004.

LEE, M.S; KIM, D; JO, K; HWANG, J.K. Nordihydroguaiaretic acid protects against high-fat diet-induced fatty liverby activating AMP-activated protein kinase in obese mice. **Biochemical and Biophysical Research Communications**. v. 401, p.92–97, 2010.

CHEN. WL,WEI. HW, CHIU. WZ, et al. Metformin regulates hepatic lipid metabolismo through activating AMP-Activated protein kinase and inducing ATGL in laying hens. **Eur J Pharmacol**. v.671(1-3), p.107-12, 2011.

CHEN, WL; CHEN, YL; CHIANG, YM; et al. Fenofibrate lowers lipid accumulation in myotubes by modulating the PPARα/AMPK/FoxO1/ATGL pathway. Biochem Pharmacol. v.84, p.522-31, 2012.

CHANG. E, PARK. CY, PARK. SW. Role of thiazolidinediones, insulin sensitizers, in non-alcoholic fatty liver disease. **J Diabetes Investig**. v.4(6), p.517-24, 2013.

SOARES E SILVA, A. et al. LPSF/GQ-02 inhibits the development of hepatic steatosis and inflammation in a mouse model of non-alcoholic fatty liver disease (NAFLD). **PloS one.** v. 10, p. e0123787, 2015.

R. MOURÃO, T. SILVA, A. SOARES et al., Synthesis and biological activity of novel acridinylidene and benzylidene thiazolidinediones. **European Journal of Medicinal Chemistry.** V.40(11), p.1129-1133, 2005.

GUPTE, AA; LIU, JZ; REN, Y; MINZE, LJ; WILES, JR; COLLINS, AR; et al. Rosiglitazone attenuates ageand diet-associated nonalcoholic steatohepatitis in male low-density lipoprotein receptor knockout mice. **Hepatology**. v.52(6): p.2001–11, 2010.

NAKATANI T, KATSUMATA A, MIURA S, KAMEI Y, EZAKI O. Effects of fi sh oil feeding and fasting on LXRalpha/RXRalpha binding to LXRE in the SREBP-1c promoter in mouse liver. Biochim Biophys Acta 2005; 1736: 77-86

HAN, JY; PARK, SH; YANG, JH; et al. Licochalcone suppresses LXRα-induced hepatic lipogenic gene espression through AMPK/Sirt1 pathway activation. **Toxicological Research**. v. 30, p19-25, 2014.

SEO, SM; KIM, JH; KIM, HJ. Honokiol activates the LKB1-AMPK signaling pathaway and attenuates the lipid accumulation in hepatocytes. **Toxicology and Applied Pharmacology**. 2015. (*article in press*)

DENG, X; ZHANG, W; O-SULLIVAN,I; et al. FoxO1 inhibits sterol regulatory element-binding protein-1c (SREBP-1c) gene expression via transcription factors Sp1 and SREBP-1c. **J Biol Chem**. v.287(24), p.20132-43, 2012.

GIRARD, J. Mechanisms of action of thiazolidinediones. (2001) **Diabetes Metab** 27: 271–8. PMID: 11452221

YKI-JARVINEN, H. Thiazolidinediones. **N Engl J Med**. v.351, p.1106–1118, 2004.

FERRÉ, P; FOUFELLE, F. Hepatic steatosis: a role for de novo lipogenesis and the transcription factor SREBP-1c. **Diabetes Obes Metab**. v.12, 2010.

LARTER, CZ; Yeh, MM; Van Rooyen, D; Brooling, J; Ghatora, K; Farrell, GC; et al. (2012). Peroxisome prolif- erator-activated receptor-a agonist,Wy14 643, improves metabolic indices, steatosis and ballooning in diabetic mice with non-alcoholic steatohepatitis. Journal of Gastroenterology and Hepatology 27 341–350 doi: 10.1111/j.1440-1746.2011.06939.x PMID: 2192964964.

