UNIVERSIDADE DE SÃO PAULO

FACULDADE DE CIÊNCIAS FARMACÊUTICAS

Programa de Pós-Graduação em Farmácia

(Fisiopatologia e Toxicologia)

Área de Fisiopatologia

“Efeitos de um novo derivado tiazolidínico (GQ-11) no processo de reparo tecidual em modelos de resistência à insulina e isquemia-reperfusão”

“Effects of a new thiazolidine compound (GQ-11) on tissue repair process in models of insulin resistance and ischemia-reperfusion”

Jacqueline Cavalcante Silva

Tese para obtenção do grau de

DOUTORA

Orientadoras:

Profa. Dra. Dulcineia S. P. Abdalla

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Original version

Evaluation Committee
PhD

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Dedicated to scientific community, to every single scientist, professor and teacher struggling for knowledge sharing and to the students putting effort to build a world with more education.

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“Things we lose have a way of coming back to us in the end, if not always in the way we expect”

Luna Lovegood

RESUMO

SILVA, J.C. **Efeitos de um novo derivado tiazolidínico (GQ-11) no processo de reparo tecidual em modelos de resistência à insulina e isquemia-reperfusão.** 2019**. 120p.** (Tese de Doutorado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo; Università degli Studi di Genova, Genova, 2019.

As tiazolidinadionas (TZDs) compreendem uma classe de fármacos hipoglicemiantes que reduzem a resistência à insulina pelos tecidos periféricos. Dados preliminares *in vivo* obtidos em nosso grupo de pesquisa mostraram que um dos novos derivados tiazolidínicos, GQ-11, além de aumentar a resposta à insulina, pode inibir citocinas pró-inflamatórias, o que a torna uma alternativa terapêutica promissora no reparo tecidual, em especial, nos casos de descompensação metabólica como ocorre na resistência à insulina e na isquemia/reperfusão. Nesse contexto, o objetivo deste trabalho foi investigar os efeitos da GQ-11 nas etapas do processo de reparo tecidual em três modelos: resistência à insulina utilizando camundongos *db/db*, epiderme humana reconstruída em matriz de colágeno glicado e isquemia/reperfusão induzida por clampeamento da aorta em ratos Wistar.

No contexto de resistência à insulina, o tratamento com GQ-11 induziu a expressão de mediadores anti-inflamatórios como IL-10, TGF-β e Arg-1 e diminuiu a expressão de citocinas pró-inflamatórias em lesões de camundongos *db/db* e em macrófagos*,* além de aumentar a capacidade de re-epitelização e a deposição de colágeno. Além disso, o tratamento também induziu a proliferação de queratinócitos e a diferenciação de fibroblastos em epiderme humana reconstruída em matriz de colágeno glicado.

No modelo de isquemia-reperfusão, o mesmo efeito anti-inflamatório da GQ-11 foi observado ao lado de efeitos anti-oxidantes através da regulação de enzimas como catalase, GPx e diminuição de TBARS. O imageamento dos animais através de tomografia por emissão de pósitrons (PET) demonstrou menor captação de ^{18}F-FDG (^{18}F-fluordesoxiglicose), indicando diminuição do processo inflamatório decorrente da reperfusão pós clampeamento aórtico.

Dessa forma, conclui-se que GQ-11, um agonista dual de PPARα/γ, tem efeito anti-inflamatório importante, podendo ser um candidato à fármaco com possível aplicação no reparo tecidual no diabetes e na prevenção da síndrome de isquemia-reperfusão desenvolvida após procedimentos cirúrgicos.

PALAVRAS-CHAVE: Tiazolidinadionas, Inflamação, Reparo Tecidual, Isquemia/Reperfusão.

ABSTRACT

SILVA, J.C. **Modulation of Inflammation and angiogenesis by a new thiazolidine compound (GQ-11) in visceral ischemia.** 2019**. 120p.** (PhD thesis) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo; Università degli Studi di Genova, Genova, 2019.

The thiazolidinediones (TZDs) class comprises drugs with hypoglycemic effects, reducing insulin resistance in peripheral tissues. Our group has demonstrated in preliminary *in vivo* studies that a new TZD, GQ-11, improves insulin resistance as well as modulates cytokines involved in inflammatory process, suggesting an interesting approach for therapeutic alternatives in tissue repair, especially in metabolic decompensation cases, as insulin resistance and ischemia-reperfusion. In this context, the aim of this study was to investigate GQ-11 effects in tissue repair in three different models: insulin resistance in *db/db* mice, reconstructed human epidermis (RHE) in glycated collagen matrix and ischemia/reperfusion induced by aorta clamping in Wistar rats.

In insulin resistance context, GQ-11 treatment upregulated the expression of anti-inflammatory mediators, such as IL-10, TGF-β and Arg-1, downregulated the expression of pro-inflammatory cytokines both in *db/db* mice wounds and in macrophage, besides increasing re-epithelization and collagen deposition. In addition, the treatment also induced keratinocytes proliferation and fibroblasts differentiation in RHE.

In ischemia-reperfusion model, the same anti-inflammatory effect was observed along with anti-oxidant properties through regulation of enzymes, such as catalase and GPx, as well as by decreasing TBARS formation. Animals imaging by positron emission tomography (PET) indicated significant less ^{18}F-

FDG uptake in animal treated with GQ-11 compared to controls, suggesting decrease of the inflammation process related to reperfusion after aorta clamping.

Concluding, the dual PPARα/γ agonist GQ-11 has an important anti-inflammatory effect, suggesting a new approach to tissue repair management in diabetes and in prevention of ischemia-reperfusion syndrome post-surgery.

KEYWORDS: Thiazolidinediones, Inflammation, Tissue Repair, Ischemia/Reperfusion

ABBREVIATION LIST

AGEs: Advanced Glycation End-products
AGE-R1/R2/R3: Advanced Glycation End-products Receptor 1/2/3
CCL-2: CC chemokine monocyte chemoattractant protein
DM: Diabetes Mellitus
ECM: Extracellular Matrix
HIF: Hypoxia-Inducible Factor
HUVEC: Human Umbilical Vein Endothelial Cell
ICAM-1: Intracellular Adhesion Molecule -1
IGF-1: Insulin-like Growth Factor-1
IL-1β: Interleukin 1β
IL-6: Interleukin-6
IL-10: Interleukin-10
I/R: Ischemia-Reperfusion
IRS: Ischemia/Reperfusion Syndrome
LDLr: Low-Density Lipoprotein Receptor
LPSF: Laboratory of Drug Design and Synthesis
NF-κB: Nuclear Factor κB
PGC-1α: Peroxisome proliferator-activated receptor γ coactivator 1-α
PHD: Oxygen-sensing Prolylhyldroxylase
PON: Paraoxinase
PPAR: Peroxisome Proliferator-Activated Receptors
RAGE: Receptor for Advanced Glycation End-products
ROS: Reactive Oxygen Species
RXR: Retinoid X Receptor
SIRT-1: Sirtuin 1
SOD: Superoxide Dismutase
TNF-α: Tumor Necrosis Factor-α
TGF-β: Transforming Growth Factor-β
TZDs: Thiazolidinediones
VCAM-1: Vascular Cell Adhesion Molecule -1
VEGF: Vascular Endothelial Growth Factor

SUMMARY

1. INTRODUCTION

The thiazolidinediones (TZDs) comprise a class of hypoglycemic drugs used in type 2 diabetes treatment, lowering glucose levels in blood through insulin sensitizing effects in peripheral tissues. Their classic insulin sensitizing effect is mediated by activation of alpha (α), gamma (γ) and/or beta/delta (β/δ) isoforms of peroxissome proliferator-activated receptors (PPARs), acting as parcial, full or dual agonists (NOLAN *et al.*, 1994; BERGER *et al.*, 1999; HAUNER, 2002).

Besides hypoglycemic effect of TZDs, some clinical studies have linked its treatment to some pleiotropic effects, such as anti-inflammatory and pro-angiogenic potential. Many studies have been reported modulation of pro-inflammatory factors by TZDs, such as Interleukin-6 (IL-6), Tumor Necrosis Factor-α (TNF-α) and CC Chemokine Monocyte Chemoattractant Protein (CCL-2), in association to its PPARγ agonism (PASCERI *et al.*, 2000, RUAN *et al.*, 2003; GLASS *et al*., 2010). Importantly, PPARγ agonists may also improve endothelial dysfunction and angiogenesis independently of the insulin sensitizing action in normoglycemic patients, especially due to induction of Vascular Endothelial Growth Factor (VEGF) and VEGF receptors expression besides repression of cell adhesion molecules expression (GENSCH *et al.,* 2007, YAMAKAWA *et al.,* 2000).

Although these beneficial effects, prolonged use of TZDs was also associated to important side effects such as fluid retention, body weight gain, peripheral edema, severe hepatotoxicity and cardiovascular risk (LEBOVITZ, 2002; PATEL, 2009; HERNANDEZ *et al.*, 2011, LOKE, *et al*., 2011, NISSEN; WOLSKI, 2010, GRAHAM *et al.*, 2010). Thus, the search for new thiazolidine

derivatives with therapeutic efficacy without inducing the reported side effects has been encouraged.

GQ-11 is a new TZD derivative, with dual agonism (PPARα/γ), synthesized by the Laboratory of Drug Design and Synthesis *(LPSF*), affiliated to the Therapeutic Innovation Research Group *(GPIT* - http://www.ufpe.br/gpit) at Federal University of Pernambuco (*UFPE*) under the supervision of Prof. Dr. Ivan da Rocha Pitta. Importantly, GQ-11 showed promising therapeutic effects in obesity-induced metabolic alterations in Low-Density Lipoprotein Receptor $(LDLr)^{-/-}$ mice, as it improves chronic inflammation and lipid profile besides its hypoglycemic potential with no weight gain or adipogenesis induction (SILVA et al., 2018). Moreover, in a previous study (Rudnicki M. et al, personal communication) it was observed that GQ-11 increased endothelial cell (Human Umbilical Vein Endothelial Cell - HUVEC) migration and VEGF expression, indicating pro-angiogenic effects in contrast to rosiglitazone anti-angiogenic action. The evidence of GQ-11 anti-inflammatory and pro-angiogenic actions suggest that it may be helpful to control and regulate processes with exacerbated inflammation and endothelial dysfunction.

2. OBJECTIVES

Considering this scenario, the aim of this study was to investigate the effects of GQ-11 on inflammation of tissue repair in different models that are described in three chapters.

In the first chapter, we describe preliminary data showing GQ-11 anti-inflammatory effects. In the second chapter, we show GQ-11 effects on tissue repair on an *in vivo* model of wound healing at insulin resistance conditions with

db/db mice while in the third chapter, GQ-11 effects are described on fibroblasts and keratinocytes proliferation and differentiation using an *in vitro* model of reconstructed human epidermis with glycated collagen. Finally, in the fourth chapter, we describe GQ-11 effects on Ischemia/Reperfusion Syndrome (IRS) damage induced in Wistar rats by supraceliac aorta clamping.

3. REVIEW

3.1. PPARs and inflammation

PPARs are mainly located in cell nucleus, where generally heterodimerizes to Retinoid X Receptor (RXR), to reach its nuclear activity, like other nuclear receptors, with different function domains: N-terminus (transcriptional activation-related), DNA-binding (RXR heterodimer-related), a mobile domain to adequate rotation of C-terminus and finally C-terminus domain (permitting co-factor interactions) (SCHULMAN et al., 1998). For that reason, it is understood that PPARs do not act by themselves, but in association to co-factors which remodel cromatin structure aiming to promote or inhibit gene transcriptions. In absence of binders (fatty acids and derivatives), PPARs create co-repressor complexes, such as NCoR, RIP140 or SMRT, suppressing gene transcription through *histona deacetilases* recruiting. In binders presence, co-activators such as p300, CBP or SRC-1, they bind to amino-terminus co-activator PPARγ-1 (PGC-1), acetylating and remodeling chromatin, reinforcing gene transcription through chromatin condensation decrease, as observed below (ONATE et al., 1995).

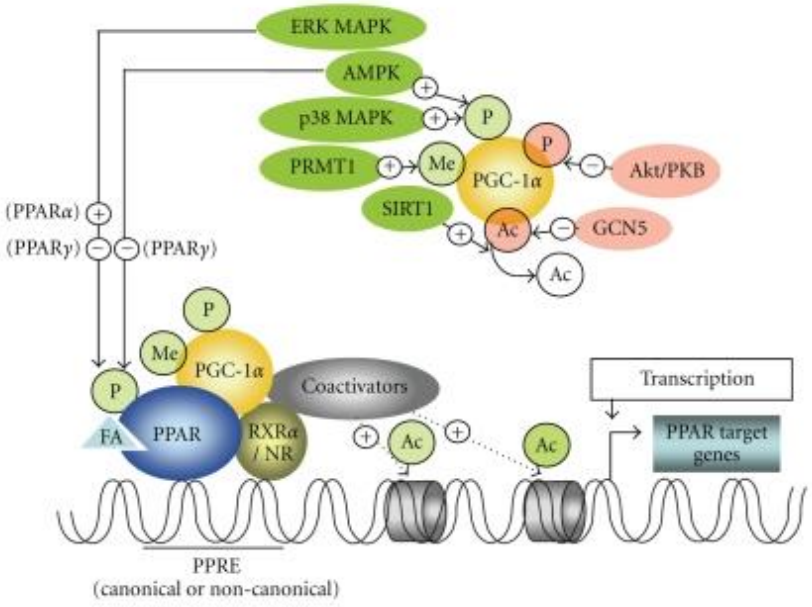

Figure 1. Activation of PPARs, co-activactors and signaling pathways (MORENO et. al., 2010).

Depending on cell type, binder-induced conformations change and a specific complex is formed between receptor and its co-activators and co-repressors, permitting the adjustment of physiological response. This also explain the diversity of gene expression changes when a nuclear receptor is activated by different binders (ONATE et al., 1995).