HUANG, Y; GUSDON, A; QU, S. Nonalcoholic fatty liver disease: molecular pathways and therapeutic strategies. Lipids in health and disease. v.12, p.171, 2013.

VARAS-LORENZO, C; MARGULIS, AV; PLADEVALL, M; et al**.** The risk of heart failure associated with the use of noninsulin blood glucose-lowering drugs: systematic re- view and meta-analysis of published observational studies**.BMCCardiovasc Disord.** v.14(1), p.1–17, 2014.

WANG, Y; WU, Z; LI, D; et al. Involvement of oxygen- regulated protein 150 in AMP-activated protein kinase-mediated alleviation of lipid-induced endoplasmic reticulum stress**. J Biol Chem**. v.286, p.11119–31, 2011.

YANG, Y; LI, W; LIU, Y; et al. Alpha-lipoic acid improves high-fat diet-induced hepaticsteatosis by modulating the transcription factors SREBP-1c, FoxO1 and NRF2 via the SIRT1/LKB1/AMPK pathway. **J Nutr Biochem**. v.25(11), p.1207-17, 2014.

BERLANGA, A; GUIU-JURADO, E; PORRAS, JA; AUGUET, T. Molecular pathways in non-alcoholic fatty liver disease. **Clinical and Experimental Gastroenterology.** v. 7, p. 221–239, 2014.

Yang J, Craddock L, Hong S, Liu ZM. AMP-activated protein kinase suppresses LXR-dependent sterol regulatory element-binding protein-1c transcription in rat hepatoma McA-RH7777 cells**. J Cell Biochem**. v. 106, p.414-26, 2009.

ZHANG, T; YAMASHITA, Y; YASUDA, M; et al**.** Ashitaba (Angelica keiskei) extract prevents adiposity in high-fat diet-fed C57BL/6 mice. **Food & function**, v.6, p.135-45, 2015.

FIGURE LEGENDS

Figure 1: Histopathological analysis of the effect of Pioglitazone and LPSF/GQ-02 on liver tissue in LDLR-/- mice. Representative images of HE (A-C) and Oil red (D-F). (A) HFD Group displaying macro and micro lipid vesicles and inflammatory infiltration (arrow). (B) Pioglitazone group had higher steatosis and inflammatory cell infiltration (arrow). (C) LPSF/GQ-02 group showed a more organized hepatic architecture and a reduction of lipid inclusions in hepatocytes. (D) HFD Group. (E) Pioglitazone group had a very similar lipid content in hepatocytes to the HFD group. (F) LPSF /GQ-02 group showed a decrease of lipid inclusions. Bars 20 µm. (G) Graph of Oil Red quantification (N=5). Letters represent significant differences in the graph (a) HFD, (b) PIO, (c) LPSF/GQ-02, $p < 0.05$.

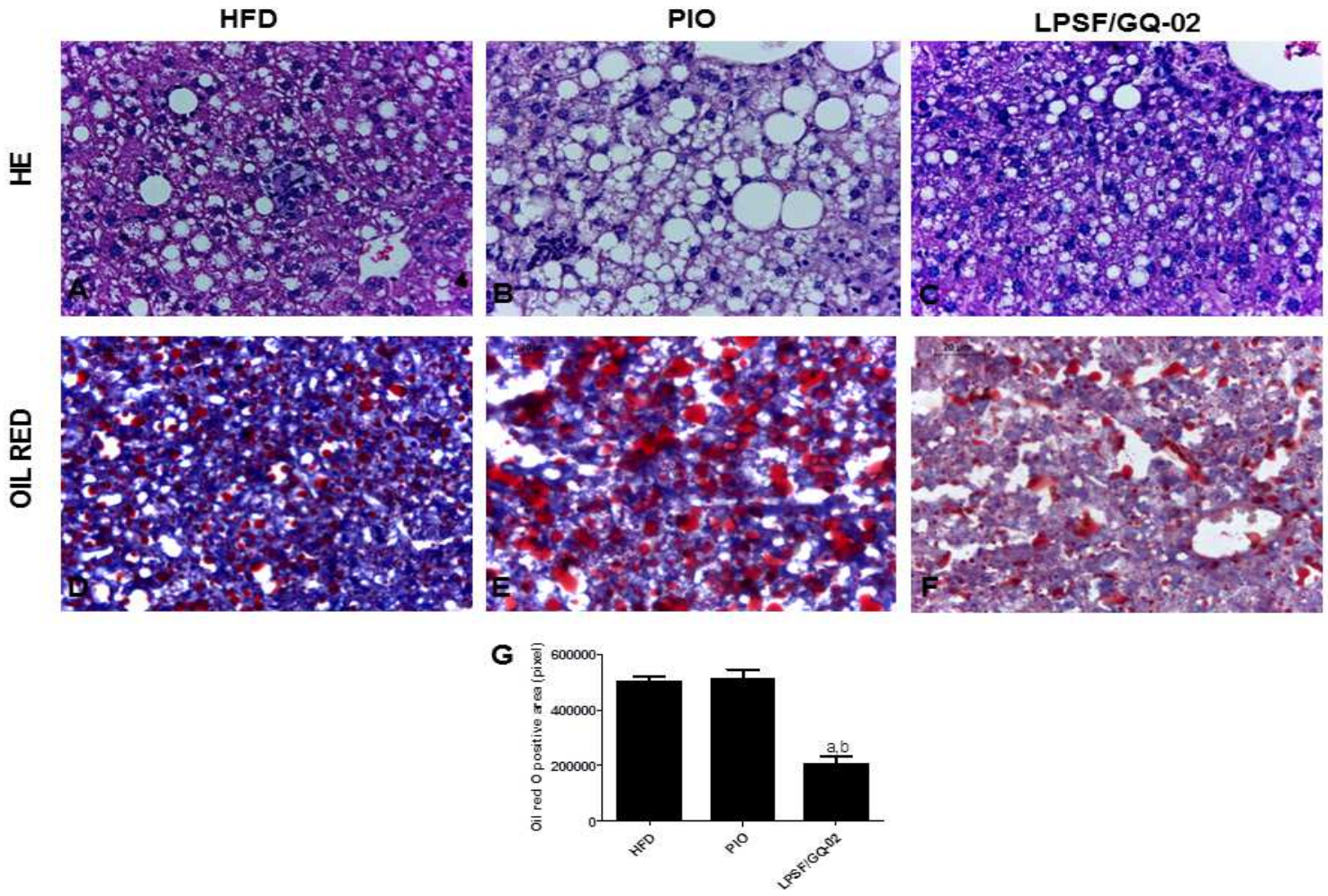
Figure 2: Immunohistochemistry Analysis for Sterol regulatory element-binding protein 1c (SREBP-1c), Acetyl-CoA carboxylase (ACC) and Adipose triglyceride lipase (ATGL) in LDLR-/- mice treated with Pioglitazone and LPSF/GQ-02. The LPSF/GQ-02 group (C) reduced the immunostaining of SREBP-1c when compared to the HFD (A) and PIO (B) groups. Pioglitazone was not effective in reducing ACC enzyme marking when compared to the HFD (E) and LPSF/GQ-02 (L) groups. The LPSF /GQ-02 (K) group exhibited an increase in ATGL immunoreactivity compared to the HFD (I) and PIO (J) groups. Bars 20 µm. (D, H, L) Graph quantifying SREBP-1c, ACC and ATGL (N=10). Letters represent significant differences in the graph: (a) HFD, (b) PIO, (c) LPSF/GQ-02, $p < 0.05$.