Modulation of PPARs action is mediated through ERK MAPK, AMPK or PGC-1α phosphorylation signaling events. PPARs phosphorylation can up or downregulate its activity, considering that activation of Peroxisome proliferator-activated receptor γ coactivator 1-α (PGC1-α) expression is Sirtuin-1 (SIRT-1)-mediated, while its inhibition is mediated by GCN5 acetylation. AMPK or p38 MAPK 6 phosphorylation increase PGC- 1α stability through arginine methylation. PGC-1α activation enable PPAR binding, finally creating an RXR and/or other receptor complex, allowing co-activators recruitment to chromatin acetylation and promoting target genes DNA codification to be transcript

(MORENO et al., 2010). From these complexes, emerge the many evidences that PPARs play a key role in lipid synthesis and catabolism regulation and insulin resistance (YU & REDDY, 2007).

Among known PPAR isoforms, PPARα is mainly expressed in liver, also found in skeletal muscle, heart and endothelium, with lipid catabolism compounds such as oxidation, binding and free fatty transport and inflammation factors mediation (KERSTEN; DESVERGNE; WAHLI, 2000). PPARγ is mainly expressed in adipose tissue, bowel, β cells and endothelium and is more related to adipogenesis, through differentiation and maturation of adipocytes (AKBIYIK *et al*., 2004, WILLSON *et al*., 2000). Different PPAR isoforms are codified by different genes in different tissues, interacting to specific binders and promoting different genes regulation (RICOTE & GLASS, 2007; BALAKUMAR *et al*., 2007).

Regarding inflammation, several studies reported PPARγ ability to stimulate monocyte differentiation to macrophages. Monocytes recruitment to injury or tissue repair sites, and their activation into resident macrophages, represent both initiation of inflammation – where macrophages execute host first protection – and its own resolution (RICOTE *et al*., 1998; GELMAN *et al*., 1999). Active silencing of inflammatory genes (TNF-α, lL-6, CCL-2, PAI-1) and transcription factors (Nuclear Factor κB: NF-κB), induced by PPARγ activation, is an important demonstration of its involvement in the control of macrophage inflammatory response (SU *et al*., 1999; GLASS *et al*., 2010).

Besides PPARγ role in inflammation process, PPARα also was described to exert anti-inflammatory effects in systemic inflammation. Some reports showed that treatment with PPARα agonists downregulates IL-6 and Interleukin 1β (IL-1β) both *in vitro* and *in vivo* (GERVOIS *et al*., 2004; MANSOURI *et al*., 2008). At

the same time, treatment with IL-1β decreases expression of PPARα and its target genes, suggesting a negative crosstalk between inflammation induced by IL-1β and PPARα expression (STIENSTRA *et al*., 2007). In addition, *in vivo* studies also reported that pre-treatment with PPARα agonists prevents upregulation of IL-1, IL-6 and TNF-α in plasma, as well as of Intracellular Adhesion Molecule -1 (ICAM-1) and Vascular Cell Adhesion Molecule -1 (VCAM-1) in aorta, suggesting an important role in regulation of systemic inflammation, even with a not well clear mechanism (MANSOURI *et al*., 2008). The ability to upregulate anti-inflammatory genes is also described, such as Il-1ra and IκBa, inhibitors of NFκB, suggesting a cooperation between PPARα transactivation and repression of inflammatory genes (KLEEMAN *et al*., 2003). Thus, finding a dual agonist of PPARα/γ might represent to find a strong anti-inflammatory agent acting in different targets to control systemic or local inflammation.

3.2. Tissue repair process

The term “tissue repair” is used to define restoration of the architecture and function of tissues when damaged by injuries. It includes two different processes: regeneration and replacement. “Regeneration” refers to the process where the growth of new cells completely restores the damaged sites to their normal state. The “replacement” process occurs when the markedly damaged or non-regenerable tissues are repaired by connective tissue implantation, a process known as scarring.

The tissue repair by regeneration involves three sequential stages: inflammation, proliferation and remodeling (MARTIN, 1997; SINGER; CLARK, 1999). Immediately after lesion, a local clot created by blood compounds provides

matrix to neutrophils, monocytes and macrophages influx, characterizing the inflammatory stage. Initially, a dynamic flux of neutrophils, with high phagocytic capacity will remove possible infections and debris, and monocytes, to be differentiated in macrophages, part of an early inflammation state, aiming specially to protect the recent damaged site. Besides intense phagocytosis, upregulation of pro-inflammatory cytokines by macrophages, such as TNF-α, IL-6 and IL-1β is described, possibly inducing some cell types proliferation. These macrophages behavior will be extended to a later inflammation state and proliferation stage, characterized by phenotypic transitions, which will base new tissues growth. Both Interleukin-10 (IL-10), VEGF and Transforming Growth Factor-β (TGF-β) upregulation will play key roles in neovascularization, extracellular matrix (ECM) synthesis and cell differentiation and maturation between later inflammation and proliferation stages (WERNER; GROSE, 2003; MIRZA; KOH, 2014).

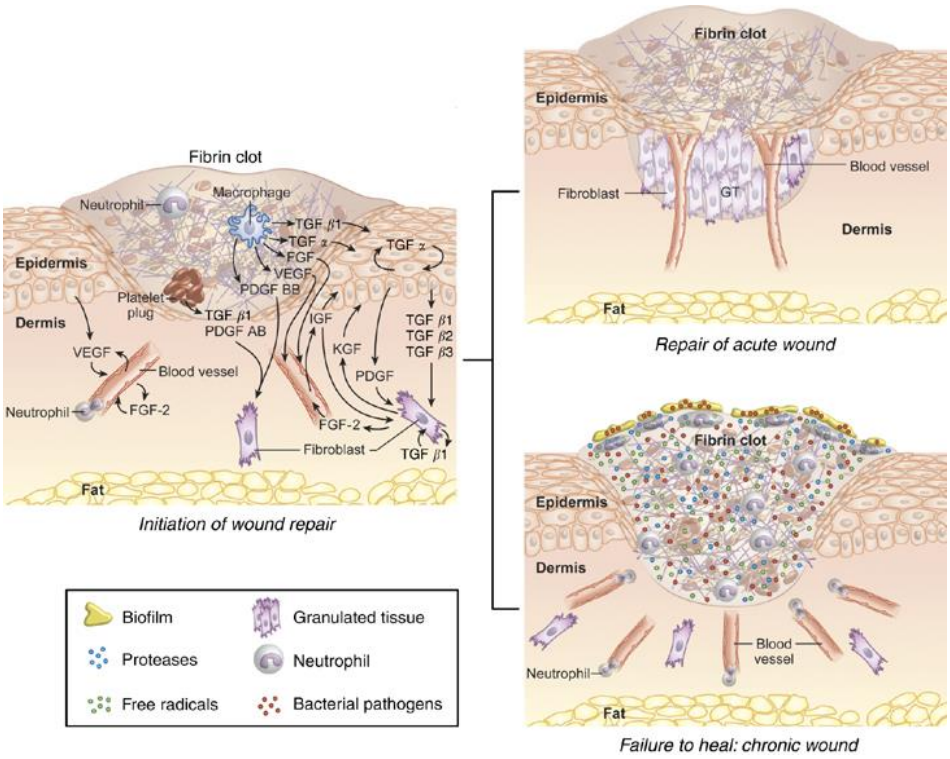

Figure 2. Cells involved in normal and deficient tissue repair process and its biomarkers (CLARK; KAUSTABH; TONESSEN, 2007).

Finally, remodeling stage is characterized by ECM collagen deposition, where the new tissue acquires traction and resistance. Balanced healing stages, including development and resolution of inflammatory process, determines tissue repair and maintenance. However, specific metabolic conditions induce cell dysfunction and exacerbated pro-inflammatory cytokines production, leading to impaired healing and delaying tissue rescue/repair, increasing the risk of complications, such as member amputations and death (SPRAVCHIKOV *et al*., 2001).

3.3. Tissue repair and inflammation in insulin resistance

Diabetes Mellitus (DM) is characterized by hyperglycemia caused by either insulin release deficiency (DM1) and/or insulin resistance (DM2), with a worldwide prevalence of 9,3% (ADA, 2016). Both genetic and environmental

factors are implicated in its incidence and etiology (RANA et al., 2007). Current DM therapies include insulin administration to DM1 patients and hypoglycemic drugs to DM2. Some therapeutic interventions aim to improve insulin sensitivity associated to anti-inflammatory and hypolipidemic effects (BERGER *et al.*, 1999; HAUNER, 2002).

In DM pathophysiology, persistent hyperglycemia might generate chains of non-enzymatic glycation – Maillard reaction – or even glucose autoxidation, resulting in reactive oxygen species (ROS) and advanced glycation end-products (AGEs) formation. AGEs constitute a variety of substances obtained from reducing sugars – capable of acting as a reducing agent due to a free aldehyde group or a free ketone group – or oxidized lipids interactions with lysine and arginine residues, amine groups from phospholipids and even nucleic acids, resulting in structure and function alterations (HORI et al, 2012). These alterations specifically contribute to important DM complications, such as retinopathies, cardiopathies, nephropathies and neuropathies (BROWNLEE et al, 1988; NORTON et al, 1996).

AGEs have direct and indirect actions through specific cell receptors in many cell types such as T cells, monocytes/macrophages, fibroblasts, smooth muscle cells and neurons. Among different AGE receptors, we can highlight RAGE – extensively characterized in oxidative stress -, AGE-R1, AGE-R2 and AGE-R3 (URIBARRI et al., 2007; VLASSARA et al., 2008).

In other hand, several reports also show that tissue repair process is impaired in insulin resistance conditions. It has been shown that skin lesions of patients with insulin resistance remain at a chronic inflammatory state of healing or have delayed transition to later healing stages. Exacerbated local cells influx,

imbalanced macrophage phenotypic transitions and increased production of pro-inflammatory mediators orchestrate an imbalance of activator and inhibitor factors, preventing ECM synthesis and remodeling, essential to normal healing process (MARHOFFER *et al*., 1992, NOVAK, *et al.*, 2013, MIRZA *et al.*, 2014). Impaired functions of neutrophils and macrophages also occur, including cell adhesion, chemotaxis, phagocytosis and cytokines production and secretion, besides dysfunctional angiogenesis (MARHOFFER *et al*., 1992). Importantly, macrophages role in diabetic tissue repair impairment shows to be substantial to new therapies development.

In early inflammatory stages, although monocytes/macrophages predominantly assume pro-inflammatory attributes, they do not seem to be fully analogues of classic M1 macrophages phenotype, assuming AG-1 upregulation – usually correlated to alternative phenotypes – besides TNF-α, IL-1 and IL-6 upregulation. Pro-inflammatory cytokines release in early inflammation is essential to efficient tissue repair when is balanced, actively participating in keratinocytes, fibroblasts and myoblasts proliferation. In this sense, appropriate pro-inflammatory cytokines release seems to be strictly involved in the ability of transition between inflammation and proliferation stages (KOH et al., 2005; BRYER & KOH, 2007).

As inflammation stage down-grade and proliferation progress – characterized more by differentiation and cell maturation than proliferation per se – macrophages acquire a new cytokine expression profile of anti-inflammatory and growth factor features, such as IL-10, TGF-β and even Insulin-like Growth Factor-1 (IGF-1) (KOH et al., 2005). Some studies show that cytokines expression regulation are mediated not only by PPAR but also by exposure to

already released cytokines (ARNOLD et al., 2007). Cell maturation and collagen production, particular from proliferation stage, are mainly controlled through TGF-β expression (BRYER & KOH, 2007). In remodeling stage, macrophages function does not seem to be very clear, in spite of TGF-β expression support and its relation to fibrosis (MIRZA & KOH, 2011).

In diabetes-related impaired processes, macrophages sustain a pro-inflammatory phenotype, prolonging inflammation stage, with exacerbated TNFα and IL-1β release, contributing to cell death and tissue damage, consequently arresting the stage transition to cell proliferation. Among the many factors implicated in inflammation resolution, PPARs modulate inflammation by binding to responsive elements, activating or inhibiting anti-inflammatory genes expression. It is known that PPARγ activity promotes expression of genes related to mitochondrial biogenesis, oxidative stress metabolism and alternative activation in macrophages (LERKHE & LAZAR, 2005). Some studies using mice with macrophage specific PPARγ-/- showed prolonged inflammation in tissue repair (MIRZA et al., 2015)

Therefore, the use of drugs that control expression and/or secretion of pro-inflammatory cytokines and chemokines involved in inflammatory stage of healing process – as PPAR agonists - is an interesting therapeutic approach.

3.4. Ischemia/reperfusion (IR), inflammation and oxidative stress.

Ischemia-Reperfusion (I/R) is defined as a pathological condition characterized by restriction of blood supply followed by flow restoration and subsequent re-oxygenation, representing the major challenge during organ transplantation and general cardiothoracic surgery (ELTZCHIG & ECKLE, 2011).

In its pathophysiology, not only the initial restriction of blood supply leads to a severe imbalance of metabolic demand but also the blood flow restoration exacerbates tissue injury and inflammation responses, contributing to a wide range of conditions (RYAN *et al*., 2011; ELTZCHIG & CARMELIET, 2011).

Metabolic substrates such as glucose and oxygen are necessary for mitochondrial ATP production. Conditions with deficient oxygen concentration and reduced aerobic glycolysis, cells switch to anaerobic metabolism, resulting in production of lactic acid following reduced cytosolic pH. An acid microenvironment within the cytosol helps cells survive to ischemia. Time is an essential component in reaching this imbalanced state and varies depending on the tissue. Cardiac cells can tolerate 20 minutes of ischemia before necrosis and hepatocytes or renal cells more than 30 minutes, while neuronal cells can tolerate no more than 20 minutes. Some tissues, such as skeletal muscle cells can tolerate 2 hours of ischemia (JENNINGS, 2013; ZHAO, 2009; ZHAO *et al*., 2003).