Figure 3: Western blotting analysis for expression of LXR, SREBP-1c, ACC, p-ACC and FAS in LDLR-/- mice treated with Pioglitazone and LPSF/GQ-02. Treatment with LPSF/GQ-02 decreased LXR expression compared with treatment with Pioglitazone (A, C). The expression of SREBP-1c cytosolic was increased when compared to the HFD and PIO (B, C) groups, while LPSF/QF-02 decreased the expression of nuclear SREBP-1c compared with the HFD and PIO groups (A, C). Treatment with Pioglitazone increases expression of the ACC enzyme when compared to the HFD and LPSF/GQ-02. On the other hand LPSF/GQ-02 was able to decrease the expression of ACC compared to the PIO group (B, C). The group treated with LPSF/GQ-02 showed an increase in the expression of the p-ACC enzyme compared to the HFD group (B, C). Expression of the FAS enzyme increased in the group treated with Pioglitazone compared to the HFD and LPSF/GQ-02 groups (B, C). Data was analyzed using ANOVA and Tukey's post hoc tests. The columns represent the mean ±

SD of the protein investigated. The results were confirmed in three different experiments (n = 5). Letters represent significant differences in the graph (a) HFD, (b) PIO, (c) LPSF/GQ-02, $p<0.05$.

Figure 4: Western blotting analysis for the expression of AMPK, p-AMPK, and FoxO1 and ATGL in LDLR-/- mice treated with Pioglitazone and LPSF/GQ-02. The groups treated with Pioglitazone and LPSF/GQ-02, respectively, had higher expressions of AMPK than the HFD group (A, B). However, only LPSF/GQ-02 was able to show a significant increase in p-AMPK compared to the HFD group (A, B). The group treated with LPSF/GQ-02 had an increased expression in the cytosol and nuclear forms of FOXO1a. By contrast, the group treated with Pioglitazone displayed an increase in the nuclear expression of FoxO1 only (A, B). The group LPSF/GQ-02 had a higher expression of ATGL than the HFD and Pioglitazone groups. The data was analyzed using ANOVA and Tukey's post hoc tests. The columns represent the mean ±S.D. of the investigated protein. The results were confirmed in three different experiments (n = 5). Letters represent significant differences in the graph: (a) HFD, (b) PIO, (c) LPSF/GQ-02, $p<0.05$.

FIGURES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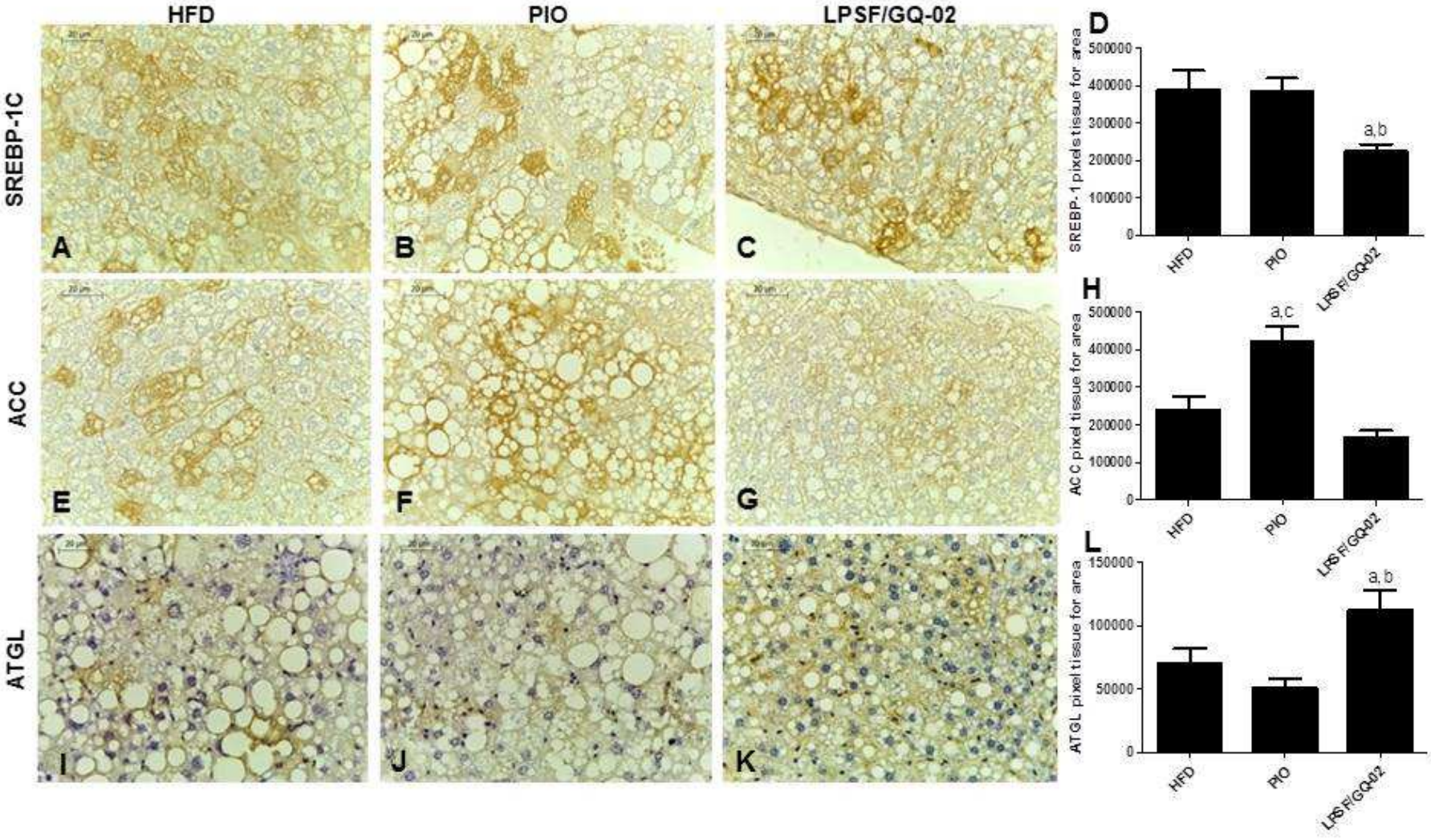
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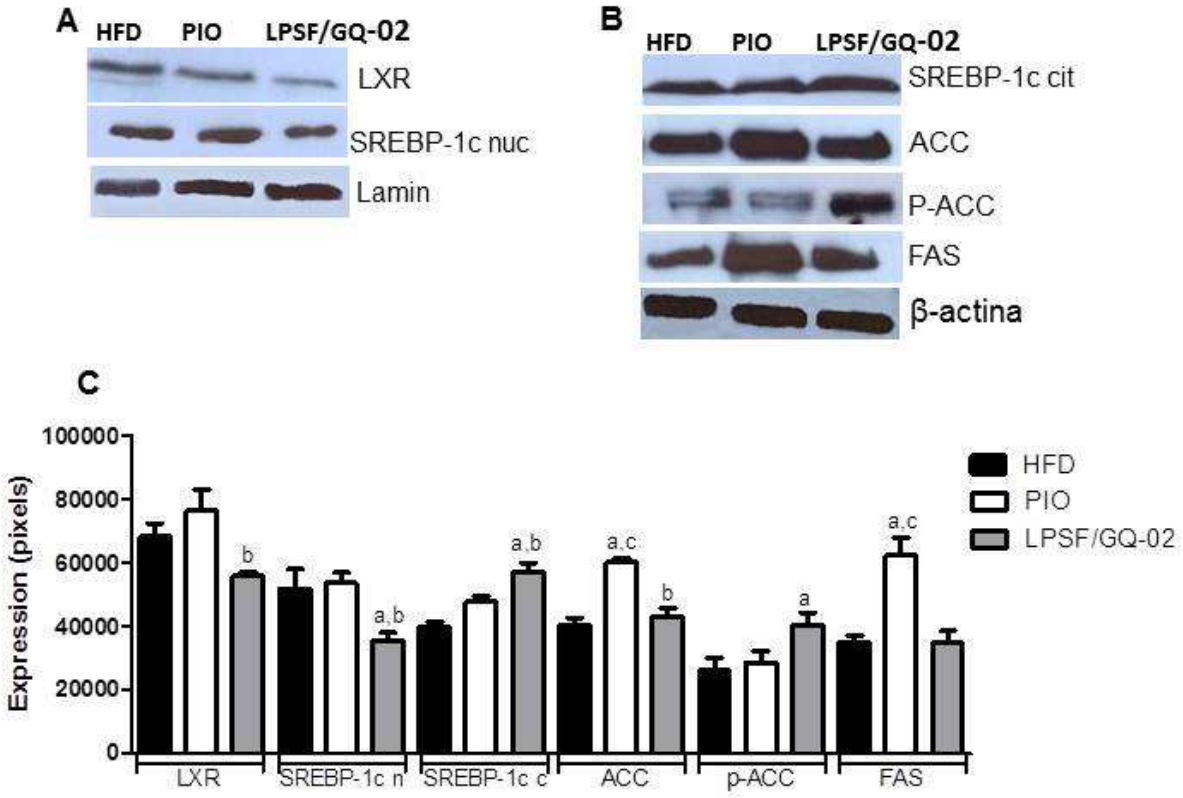
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