Ischemia condition is also associated to the inhibition of oxygen-sensing prolylhyldroxylase (PHD) and paraoxonase (PON) enzymes, once they require oxygen as a cofactor, which in turn leads to a post-translational activation of hypoxia and inflammatory signaling cascades, controlling transcription factors such as hypoxia-inducible factor (HIF) and NF-κb, sequentially inducing IL-6 and TNF-α (SEMENZA, 2007, KARHAUSEN *et al*., 2004; KAELIN & RATCLIFFE, 2008). Particularly, at this point it is reported increased permeability of capillaries and arterioles, following increased diffusion and fluid filtration across the tissues by activated endothelial cells, associated by itself to increased ROS and decreased Nitric Oxide (NO) production (RODRIGUEZ-LARA *et al*., 2016; POWERS *et al*., 2006).

In response to tissue damage, exacerbated free radicals release, oxidative stress induction as well as pro-inflammatory cytokines expression and activation of cell death programs are carried by reperfusion after ischemia. Therefore, the critical points of I/R are inflammation, headed by TNF-α and IL-6, and oxidative stress, headed by production of pro-oxidant elements, depletion of Superoxide Dismutase (SOD), accumulation of free radicals and redox signaling disruption (BOUWMEESTER T *et al*., 2004; HOUSENLOY DJ & YELLON DM, 2006).

The increase of cytokines/inflammatory mediators is followed by a substantial increase of leukocytes, neutrophils and macrophages recruitment besides important expression increase of ICAM-1 in endothelial cells. Finally, I/R damage causes cell necrosis by cytoskeletal proteolysis and membrane lipoperoxidation-induced structural lesions (ABDUL-HUSSIEN et al., 2010).

PPARγ agonists have been related to I/R protective effects through inhibition of intracellular cell adhesion molecules expression, reduction in neutrophil and macrophages infiltration, modulating inflammatory response and subsequent reduction of oxidative stress in endothelium, playing the main role in inflammatory process modulation during reperfusion (PANE *et al*., 2017). Consistently, previous studies also showed anti-inflammatory effects promoted by GQ-11, through TNF-α, CCL-2 and IL-1β modulation in obese mice (SILVA *et al*., 2018). Evidences suggest that IL-1β plays a key role in pathophysiology of hepatic I/R injury, some of them attesting that neutrophils and macrophages can contribute to the cytokine maturation, independently of inflammasome (SADATOMO *et al*., 2017).

Many studies support inflammation and oxidative stress as interdependent and connected processes, which co-exist in inflamed sites. Here we understand

that inflammatory conditions induce ROS formation, leading to exacerbated oxidative damage. At the same time, increased ROS and oxidative stress products enhance pro-inflammatory responses. Therefore, regulation of both processes are important targets in the search for I/R treatments. Some studies recommend aggressive use of nonsteroidal anti-inflammatory drugs in these cases. However, prolonged use of these drugs is related to cardiovascular morbidity and mortality (JENNINGS, 2013; ZHAO *et al*., 2003). Moreover, commercial TZDs are related to important side effects – as discussed before - suggesting that alternative PPAR agonists could be important mediators in these conditions.

4. CHAPTER I: "GQ-11: A new PPAR agonist improves obesity-induced metabolic alterations in LDLr$^{-/-}$ mice".

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ARTICLE

GQ-11: A new PPAR agonist improves obesity-induced metabolic alterations in LDLr$^{-/-}$ mice

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Abstract

Background Obesity and insulin resistance/diabetes are important risk factors for cardiovascular diseases and demand safe and efficacious therapeutics.

Objective To assess the effects of a new thiazolidine compound—GQ-11—on obesity and insulin resistance induced by a diabetogenic diet in LDL receptor-deficient (LDLr$^{-/-}$) mice.

Methods Molecular docking simulations of GQ-11, PPARα and PPARγ structures were performed. Male C57BL/6J LDLr$^{-/-}$ mice fed a diabetogenic diet for 24 weeks were treated with vehicle, GQ-11 or pioglitazone or (20 mg/kg/day) for 28 days by oral gavage. Glucose tolerance test, insulin, HOMA-IR, adipokines (leptin, adiponectin) and the lipid profile were assessed after treatment. Adipose tissue was analysed by X-ray analysis and morphometry; gene and protein expression were evaluated by real-time PCR and western blot, respectively.

Results GQ-11 showed partial agonism to PPARγ and PPARα. In vivo, treatment with GQ-11 ameliorated insulin sensitivity and did not modify subcutaneous adipose tissue and body weight gain. In addition, GQ-11 restored adipokine imbalance induced by a diabetogenic diet and enhanced *Glut-4* expression in the adipose tissue. Improved insulin sensitivity was also associated with lower levels of MCP-1 and higher levels of IL-10. Furthermore, GQ-11 reduced triglycerides and VLDL cholesterol and increased HDL-cholesterol by upregulation of *Apoa1* and *Abca1* gene expression in the liver.

Conclusion GQ-11 is a partial/dual PPARα/γ agonist that demonstrates anti-diabetic effects. Additionally, it improves the lipid profile and ameliorates chronic inflammation associated with obesity in atherosclerosis-prone mice.

Introduction

Obesity, along with insulin resistance, represents one of the biggest issues in public health worldwide [1]. Societal changes and the global nutrition transition in addition to less physical activity have driven the increase in adiposity in the obesity epidemic [2]. For a long time, adipose tissue was known simply as an energy storage site, but more recently, its endocrine function has been well established and characterized by the secretion of adipokines, which affect several biological processes in the adipose tissue and distant organs, such as the liver, brain and heart. In particular, adipokines modulate insulin sensitivity, metabolic regulation and inflammation [3, 4].

Inflammation is considered a hallmark of obesity. Several studies in experimental models and human populations demonstrate that chronic, low-grade inflammation characterized by increased pro-inflammatory cytokines is

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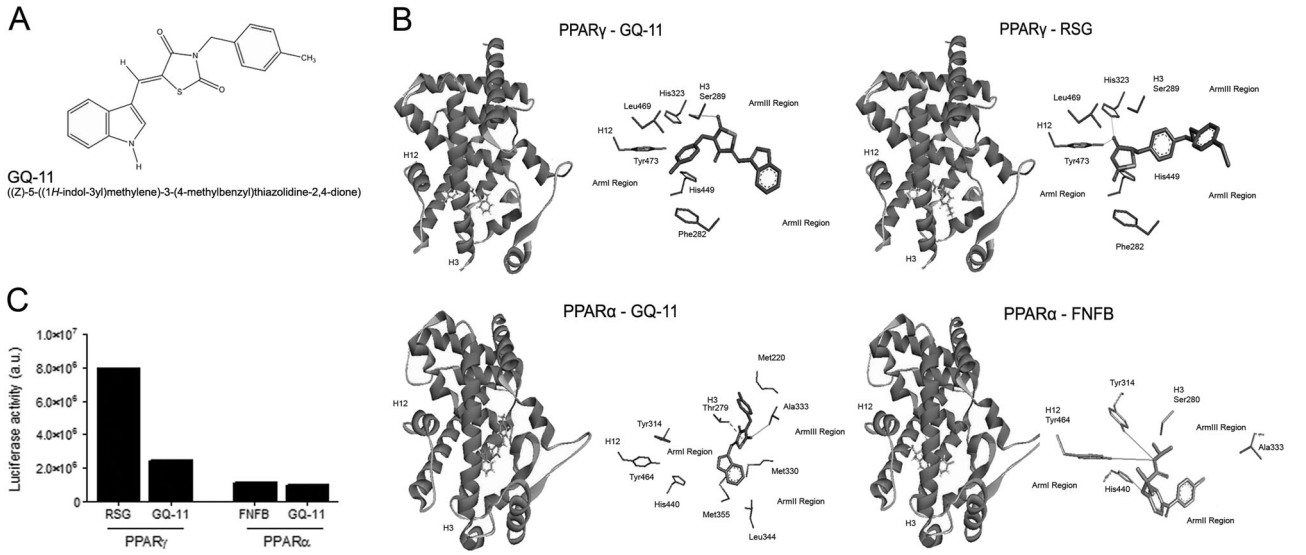


Fig. 1 GQ-11 is a partial/dual PPAR agonist that shows distinct binding interactions with PPARα and PPARγ. **a** Chemical structure of GQ-11. **b** Molecular docking studies of GQ-11 to PPARγ and PPARα. Docking simulation was performed with 3D structures of PPARγ and PPARα (Protein Data Bank under ID 4CI5) in GOLD 5.1 software aiming to predict the binding mode of GQ-11 and compared to RSG and FNFB. **c** In vitro activation of PPARγ and PPARα by GQ-11. RSG or FNFB was used as classical PPARγ and PPARα, respectively. PPARS activation was evaluated by luciferase reporter assay using the human cell line HEK-293. RSG Rosiglitazone, FNFB fenofibrate

associated with insulin resistance [3–5]. Notably, pro-inflammatory cytokines, such as the chemokine monocyte chemotactic protein-1 (MCP-1), are upregulated in adipose tissue by a high-fat diet prior to the development of systemic insulin resistance [6]. Thus, since inflammation in adipose tissue likely contributes to systemic inflammation and metabolic abnormalities induced by obesity, it might represent an important target for pharmacological approaches to treat obesity and insulin resistance.

Thiazolidinedione (TZDs) drugs are insulin-sensitizing agents commonly used for the treatment of type 2 diabetes. These drugs are synthetic ligands of peroxisome proliferator-activated receptors γ (PPARγ), which are ligand-activated nuclear receptors involved in the regulation of lipid and glucose metabolism [7]. Indeed, activation of PPARs affects the expression of target genes such as *adiponectin* and *apoA1* and increases glucose transporter type 4 (GLUT-4) translocation to the cell membrane [8], promoting adipose and whole-body homeostasis of fatty acids and triacylglycerols, glucose control and insulin sensitivity [9]. In addition to these effects on lipid and glucose metabolism, TZDs also show anti-inflammatory properties in diabetic patients [10, 11]. However, long-term use of TZDs might have important adverse effects, mainly weight gain and bone loss, which has stimulated the search for new TZDs with preserved efficacy and minimal side effects [12–14]. Therefore, the aim of this study was to investigate the pharmacological potential of GQ-11, a new thiazolidine compound, as a novel anti-diabetic drug candidate in a diet-induced obesity (DIO) model using low-density lipoprotein receptor-deficient ($LDLr^{-/-}$) mice.

Material and methods

Materials

GQ-11 [(Z)-5-((1*H*-indol-3-yl)methylene)-3-(4-methylbenzyl)thiazolidine-2,4-dione] (Fig. 1a) was synthesized as previously described [15] in the Laboratory of Drug Design and Synthesis of the Federal University of Pernambuco (Recife, Pernambuco, Brazil). Synthesis details are provided in the Supplementary Information (SI—1.1). Rosiglitazone (RSG) was obtained from Cayman Chemical (Ann Arbor, MI, USA) and fenofibrate (FNFB) and pioglitazone (PIO) were purchased from Sigma-Aldrich (St Louis, MO, USA). Tissue culture media, serum, supplements and other reagents were purchased from Life Technologies (Carlsbad, CA, USA) unless otherwise stated.

Molecular modelling

Molecular docking studies were carried out using GOLD 5.1 software [16] aiming to predict the binding mode of GQ-11 and RSG for comparison. The 3D structures of PPARγ and PPARα used in this study were obtained from the Protein Data Bank under ID 4CI5 [17]; they were prepared by removing crystallographic ligand and water molecules and adding hydrogen atoms. The binding site was defined as 5 Å around a crystallographic ligand. A genetic algorithm was employed to generate 100 poses for each studied ligand, and all torsions and ring flipping were allowed in pose calculations. GOLDScore was used as the

scoring function in order to rank the most suitable poses, and the highest ranked pose inside the most populated cluster (poses were clustered according to default RMSD values) was selected in order to analyse the most representative binding mode.

Transcriptional transactivation assay

The detailed transcriptional transactivation assay is described in the Supplementary Information (SI—1.2); pCMX expression plasmids for PPARα and PPARγ and the minimal promoters containing multiple binding sites for PPAR and the GAL4 response element (UAS-LUC) (kindly provided by Dr. A. Castrillo, Instituto de Investigaciones Biomédicas Alberto Sols, Consejo Superior de Investigaciones Científicas-CSIC) were used [18].

Animals and treatment

Male homozygous C57BL/6J LDLr$^{-/-}$ mice (22–25 g at the beginning of the study) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The study protocols were approved by the Ethics Committee for Animal Studies of the Faculty of Pharmaceutical Sciences, University of São Paulo (Protocol Number: 377/2012) and followed the rules of the Guide for the Care and the Use of Laboratory Animals [19] published by the US National Institutes of Health (NIH Publication No. 85-23, updated in 2011). The mice were maintained in plastic cages at 22 °C on a 12 h light–dark cycle and given free access to food and water during all the experiments. The mice were fed a diabetogenic diet containing 36% fat and 36% carbohydrate for 24 weeks, as previously described [20]. After this period, mice were randomly allocated into three groups ($n = 6$ per group—no randomization method was used) and received oral treatment with vehicle (NaCl 0.9 and 0.025% Tween 20, control group), PIO (20 mg/kg/day) or GQ-11 (20 mg/kg/day) for 4 weeks. Sample size calculation was based on evidence from previous preclinical studies for testing TZDs anti-diabetic effects [20–22]; no sample size calculations were performed. Glucose tolerance tests and HOMA-IR were performed after 4 weeks of treatment, as previously described [23, 24]. Detailed methods are described in Supplementary Information (SI—1.3 and 1.4) as well as the diabetogenic diet (SI, Table 1).