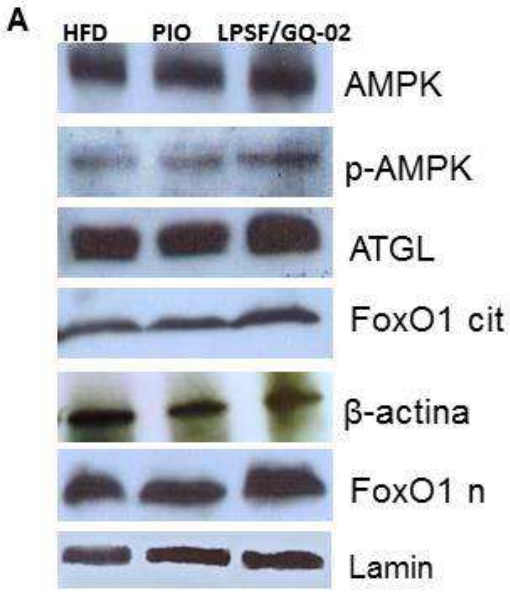
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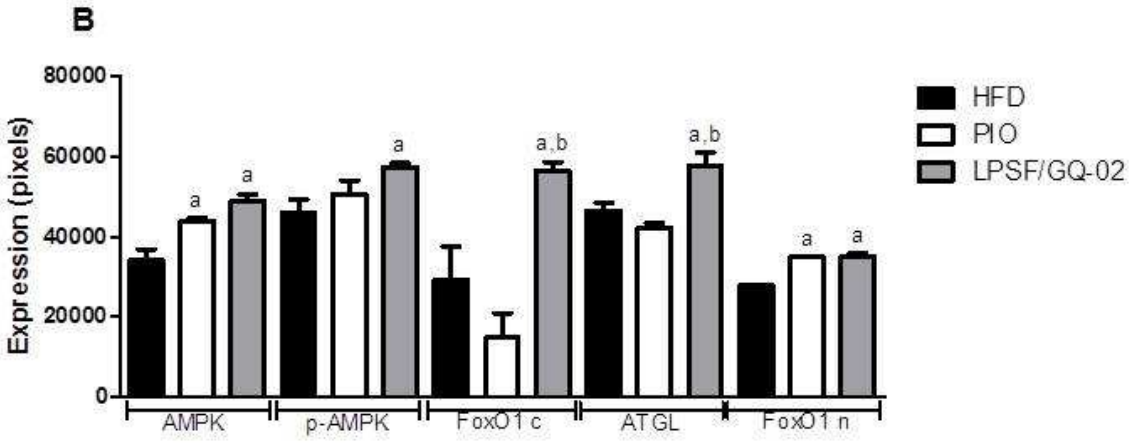
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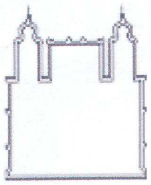
HFD
PIO
LPSF/GQ-02