Determination of bone mineral density and subcutaneous adipose tissue

Mice were anesthetized with 3% isoflurane in oxygen, placed in a prone position into MSFX-Pro equipment (Bruker BioSpin Corporation, Billerica, MA, USA) and then subjected to a whole-body scan using dual-energy X-ray absorptiometry. Images were obtained in accordance with the settings described (SI, Table 2). Bone mineral density analysis was performed on images obtained perpendicularly to the left femur at a specific region drawn between the femur and hip. Bone density was calculated using an algorithm provided by the Bruker Molecular Imaging software.

Biochemical determinations

Blood was collected by cardiac puncture and serum was separated by centrifugation. Triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were determined using commercial kits (Labtest, Lagoa Santa, MG, Brazil). As previously described [25], very low-density lipoprotein cholesterol (VLDL-C) levels were estimated by the Friedewald formula [26]. Glucose concentrations were measured using Accu-Chek Performa (Roche Diagnostics Corporation, Indianapolis, IN, USA). Serum interleukin-10 (IL-10) and MCP-1 were measured by flow cytometry using the Cytometric Bead Array (CBA) inflammatory kit (BD, East Rutherford, NJ, USA). Serum adipokines and insulin were measured by enzyme-linked immunosorbent assay (ELISA) with commercial kits at the end of the treatment period (EMD Millipore Mouse ELISA Kit, Billerica, MA, USA).

Adipocyte size measurements

Subcutaneous adipose tissue was collected after euthanasia, fixed for 24 h in 4% formalin solution, paraffin-embedded and sectioned. The sections were stained with haematoxylin and eosin. Adipocytes were analysed/measured with ImageJ software (NIH-Fiji, Bethesda, MD, USA) and Adiposoft plugin (University of Navarra, Pamplona, Spain) in six representative images per mouse.

Quantitative real-time PCR analysis

Total RNA was isolated from murine epididymal adipose tissue (eWAT) and liver using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and 1 µg of RNA was reverse-transcribed into cDNA using the high-capacity cDNA reverse transcription kit, according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR was performed in an ABI 7500 Fast Real-Time PCR using SYBR green master mix (Applied Biosystems, Foster City, CA, USA) and specific primer pairs as listed (SI, Table 3). Expression levels of each target gene were normalized to *Rpl-4* rRNA relative expression as internal efficiency controls. The mRNA fold change was calculated using the $2^{(-\text{Delta Delta } C(t))}$ method

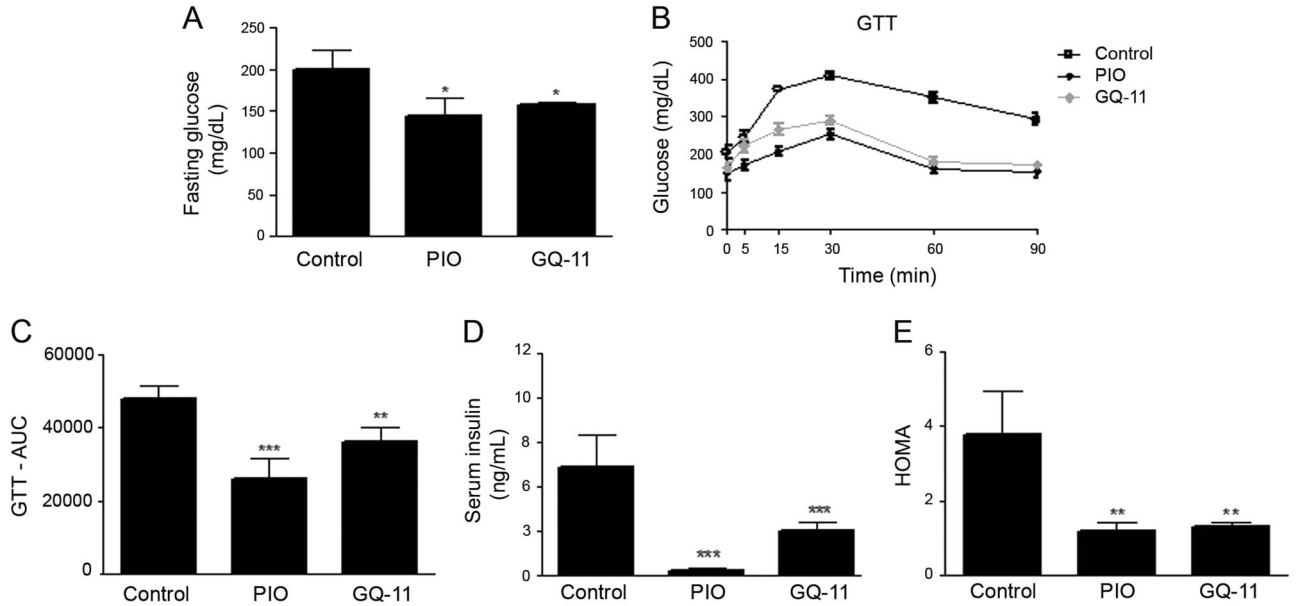


Fig. 2 GQ-11 ameliorates insulin resistance in LDLr$^{-/-}$ mice with diet-induced obesity. After 20 weeks of a diabetogenic diet, low-density lipoprotein receptor-deficient (LDLr$^{-/-}$) mice were treated by oral gavage with vehicle (0.9% NaCl and 0.025% Tween 20), pioglitazone (PIO, 20 mg/kg/day) or GQ-11 (20 mg/kg/day) for 28 days, and insulin-mediated glucose homeostasis was assessed. **a** Fasting glucose, **b** glucose tolerance test (GTT), **c** area under the curve (AUC) of GTT, **d** serum insulin levels, **e** HOMA-IR analysis for insulin resistance. Data are expressed as mean ± S.D. of six mice per group. Statistical analyses were performed using ANOVA/Tukey's multiple comparison tests. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ vs. control group (vehicle treatment). PIO pioglitazone

[27], and the values are expressed as fold increases relative to the control group.

Western blot

The total protein of eWAT was extracted with RIPA buffer (Sigma-Aldrich, St Louis, MO, USA) with a protease, proteasome and phosphatase inhibitor cocktail. Next, 20 µg of total protein was resolved by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane using the Bio-Rad transfer system (Bio-Rad, Richmond, CA, USA). The membranes were blocked with 5% non-fat-milk in TBST for 2 h at room temperature with gentle shaking and incubated overnight at 4 °C with primary antibodies against IL-10, MCP-1 (Abcam, Cambridge, UK, 1:1000 #AB189392 and 1:2000 #AB7202, respectively), and β-actin (Sigma-Aldrich, St Louis, MO, USA, 1:40,000). After washing, membranes were incubated with a secondary antibody (sheep anti-rabbit IgG 1:3000, horseradish peroxidase-conjugated; Amersham Biosciences, Piscataway, NJ, USA). Membranes were developed using enhanced chemiluminescence reagents (Bio-Rad, Richmond, CA, USA).

Statistical analyses

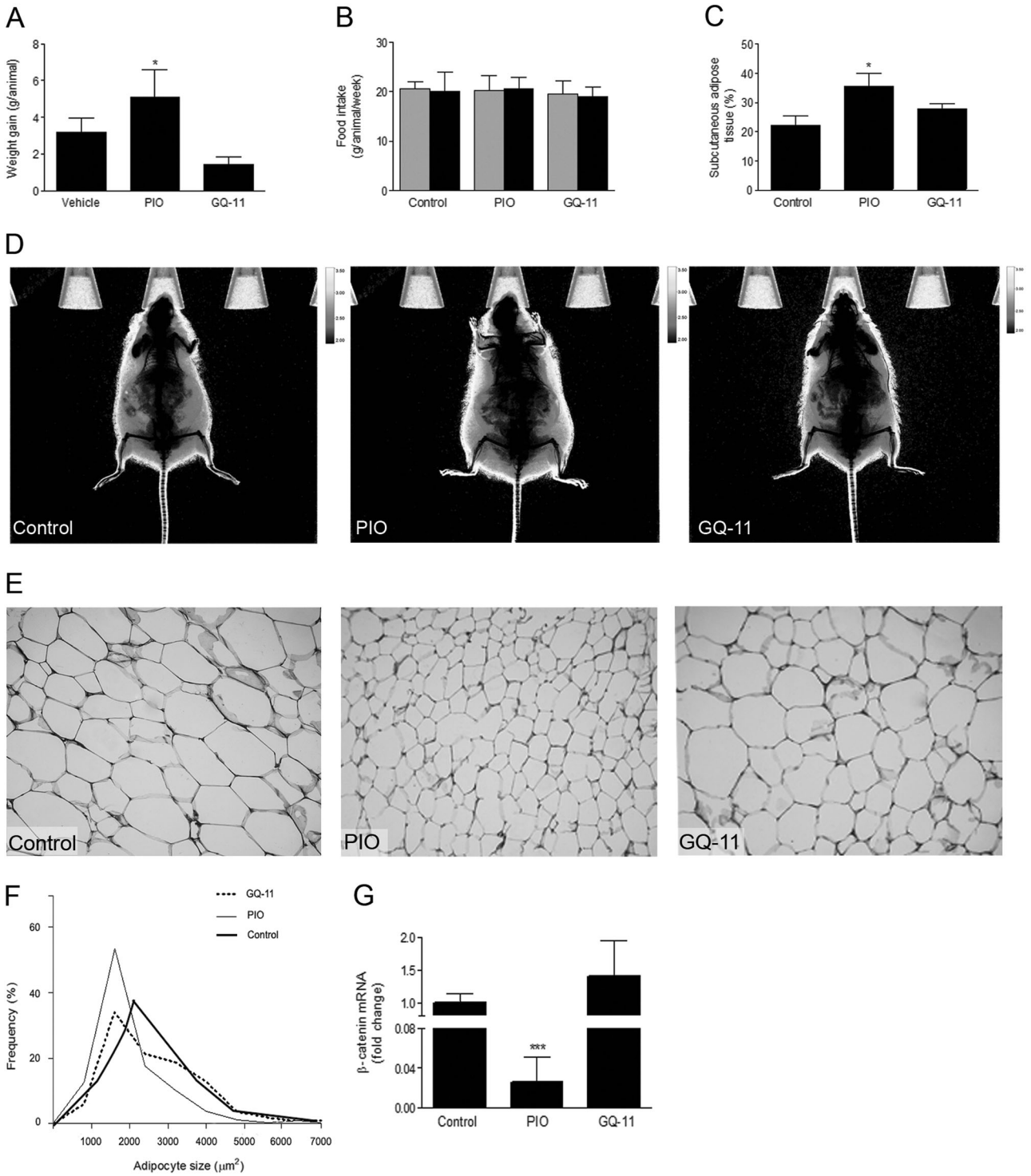
Statistical analyses were performed using GraphPad Prism software, version 5.0. One-way analysis of variance (ANOVA) followed by Tukey's test was used to calculate statistical significance as appropriate. All data in this study are expressed as mean ± standard deviation (S.D.). P values < 0.05 were considered significant. No blinding was adopted for analysis of the results.

Results

GQ-11 is a partial/dual PPAR agonist that shows distinct binding interactions with PPARα and PPARγ

To explore if GQ-11 might interact with and activate PPARs, a docking study was conducted, and the interactions of GQ-11 with PPARs were compared to RSG and fenofibrate. As illustrated in Fig. 1b, while RSG forms hydrogen bonds with PPARγ polar residues His323 and Tyr473, GQ-11 interacts with the hydrophobic residues Phe282 and Leu469 of PPARγ arm I and forms a hydrogen bond with Ser289. Furthermore, we assessed the binding affinity of GQ-11 for PPARα. Our in-silico docking simulations demonstrated that GQ-11 interacts with PPARα at the H3 region via a non-classic hydrogen bond—a hydrogen bond with Ala333—and forms additional hydrophobic interactions with Met330, Leu344 and Met355 of the Arm II region and with Met220 of the Arm III region.

Next, we evaluated whether GQ-11 stimulated the transcriptional activity of PPARs using a gene reporter



assay with HEK-293 cells. Compared to RSG treatment, GQ-11 induced weak transactivation of PPARγ (Fig. 1c), indicating that GQ-11 is a partial agonist of PPARγ. Similar results were obtained with PPARα and confirm that GQ-11 exhibited weak PPARγ and PPARα agonistic activities.

GQ-11 improves hyperglycaemia and insulin sensitivity in a mouse model of DIO

As TZDs are mainly used for the treatment of insulin resistance, we studied the effects of GQ-11 on insulin-mediated glucose homeostasis using a mouse model of

◀ **Fig. 3** Effects of GQ-11 treatment on body weight, food intake and adiposity in LDLr$^{-/-}$ mice with diet-induced obesity. Low-density lipoprotein receptor-deficient (LDLr$^{-/-}$) mice received a diabetogenic diet for 20 weeks and were subsequently treated by oral gavage with vehicle (0.9% NaCl and 0.025% Tween 20), pioglitazone (PIO, 20 mg/kg/day) or GQ-11 (20 mg/kg/day) for 28 days. **a** Body weight gain was measured at the end of the study protocol. **b** Food intake was determined weekly, and the graph shows food consumption at the beginning (grey bars) and the end of the study protocol (black bars). **c** Subcutaneous adipose tissue was evaluated by dual-energy X-ray absorptiometry at the end of the experiment. **d** Representative images obtained by X-ray for the control, PIO and GQ-11 treatments showing delimitation areas of subcutaneous adipose tissue (**e**). Representative HE–stained images of subcutaneous adipocytes (magnification ×4). **f** Adipocyte size was measured using ImageJ/Adiposoft. **g** β-Catenin mRNA expression in eWAT was evaluated by real-time PCR. Data are expressed as mean ± S.D. of six mice per group. Statistical analyses were performed using ANOVA/Tukey's multiple comparison tests. *$P < 0.05$ and ***$P < 0.001$ vs. control group (vehicle treatment). PIO pioglitazone

DIO, which was previously described and validated [20]. Similar to treatment with PIO, GQ-11 ameliorated insulin resistance compared to control mice, as indicated by decreased fasting glucose (Fig. 2a), improved glucose tolerance (Fig. 2b, c) and lower serum insulin levels after treatment period (Fig. 2d). Accordingly, lower HOMA-IR values were also observed in PIO and GQ-11-treated mice (Fig. 2e), supporting the hypothesis that GQ-11 has anti-diabetic properties.

GQ-11 does not increase body weight and adiposity

Considering that increase in body weight is an important adverse effect reported with TZD use, we analysed the effects of GQ-11 on body weight gain and adiposity after 28 days of treatment. Fig. 3a shows that PIO-treated mice gained more weight than did those mice treated with GQ-11. Food intake was not altered by either PIO or GQ-11 treatments (Fig. 3b), indicating that body weight gain was not caused by changes in food consumption. Notably, while PIO treatment was also associated with an increase in subcutaneous adipose tissue, GQ-11 did not modify adipose mass compared to the control group (Fig. 3c). This finding is illustrated by X-ray imaging of adipose tissue (Fig. 3d). The morphometric analysis of the subcutaneous adipose tissue revealed smaller adipocytes with PIO treatment (Fig. 3e, f). In contrast, as showed by HE staining and size analysis, no differences were observed between the GQ-11 and control groups, indicating that GQ-11 did not impact adipocyte morphology or adipose tissue expansion.

TZDs can disrupt the Wnt/β-catenin pathway leading to decreased β-catenin expression, which has been associated with increased adipogenesis [28]. Thus, we examined the expression of the β-catenin gene in the eWAT of obese mice. In fact, PIO down-regulated β-catenin mRNA expression, but no differences in *β-catenin* mRNA levels were observed between the GQ-11 and the control groups (Fig. 3g), which suggests that PIO interferes in the Wnt/β-catenin pathway. Considering that disruption of this pathway by PPARγ agonists can also affect osteogenesis, the bone density of treated mice was evaluated by X-ray. We found that GQ-11 did not affect bone density values whereas a decline in bone density was detected with PIO treatment (Fig. S1).

GQ-11 alters the levels of adipokines and *Glut-4*

To explore whether the improvements in insulin sensitivity are associated with changes in the adipokines, we examined the effects of GQ-11 on adiponectin and leptin levels. As shown in Fig. 4a, b, GQ-11 increased adiponectin mRNA expression in eWAT what was accompanied by elevated adiponectin levels in blood serum. In contrast, leptin transcription was suppressed by both TZDs in adipose tissue and decreased in blood serum. Alterations in *Glut-4* gene expression can also affect not only adipose tissue but also systemic insulin sensitivity. For this reason, we assessed whether GQ-11 might affect *Glut-4* expression in adipose tissue. As shown in Fig. 4a, GQ-11 and PIO upregulated *Glut-4* gene expression in eWAT. Thus, TZDs regulate expression of adipokines and *Glut-4* at the transcriptional level, which results in an increase in serum levels of adiponectin, with a concomitant reduction in leptin and ultimately reflects in improved whole-body insulin sensitivity.

GQ-11 ameliorates chronic inflammation associated with obesity

To study the effects of GQ-11 on the inflammatory state of obese mice, we analysed the mRNA and protein levels of anti- and pro-inflammatory cytokines in eWAT as well as their systemic levels. Fig. 5a, b show that both mRNA and protein levels for IL-10 were elevated in adipose tissue under GQ-11 treatment. In addition, MCP-1 transcription and protein expression were down-regulated in adipose tissue from GQ-11-treated mice compared to controls. Moreover, the serum concentration of MCP-1 was lower whereas IL-10 levels were higher under GQ-11 treatment (Fig. 5c) in relation to controls. Conversely, no significant effects were observed with PIO treatment. Therefore, these data suggest that, unlike PIO, GQ-11 can ameliorate the local and systemic inflammation induced by obesity.

GQ-11 improves the lipid profile in obese mice

As insulin resistance is associated with dyslipidaemia, we also investigated the lipid profile of mice treated with GQ-11. As illustrated in Fig. 6a, b, both PIO and GQ-11

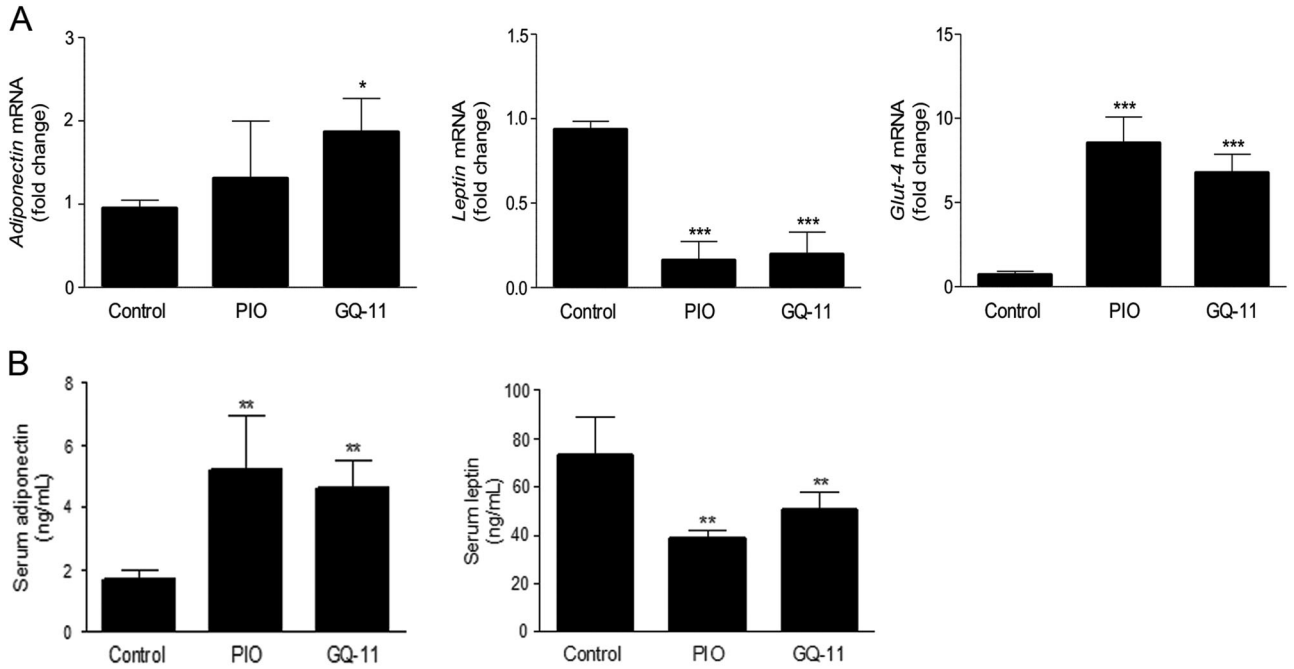


Fig. 4 GQ-11 induces adiponectin and *Glut-4* expression and down-regulates leptin in LDLr$^{-/-}$ mice with diet-induced obesity. **a** *adiponectin, leptin* and *Glut-4* mRNA expression were quantified by real-time PCR. **b** Levels of adiponectin and leptin were assessed in blood serum by ELISA. Data are expressed as mean ± S.D. of six mice per group. Statistical analyses were performed using ANOVA/Tukey's multiple comparison tests. *$P < 0.05$ **$P < 0.01$ and ***$P < 0.001$ vs. control group (vehicle treatment). PIO pioglitazone

treatments exerted no effect on total cholesterol and LDL-cholesterol, whereas both induced a decrease in VLDL cholesterol. Moreover, triglycerides were also decreased in the blood plasma of GQ-11-treated mice (Fig. 6a). Importantly, a significant increase in HDL-cholesterol was induced by GQ-11 in contrast to PIO that decreased HDL-cholesterol by 27.5% compared with the control (Fig. 6b).

To gain insight into the underlying mechanisms of increased HDL-cholesterol induced by GQ-11, we further investigated the mRNA expression of genes involved in lipid metabolism in the liver. Unlike PIO, GQ-11 upregulated the mRNA levels of apolipoprotein A-I (*Apoa1*), ATP-binding cassette transporter (*Abca1*) and class B scavenger receptor type 1 (*Srb1*) (Fig. 6c, d). No changes were observed in *Abcg8* transcript levels (Fig. 6d). These findings suggest that GQ-11 might interfere with HDL biogenesis or clearance.

Discussion

In this study, we demonstrate that GQ-11, a novel thiazolidinedione, is a weak dual agonist of PPARγ and PPARα with anti-diabetic properties. GQ-11 improved important metabolic alterations provoked by DIO, such as insulin resistance, inflammation and dyslipidaemia. Our data also indicate that treatment with GQ-11 does not cause further increases in body weight gain and adiposity. Thus, our findings support the notion that GQ-11 is a potential novel drug candidate for the treatment of obesity-related metabolic complications.

It has been proposed that the anti-diabetic effect of PPARγ agonists could be mediated through improvements in the adipokine imbalance induced by obesity—especially on leptin and adiponectin levels [29, 30]. Consistent with other TZDs drugs, GQ-11 showed glucose-lowering and insulin-sensitizing properties which were associated with an increase of adiponectin and decrease of leptin levels. Additionally, GQ-11 glucose-lowering effects can be also mediated by the upregulation of *Glut-4* expression in adipose tissue, which is a key regulator of whole-body glucose homeostasis [31] and a target of TZDs effects [8, 32].

Although GQ-11 presents similar anti-diabetic properties as compared to PIO, our data provide evidence that this novel TZD also has distinct biological effects. First, GQ-11 presented an anti-inflammatory effect, which can be related to the metabolic improvement observed with this TZD. In the adipose tissue, increased MCP-1 expression leads to exacerbated recruitment of macrophages and the induction of classical activation (M1), contributing to insulin resistance development [33]. In contrast, IL-10, an anti-inflammatory cytokine expressed by alternatively activated macrophages (M2), ameliorates insulin resistance [34]. Thus, by diminishing MCP-1 and increasing IL-10 levels, GQ-11 may reduce inflammation promoting greater insulin sensitivity. Second, GQ-11 improved lipid profile by

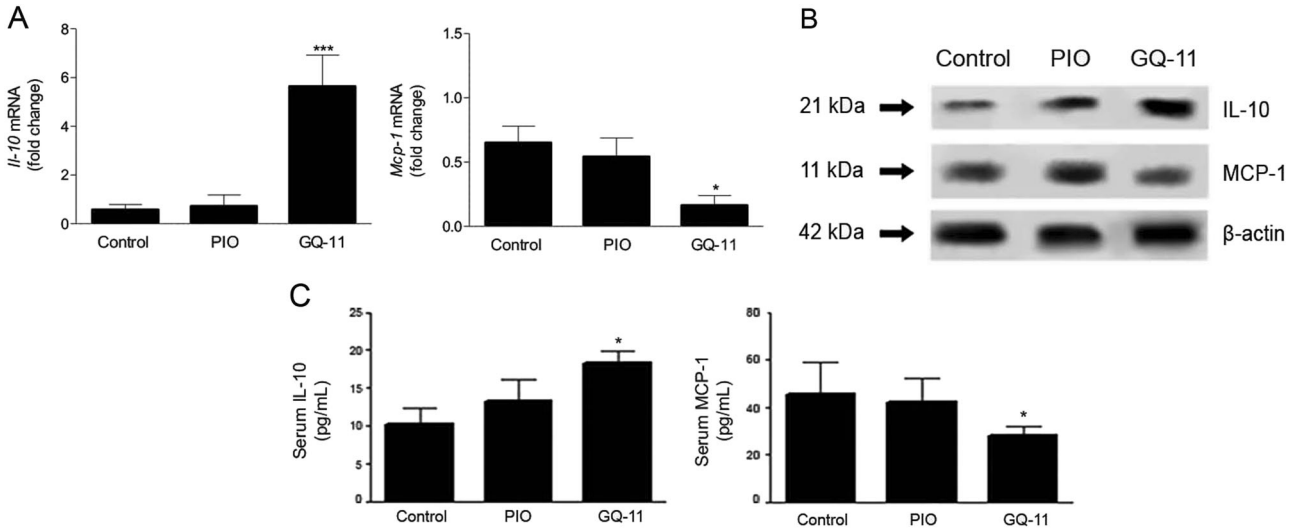


Fig. 5 Anti-inflammatory effects of GQ-11 treatment in LDLr$^{-/-}$ mice with diet-induced obesity. **a** mRNA levels of interleukin 10 (*Il-10*) and macrophage chemotactic protein-1 (*Mcp-1*) quantified by real-time PCR. **b** Representative western blot of IL-10 and MCP-1 proteins in eWAT. β-Actin was used as a loading control. **c**. Levels of IL-10 and MCP-1 were measured in blood serum by ELISA. Data are expressed as mean ± S.D. of six mice per group. Statistical analyses were performed using ANOVA/Tukey's multiple comparison tests. $^{*}P < 0.05$ and $^{***}P < 0.001$ vs. control group (vehicle treatment). PIO pioglitazone

decreasing VLDL cholesterol and increasing HDL-cholesterol levels while PIO induced a substantial decrease of HDL and increased triglycerides levels. These divergent effects of these TZDs may be, at least partially, due to their effects on hepatic lipid metabolism. Remarkably, GQ-11 upregulated mRNA levels of *apoA1*, *Abca1* and *Sr-b1* in the liver pointing to increased HDL metabolism. In line with these data, previous studies demonstrate that enhanced *Abca1* expression might predict higher secretion of cellular free cholesterol and phospholipids to apoA1, increasing HDL biogenesis [35]. In contrast, increased hepatic *Sr-b1* was previously associated with lower plasma HDL [33]. Of note, SR-B1 protein is mainly regulated at a post-transcriptional level [36, 37], which suggests that increased mRNA levels may not be reflected in enhanced SR-B1 protein expression. Although the mechanisms underlying improved lipid metabolism induced by GQ-11 remain to be determined, one plausible explanation implicates PPARα-mediated contribution, as activation of this transcription factor induces HDL synthesis and the reverse cholesterol transport besides to decrease triglycerides [38]. Considering that dyslipidaemia is a recognized risk factor for cardiovascular disorders and non-alcoholic fatty liver disease—commonly associated with obesity [39, 40]—our data indicate that GQ-11 may prevent future events and co-morbidities in obesity and type 2 diabetes by improving lipid metabolism.