6 CONCLUSÃO

O tratamento com a molécula LPSF/GQ-02 foi eficaz em reduzir a esteatose hepática através da inibição da via lipogênica LXR/SRBP-1c/ACC/FAS, bem como através da ativação da lipolítica AMPK/FoxO1/ATGL, indicando uma maior β-oxidação de ácidos graxos e uma maior geração de ATP. Portanto, o presente estudo sugere uma possível ação hipolipemiante da LPSF/GQ-02, podendo esta ser considerada uma molécula promissora para o tratamento NAFLD.

Anexos

ANEXO 01: Parecer do comitê de Ética



MINISTÉRIO DA SAÚDE / FUNDAÇÃO OSWALDO CRUZ
VICE-PRESIDÊNCIA DE PESQUISA E DESENVOLVIMENTO TECNOLÓGICO
Comissão de Ética no Uso de Animais
CEUA-FIOCRUZ

CERTIFICADO

Certificamos que o protocolo intitulado :

" Estudo comparativo de tratamentos com glitazonas sobre o processo aterosclerótico em camundongos C57BL/6J. "

número P-510/08, proposto por Christina Alves Peixoto, foi licenciado pelo Nº L-010/09.

Sua licença de Nº L-010/09 autoriza o uso anual de :

- 120 Mus musculus

Esse protocolo está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi APROVADO pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA - FIOCRUZ). Na presente formatação, este projeto está licenciado e tem validade até 21 de janeiro de 2013.

Rio de Janeiro, 21/01/2009

Dra. Norma Vollmer Labarthe
Coordenadora da CEUA
FIOCRUZ

ANEXO 02: Normas da revista do artigo

TOXICOLOGY AND APPLIED PHARMACOLOGY

AUTHOR INFORMATION PACK

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DESCRIPTION

Toxicology and Applied Pharmacology publishes original scientific research of relevance to animals or humans pertaining to the action of **chemicals**, **drugs**, or **chemically-defined natural products**.

Regular articles address **mechanistic approaches** to **physiological**, **pharmacologic**, **biochemical**, **cellular**, or **molecular understanding** of **toxicologic/pathologic lesions** and to methods used to describe these responses. Safety Science articles address outstanding state-of-the-art preclinical and human translational characterization of drug and chemical safety employing cutting-edge science. Highly significant Regulatory Safety Science articles will also be considered in this category. Papers concerned with alternatives to the use of experimental animals are encouraged.

Short articles report on high impact studies of broad interest to readers of *TAAP* that would benefit from rapid publication. These articles should contain no more than a combined total of four figures and tables. Authors should include in their cover letter the justification for consideration of their manuscript as a short article.

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Toxicologists, pharmacologists, cancer researchers, epidemiologists, molecular and cell biologists

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INTRODUCTION

Toxicology and Applied Pharmacology publishes original scientific research of relevance to animals or humans pertaining to the action of chemicals, drugs, or chemically-defined natural products.

Regular articles address mechanistic approaches to physiological, pharmacologic, biochemical, cellular, or molecular understanding of toxicologic/pathologic lesions and to methods used to describe these responses. Safety Science articles address outstanding state-of-the-art preclinical and human translational characterization of drug and chemical safety employing cutting-edge science. Highly significant Regulatory Safety Science articles will also be considered in this category. Papers concerned with alternatives to the use of experimental animals are encouraged.

Short articles report on high impact studies of broad interest to readers of *TAAP* that would benefit from rapid publication. These articles should contain no more than a combined total of four figures and tables. Authors should include in their cover letter the justification for consideration of their manuscript as a short article.

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cm centimeter
cpm counts per minute
mm millimeter
dpm disintegrations per minute
um micrometer
sc subcutaneous
nm nanometer
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g gram
ip intraperitoneal
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SE standard error
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