It is also worth to highlight that GQ-11 did not affect body weight gain. Excessive weight gain is associated with use of classical TZDs leading to increased adiposity mainly in subcutaneous adipose tissue [41]. PPARγ is considered to be as the master regulator of adipogenesis [42, 43] and is essential for preadipocytes differentiation. Given the absence of effect on weight gain, adipocyte size or adiposity observed in GQ-11-treated LDLr$^{-/-}$ mice, our data imply that this new TZD might not affect adipogenesis. It is known that the canonical Wnt/β-catenin pathway is involved in the differentiation and proliferation of osteoblasts and adipocytes [28, 44], and its suppression, induced by PPARγ activation, may induce not only adipogenesis but also bone loss [45, 46]. Remarkably, GQ-11 preserved mouse bone density in our study, which reinforces the concept that this novel TZD may not affect differentiation of mesenchymal stem cells that can ultimately reflect in therapeutic benefits.

Finally, according to our findings, the distinct biological profile observed with GQ-11 result from the different binding interaction with PPARs. It is well established that TZDs form strong hydrogen bonds with PPARγ residues His323 (at helix 5), His449 (at helix 10) and Tyr473 (at helix 12), leading to AF2 surface stabilization and contributing to full agonism of PPARγ [46]. Consistent with other partial agonists [47], GQ-11 makes interactions with residues from helix 3 (Phe282) and helix 12 (Leu469) and only hydrogen bond with the PPARγ residue Ser289 at helix 3, which could reflect in weak PPARγ agonistic activities. Notably, GQ-11 also interacts and weakly activates PPARα. Considering that concomitant activation of PPARγ and PPARα can affect insulin sensitivity, inflammation and lipid metabolism, it is conceivable that the beneficial effects promoted by GQ-11 may, at least in part, result from its partial dual agonism.

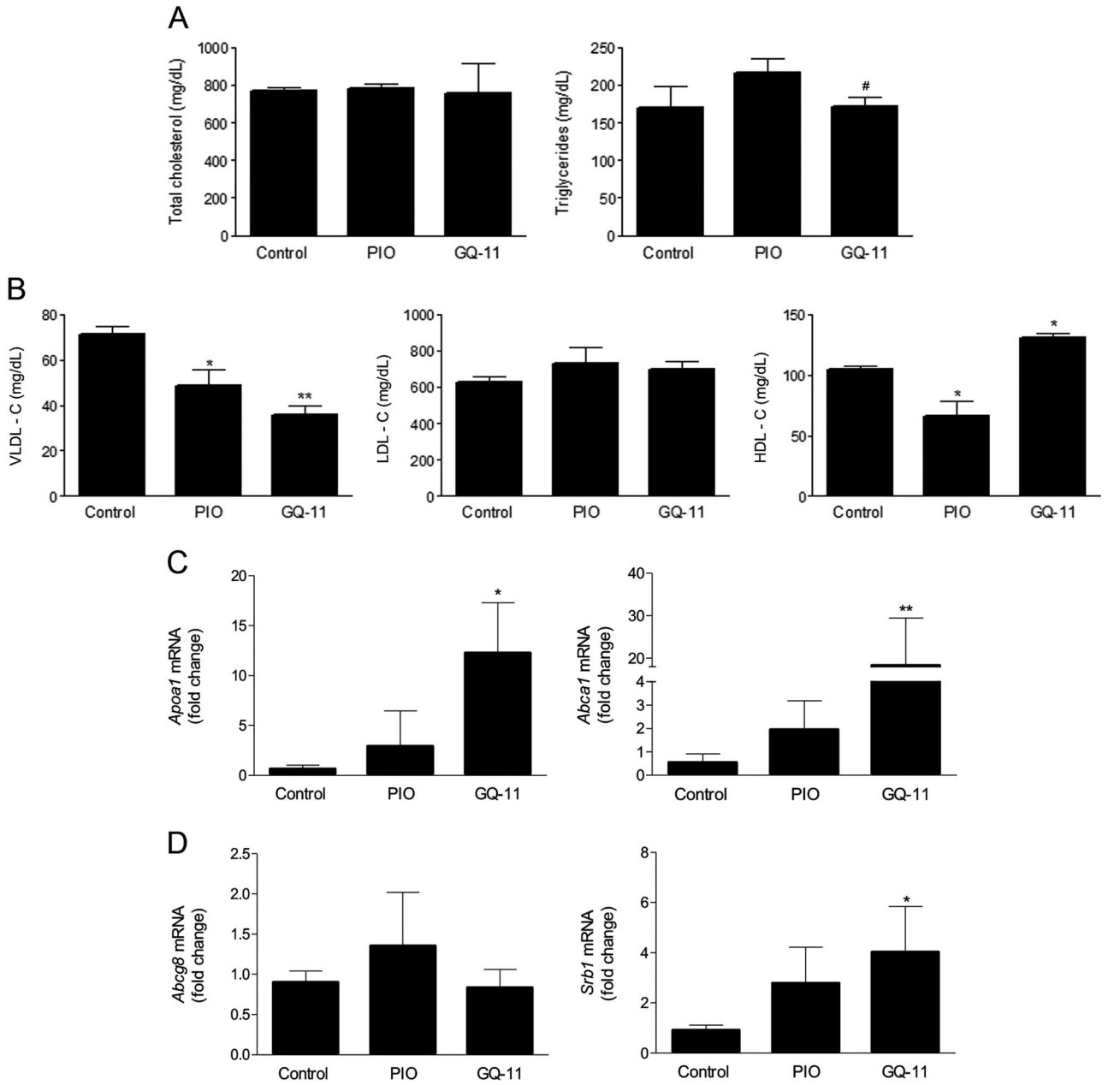


Fig. 6 Effects of GQ-11 treatment on plasma lipid profile and mRNA levels in livers of LDLr$^{-/-}$ mice with diet-induced obesity. **a** Total cholesterol and triglycerides. **b** Very low-density lipoprotein cholesterol (VLDL-C), low-density lipoprotein (LDL-C) cholesterol and high-density lipoprotein cholesterol (HDL-C). Lipid profile was evaluated in blood serum at the end of the treatments. Hepatic mRNA expression of *Apoa1*and *Abca1* **c** and *Abcg8* and *Sr-b1* **d** was evaluated by real-time PCR. Data are expressed as mean ± S.D. of six mice per group. Statistical analyses were performed using ANOVA/Tukey's multiple comparison tests. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control group (vehicle treatment) $^{\#}P < 0.05$ vs. pioglitazone

Despite the link that we observed between PPARs agonism and GQ-11 biological effects, we cannot fully exclude that GQ-11 anti-diabetic effects may also be mediated by inhibition of PPARγ phosphorylation induced by Cdk5, activated by p35/25—targets of pro-inflammatory cytokines [10]. As a consequence, its phosphorylation consists of an important link between adiposity and insulin resistance as well as obesity-related health problems [5, 48]. Previous studies report that inhibition of PPARγ phosphorylation by TZDs enhances adiponectin secretion and improves glucose tolerance [30]. Thus, additional studies determining whether GQ-11 anti-diabetic effects result from mechanisms independent of classical receptor transcriptional agonism will be crucial to expanding our knowledge of its mechanism of action.

Altogether, this study reveals that GQ-11 is a weak dual agonist of PPARγ and PPARα and presents glucose-lowering and insulin-sensitizing properties in a mouse model of DIO. The pleiotropic effects of GQ-11 on inflammation and dyslipidaemia as well as the absence of effects on body weight gain and bone density indicate that GQ-11 can present some therapeutic advantages when compared with classical TZDs. Thus, the GQ-11 effects showed here provides important insights regarding the pharmacological potential of GQ-11 as a promising novel anti-diabetic drug candidate.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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5. CHAPTER II: “New Peroxisome Proliferator-Activated Receptor Agonist (GQ-11) Improves Wound Healing in Diabetic Mice”.

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Advances in Wound Care

New Peroxisome Proliferator-Activated Receptor Agonist (GQ-11) Improves Wound Healing in Diabetic Mice

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Objective: Chronic wounds associated with diabetes are an important public health problem demanding new treatments to improve wound healing and decrease amputations. Monocytes/macrophages play a key role in sustained inflammation associated with impaired healing and local administration of peroxisome proliferator-activated receptor (PPAR)γ agonists may modulate macrophage, improving healing. In this study, we investigated the effects of GQ-11, a partial/dual PPARα/γ agonist, on macrophage function and wound healing in diabetes.

Approach: Wounds were surgically induced at the dorsum of C57BL/6J and BKS.Cg-Dock7^{m} +/+ Lepr^{db}/J (db/db) mice and treated with hydrogel (vehicle), pioglitazone or GQ-11, for 7 or 10 days, respectively. After treatment, wounds were analyzed histologically and by quantitative PCR (qPCR). In addition, bone marrow-derived macrophages (BMDM) were cultured from C57BL/6J mice and treated with vehicle, pioglitazone, or GQ-11, after challenge with lipopolysaccharide or interleukin-4 to be analyzed by qPCR and flow cytometry.

Results: GQ-11 treatment upregulated anti-inflammatory/pro-healing factors and downregulated pro-inflammatory factors both in wounds of db/db mice and in BMDM.

Innovation: Wounds of db/db mice treated with GQ-11 exhibited faster wound closure and re-epithelization, increased collagen deposition, and less Mac-3 staining compared with vehicle, providing a new approach to treatment of diabetic wound healing to prevent complications.

Conclusion: GQ-11 improves wound healing in db/db mice, regulating the expression of pro- and anti-inflammatory cytokines and wound growth factors, leading to increased re-epithelization and collagen deposition.

Keywords: wound closure, diabetes, biomarkers, cell biology

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INTRODUCTION

Prevalence of diabetes among adults >18 years of age is 8.8% worldwide and is a major cause of blindness, kidney failure, heart attacks, stroke, and lower limb amputation.[1] Chronic wounds associated with diabetes is an important health problem, affecting up to 25% of diabetic patients, and 14–24% of these wounds will lead to amputation.[2]

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Hyperglycemia and hyperlipidemia likely contribute to many of the complications associated with diabetes,[3,4] and may contribute to several characteristics of diabetic wounds: a persistent inflammatory response with prolonged monocytes/macrophages (Mo/Mp) accumulation, increased pro-inflammatory cytokine release, and impaired growth factor release as well as impaired angiogenesis and extracellular matrix.[5,6]

Mo/Mp have been established as key regulators of wound healing.[7,8] In normal wound healing, these cells adopt a pro-inflammatory phenotype in the early stages of healing that is efficient for killing pathogens and clearing the wound of damaged tissue. During the later stages of healing, they assume a pro-healing phenotype associated with the release of anti-inflammatory cytokines and growth factors that promote angiogenesis, cell proliferation/differentiation, and collagen deposition as well as wound closure. Importantly, wound macrophages in diabetic mice exhibit an impaired phenotype transition resulting in a sustained pro-inflammatory phenotype, which may be an important contributor to the persistent inflammatory response in diabetic wounds. Associated with the impaired phenotype transition are increased levels of interleukin (IL)-1β and tumor necrosis factor (TNF)-α along with decreased levels of vascular endothelial growth factor (VEGF) and transforming growth factor (TGF)-β, which likely contribute to impaired healing of wounds of diabetic mice.[5,9,10]

A number of factors have been postulated to induce the switch of macrophages from pro-inflammatory to noninflammatory and promote the resolution of inflammation.[5,9,10]

Among them, peroxisome proliferator-activated receptors (PPARs) may help to resolve inflammation by transrepression of pro-inflammatory genes and activation of anti-inflammatory genes expression in Mo/Mp and other cells.[11] Importantly, local administration of PPARγ agonists to wounds of diabetic mice has been shown to promote a noninflammatory Mp phenotype and improve wound healing, suggesting a promising approach to downregulate inflammation and improve healing in chronic wounds.[12]

CLINICAL PROBLEM ADDRESSED

Common PPARγ agonists such as thiazolidinediones (TZDs) are used to treat insulin resistance associated with diabetes, and while they induce a pleiotropic anti-inflammatory effect, they have important adverse effects, including weight gain, bone loss, and cardiovascular events,[13,14] encouraging the search for new TZDs with preserved efficacy and reduced side effects. GQ-11 is a new thiazolidine compound, exhibiting partial/dual PPARα/γ agonism with classical antidiabetic effects of TZDs, improves lipid profile and reduces chronic inflammation associated with obesity in mice with reduced side effects.[15] Therefore, the aim of this study was to determine whether GQ-11 reduces inflammation and improves wound healing in diabetic mice in basic research, providing better understanding of underlying mechanisms, improving diabetic wound healing clinical trials and purposing a new approach to diabetic wound healing treatment to prevent its complications such as amputations.

MATERIALS AND METHODS

GQ-11 synthesis

GQ-11 [(Z)-5-((1*H*-indol-3-yl)methylene)-3-(4-methylbenzyl)thiazolidine-2,4-dione] was synthesized as previously described,[15] in the Laboratory of Drug Design and Synthesis of the Federal University of Pernambuco (Recife, Pernambuco, Brazil).

Animals and treatment

Male homozygous C57BL/6J and BKS.Cg-Dock7^{m} +/+ Lepr^{db}/J (db/db) mice were purchased from Jackson Laboratory (Bar Harbor, ME) and maintained in the animal facility at College of Medicine Research Building—University of Illinois at Chicago. The study protocols were approved by the Animal Care Committee of University of Illinois at Chicago (No. 15-180) and followed the rules of the Guide for the Care and the Use of Laboratory Animals, published by the U.S. National Institutes of Health (NIH).[16] Mice were maintained in plastic cages at 22°C, 12 h light–dark cycle, and given free access to food and water during all the experiments. Sample size calculation was based on evidence from previous preclinical studies for testing TZDs antidiabetic effects.[15,17,18]

At 10 weeks of age, animals were anesthetized with isoflurane (3%), the skin shaved and cleaned with 70% alcohol, and four full thickness wounds were created on the dorsum using 8 mm biopsy punch. On day 3 postinjury, after initial inflammatory response was allowed to proceed normally, animals were randomly allocated into three treatment groups ($n = 6$/group): vehicle (F-127® Pluronic Gel, Sigma-Aldrich, Cat No. P2443, 25% in phosphate-buffered saline [PBS], 0.1% dimethyl sulfoxide [DMSO]), pioglitazone (Sigma-Aldrich, Cat No. E6910, powder, vehicle diluted, 2 mM), or GQ-11

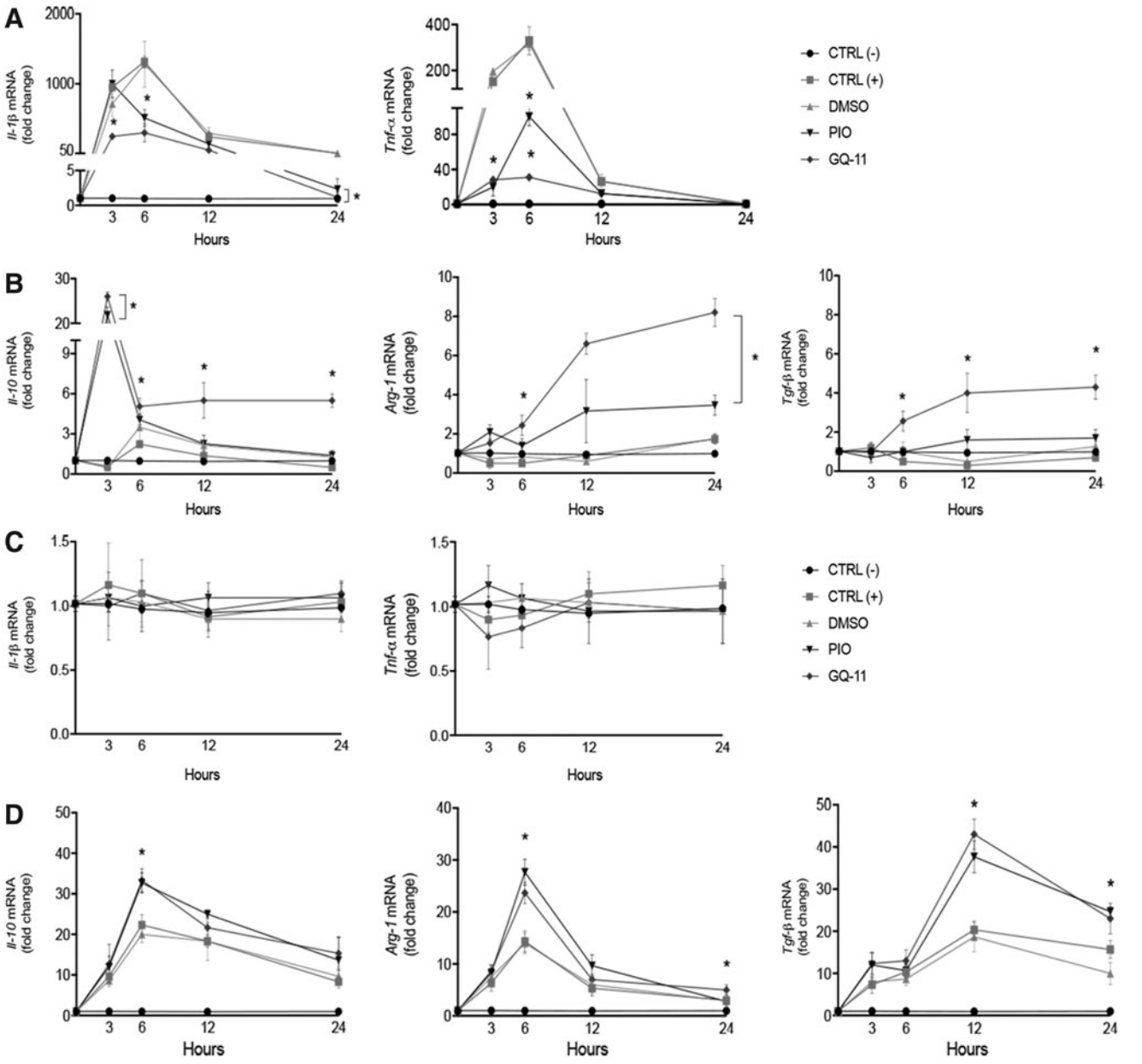

Figure 1. GQ-11 increases anti-inflammatory cytokine expression and decreases pro-inflammatory cytokine expression in BMDM. **(A)** Fold change of *Il-1*β and *Tnf-*α after challenge with LPS. **(B)** Fold change of *Il-10*, *Arg-1*, and *Tgf-b* after LPS challenge. **(C)** Fold change of *Il-1*β and *Tnf-*α after IL-4 challenge. **(D)** Fold change of *Il-10*, *Arg-1* and *Tgf-b* after IL-4 challenge. BMDM were pretreated for 24 h with vehicle (DMSO 0.01%), pioglitazone (PIO 10 μM), or GQ-11 (10 μM) and challenged with LPS (100 ng/mL) or IL-4 (20 ng/mL). mRNA expression of BMDM was quantified by qPCR. Negative controls [CTRL (–)] are represented by nontreated and nonchallenged cells. Positive controls [CTRL (+)] are represented by nontreated but challenged cells. Data are expressed as the mean ± SD of biological triplicates. Statistical analyses were performed using ANOVA/Tukey's multiple comparison tests. $*p < 0.05$. ANOVA, analysis of variance; BMDM, bone marrow-derived macrophages; DMSO, dimethyl sulfoxide; IL, interleukin; LPS, lipopolysaccharide; mRNA, messenger RNA; qPCR, quantitative PCR; SD, standard deviation; TGF, transforming growth factor; TNF, tumor necrosis factor.

(powder, vehicle diluted, 2 mM). For each group, 50 μL of drug or vehicle gel was applied topically with a pipette on the wound surface. For C57BL/6J mice, treatment was performed daily for 4 days and for db/db mice, treatment was performed daily for 7 days. To ensure the gel stayed on the wound and moist wound healing, Tegaderm® film was applied over the wounds and changed daily. As a control for gene expression evaluation, a separate group with three C57BL/6J mice was submitted to the same wounding procedures. These control mice were euthanized and wounds harvested for analysis on day 3 postsurgery.

For all experimental mice, wound closure was assessed by digital images taken daily at a distance

of 10 cm from the lesions and a scale was placed in the field of view to calibrate the images. At the end of the treatment period (7 days for wild type, 10 days for db/db), animals were euthanized through cervical dislocation under isoflurane anesthesia (3%). Wounds were harvested and prepared for messenger RNA (mRNA) analysis or histological analysis.

Histology and hematoxylin-eosin/Trichrome staining

Samples used for histology were fixed in 4% paraformaldehyde (4°C) for 4 h and then immersed in 15% sucrose for 48 h and in 30% sucrose for 24 h (all at 4°C). After sucrose protection, wounds were embedded in Optimum Cutting Temperature medium (Thermo Fisher Scientific, Cat No. 2373571) on dental wax to prevent folding, and frozen in 2-methylbutane cooled with liquid nitrogen. Samples were stored at –80°C until sectioning.

Frozen wounds were sectioned in a cryostat (Leica Biosystems). Both chamber and specimen head temperatures were set at –32°C. Sections (10 μm) were collected on Superfrost Plus microscope slides (Thermo Fisher Scientific, Cat No. 4951PLUS4) and stored at –20°C. Each wound was sectioned from one edge through the center of the wound, and sections showing the largest wound diameter were deemed to represent the center of the wound and used for further analysis. Hematoxylin-eosin (HE) and Trichrome staining were performed according to our standard protocol.[5,19]

Immunohistochemistry

For Mac-3 staining, slides were defrosted for 30 min, washed in PBS for 5 min then immersed in citrate buffer (pH = 6), warmed in a microwave for 5 min, and cooled for 25 min at room temperature. Sections were then marked with PAP pen, washed with PBS (twice, 2 min each), glycine (three times, 2 min each), and PBS again (twice, 2 min each). Next, sections were incubated in hydrogen peroxide (0.3%, 5 min), washed with PBS (twice, 2 min each), and blocked with PBS-bovine serum albumin solution (0.2%, 30 min). After blocking, sections were incubated with primary antibody against Mac-3 (2 h, room temperature—Biolegend, Inc., Cat No. 108501, dilution 1:50), washed with PBS (three times, 2 min each), incubated with secondary antibody (30 min, at room temperature—Invitrogen, Cat No. 11481782, 1:200 dilution), and washed again with PBS (once, 30 min, room temperature). Then, sections were incubated with horseradish peroxidase (30 min, at room temperature), washed with PBS (once, 10 min, room temperature), and developed with DAB Kit (Vector Labs, Cat No. SK4100) for 3 min, under microscope observation. Sections were then washed with PBS for 2 min and counterstained with QS Hematoxylin (Vector Labs, Cat No. H3404,) for 5 s. A final wash in distilled water was then performed before mounting samples in Vectashield® (Vector Labs, Cat No. H1000).

Image analysis for wound measurements

Images of the wounds surface were analyzed using a Java-based image processing software (ImageJ, NIH). The percentage of wound closure was calculated (% wound closure = [100 × (wound size "post-surgery" – wound size "after treatment period")]/ wound size "postsurgery"). Histology images were acquired using a Nikon Instruments 80i microscope with a 2×/0.06 objective and a DS-QI1 digital camera, and were analyzed using the NIS-Elements® Advanced (Nikon Corporation) software. Data were processed on Excel (Microsoft) and plotted on GraphPad Prism for graphs and statistics. For histology analysis, the percentage of re-epithelization [(distance traversed by epithelium over wound from wound edge/distance between wound edges) × 100] was calculated for two sections per wound and was averaged over sections to provide a representative value for each wound. Average granulation thickness was measured in the same sections by dividing the wound bed area by wound length. In the slides stained with Trichrome, the percentage of blue stain was measured in granulation tissue area using NIS-Elements Advanced (Nikon Corporation) software.

Cell culture

Bone marrow-derived macrophages (BMDM) were isolated and differentiated as previously described.[20] In brief, femurs were harvested from C57Bl/6J male mice, bone marrow was flushed, and monocytes were seeded in 15 cm dishes and differentiated with macrophage colony-stimulating factor (10 ng/mL) added to Roswell Park Memorial Institute Medium high glucose (10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin), for 7 days at 37°C, 5% carbon dioxide.

After differentiation, 6×10^4 cells were seeded in 24-well plates and after allowing 12 h for attachment, cells were treated with vehicle (DMSO, Sigma-Aldrich, Cat No. 276855, 0.01%), 10 μM pioglitazone (Sigma-Aldrich, Cat No. E6910), or 10 μM GQ-11. After 24 h treatment, cells were challenged with 100 ng/mL lipopolysaccharide (LPS) (Sigma-Aldrich, Cat No. L2630) or 20 ng/mL IL-4 (Sigma-Aldrich, Cat No. I1020). Cells and medium were collected in the intervals of 3, 6, 12, and 24 h after challenge.

Cytokine assay

Levels of cytokines/chemokines secreted by BMDM were measured in cell culture medium by

cytometric bead array (CBA) Mouse Inflammation Kit (BD Biosciences, Cat No.552364). Levels of IL-6, IL-10, monocyte chemoattractant protein-1 (MCP-1), and TNF-α were measured following the manufacturer instructions.

Quantitative PCR analysis

mRNA was isolated from wounds and from BMDM using TRIzol Reagent (Invitrogen, Cat No. 15596026). One microgram of RNA was reverse-transcribed into complementary DNA (cDNA) using the high-capacity cDNA SuperScript Vilo® Kit, according to the manufacturer's instructions (Thermo Fisher Scientific, Cat No. 11706050). Quantitative PCR (qPCR) was performed in an ABI 7500 Fast Real-Time PCR using SYBR green master mix (Thermo Fisher Scientific, Cat No. 4385610) and primers for *Arg-Il-6*, *Il-10*, *Tgf-*β, *Tnf-*α, *Il-1*β, *Vegf*, *PPAR*γ, *PPAR*α (primer sequences available in Supplementary Table S1; Supplementary Data available online at www.libertpub.com/wound). Expression levels of each target gene were normalized to *Rpl-4* and mRNA relative expression as internal efficiency controls. The mRNA fold change was calculated using the 2(-Delta Delta C(t)) method,[21] and values expressed as fold increases relative to the control group.

Statistical analyses

Statistical analyses were performed using GraphPad Prism software, version 5.0. One-way analysis of variance followed by Tukey's test was used to calculate statistical significance as appropriate. All data in this study are expressed as the mean ± standard deviation. Values of $p < 0.05$ were considered significant.

RESULTS

GQ-11 decreases expression and release of pro-inflammatory cytokines and induces expression of anti-inflammatory cytokines and growth factors in BMDM

We first sought to determine whether pretreatment of cultured macrophages with pioglitazone or GQ-11 altered responses to either LPS or IL-4. When cells were challenged with LPS, pretreatment with pioglitazone and GQ-11 blunted induction of the pro-inflammatory cytokines *Il-1*β and *Tnf-*α (Fig. 1A). Importantly, both pioglitazone and GQ-11 upregulated the expression of *Il-10*, *Arg-1* and *Tgf-*β in BMDM challenged by LPS, indicating that even in the presence of a strong inflammatory stimulus these drugs can maintain a macrophage anti-inflammatory phenotype (Fig. 1B). Accordingly, pretreatment with pioglitazone and GQ-11 did not alter the expression of pro-inflammatory cytokines when BMDM were challenged with IL-4 (Fig. 1C), but upregulated the expression of *Il-10*, *Arg-1*, and *Tgf-*β, compatible with the anti-inflammatory phenotype (Fig. 1D).

When measuring cytokine/chemokine protein levels in cell culture medium, pretreatment with pioglitazone or GQ-11 blunted the release of the pro-inflammatory cytokines IL-6, MCP-1, and TNF-α, after the challenge with LPS (Fig. 2A). Although pro-inflammatory cytokine release was below detectable levels in the medium of cells challenged with IL-4, pretreatment with pioglitazone or GQ-11 was able to increase IL-10 release, after challenge with either LPS or IL-4 (Fig. 2B, C).

GQ-11 increases expression of anti-inflammatory cytokines and growth factors in wounds of both nondiabetic and diabetic mice

To determine whether GQ-11 can ameliorate inflammation *in vivo*, we applied GQ-11 topically to wounds in nondiabetic and diabetic mice. We first assessed the effect of GQ-11 on expression of PPARs in wound tissue, and found that GQ-11 treatment upregulated the expression of *Ppar-*α and *Ppar-*γ, and pioglitazone upregulated expression of *Ppar-*γ, in wounds of both nondiabetic and diabetic mice compared with vehicle control treatment (Fig. 3A), confirming targeting of the PPAR pathway by these drugs.

In addition, treatment with GQ-11 or pioglitazone upregulated the expression of anti-inflammatory cytokines and growth factors in wounds, including *Il-10*, *Tgf-*β, and *Vegf* in both nondiabetic and diabetic mice compared with vehicle controls (Fig. 3B), indicating that GQ-11 can induce anti-inflammatory cytokines and growth factors associated with tissue repair. Whereas there was no effect of either GQ-11 or pioglitazone treatment on expression of pro-inflammatory cytokines in wounds of nondiabetic mice compared with controls, GQ-11 downregulated *Il-6* in wounds of diabetic mice compared with control or pioglitazone groups (Fig. 3C). The lack of effect of GQ-11 on *Il-1*β and *Tnf-*α expression may be due to the time point examined; the expression of these cytokines was significantly lower in all treatment groups at the time point examined compared with the positive controls collected on day 3 postinjury.

GQ-11 accelerates wound closure in diabetic mice

To determine whether GQ-11-induced alterations in gene expression were associated with improved wound healing, we first assessed the

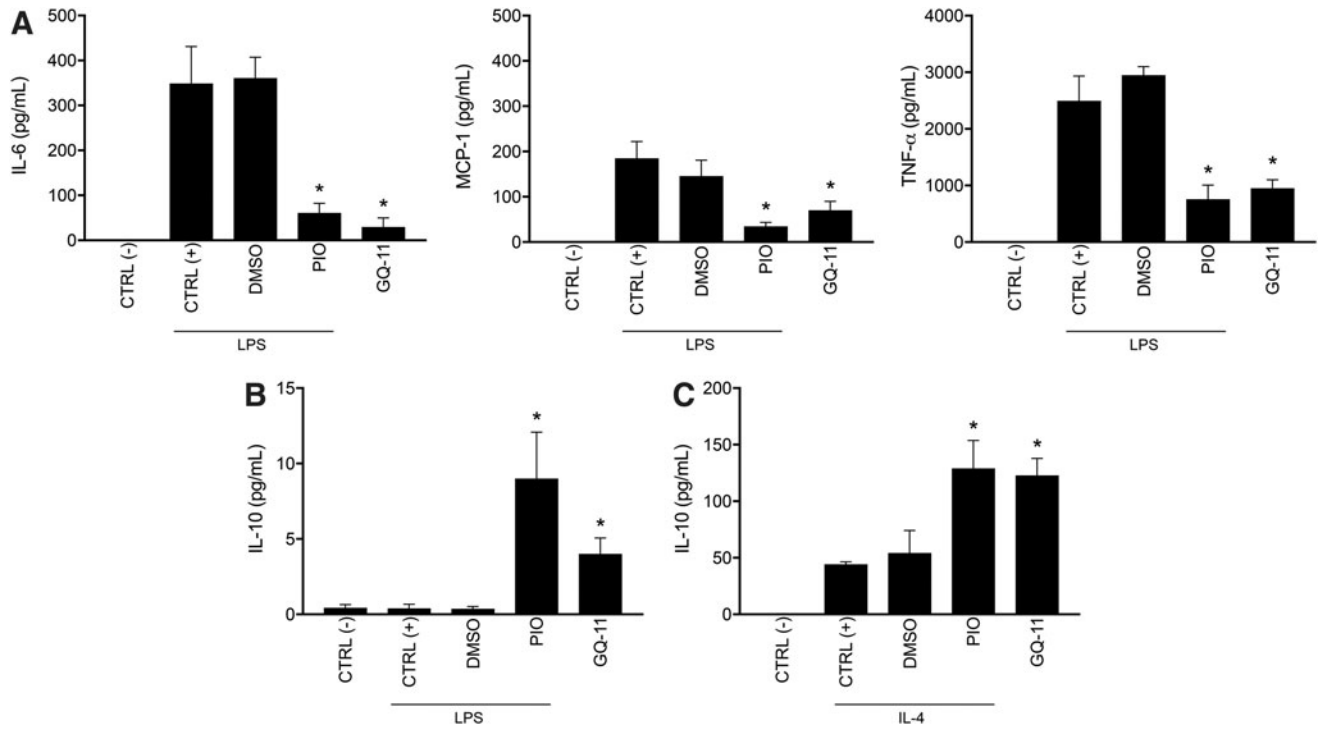

Figure 2. GQ-11 decreases pro-inflammatory cytokine release and increases anti-inflammatory cytokine release by BMDM. **(A)** Concentrations of IL-6, MCP-1, and TNF-α protein of BMDM supernatant. **(B)** IL-10 concentration from supernatant of BMDM pretreated for 24 h with vehicle (DMSO 0.01%), pioglitazone (PIO 10 μM), or GQ-11 (10 μM) and challenged with LPS (100 ng/mL). **(C)** IL-10 concentration in supernatant of BMDM pretreated for 24 h with vehicle (DMSO 0.01%), pioglitazone (PIO 10 μM), or GQ-11 (10 μM) and challenged with IL-4 (20 ng/mL). Total protein of BMDM supernatant was accessed by flow cytometry, with a CBA. Negative controls [CTRL (–)] are represented by nontreated and nonchallenged cells. Positive controls [CTRL (+)] are represented by nontreated but challenged cells. Data are expressed as the mean ± SD of biological triplicates. Statistical analyses were performed using ANOVA/Tukey's multiple comparison tests. *$p < 0.05$. CBA, cytometric bead array; MCP-1, monocyte chemoattractant protein-1.

effects of GQ-11 on wound closure using external measurements. In nondiabetic mice, there was no effect of GQ-11 on wound closure (Fig. 4A). However, in diabetic db/db mice, we observed a substantial increase of about 30% in wound closure on day 10 postwounding in animals treated with GQ-11, compared with pioglitazone or control groups (Fig. 4B; Supplementary Table S2). Interestingly, the improvement was only apparent at the later stage of wound closure, indicating an effect during the resolution of inflammation.

Wound healing was also assessed through histological analysis. HE staining of wound cryosections allowed us to measure granulation tissue thickness and re-epithelization. Again, no difference in either granulation tissue thickness or re-epithelization was observed between treatment groups in nondiabetic mice (Fig. 5A). In db/db mice, treatment with GQ-11 significantly increased re-epithelization to 83% compared with 45% for controls and 59% for pioglitazone treatment, consistent with the wound closure data. GQ-11 also induced a trend of an increase in granulation tissue thickness, but this did not reach statistical significance (Fig. 5B; Supplementary Table S2).

GQ-11 increases collagen deposition in diabetic mice

In addition to measuring re-epithelization in HE-stained cryosections, Trichrome staining allowed collagen deposition in wounds to be assessed in both nondiabetic and diabetic mice. Treatment of wounds in nondiabetic mice did not alter collagen deposition compared with controls (Fig. 5C). In contrast, GQ-11 treatment of wounds in diabetic mice increased collagen deposition by about 50%, compared with control and pioglitazone groups (Fig. 5D; Supplementary Table S2).

GQ-11 may decrease macrophage infiltration in wounds of db/db mice

Qualitatively, we observed that in wounds of both nondiabetic and diabetic mice, those treated with GQ-11 showed less macrophage infiltration compared with pioglitazone and vehicle in nondiabetic mice (Fig. 6A), and compared with vehicle in diabetic mice (Fig. 6B). These findings indicate that

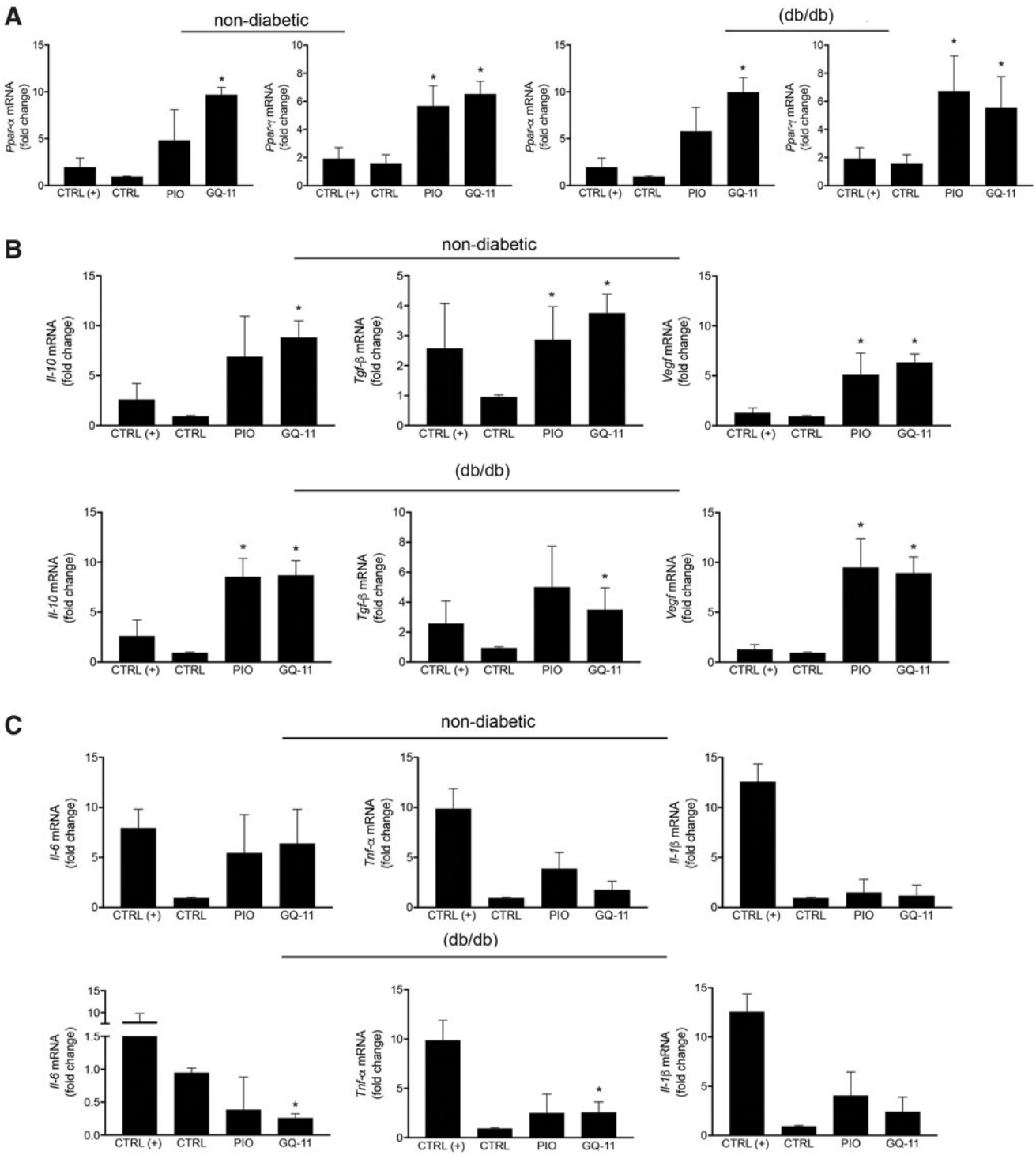

Figure 3. GQ-11 increases the expression of anti-inflammatory cytokines and decreases the expression of pro-inflammatory cytokines in wounds of db/db mice. **(A)** Fold change of *Ppar-*α and *Ppar-*γ in wounds of nondiabetic and diabetic db/db mice. **(B)** Fold change of *Il-10, Tgf-*β, and *Vegf* in nondiabetic and db/db mice. **(C)** Fold change of Il-6, *Tnf-*α, and *Il-1*β in nondiabetic and db/db mice. mRNA expression was quantified by qPCR. Data are expressed as the mean $\pm$ SD of six mice per group. Statistical analyses were performed using ANOVA/Tukey's multiple comparison tests. $^*p < 0.05$.

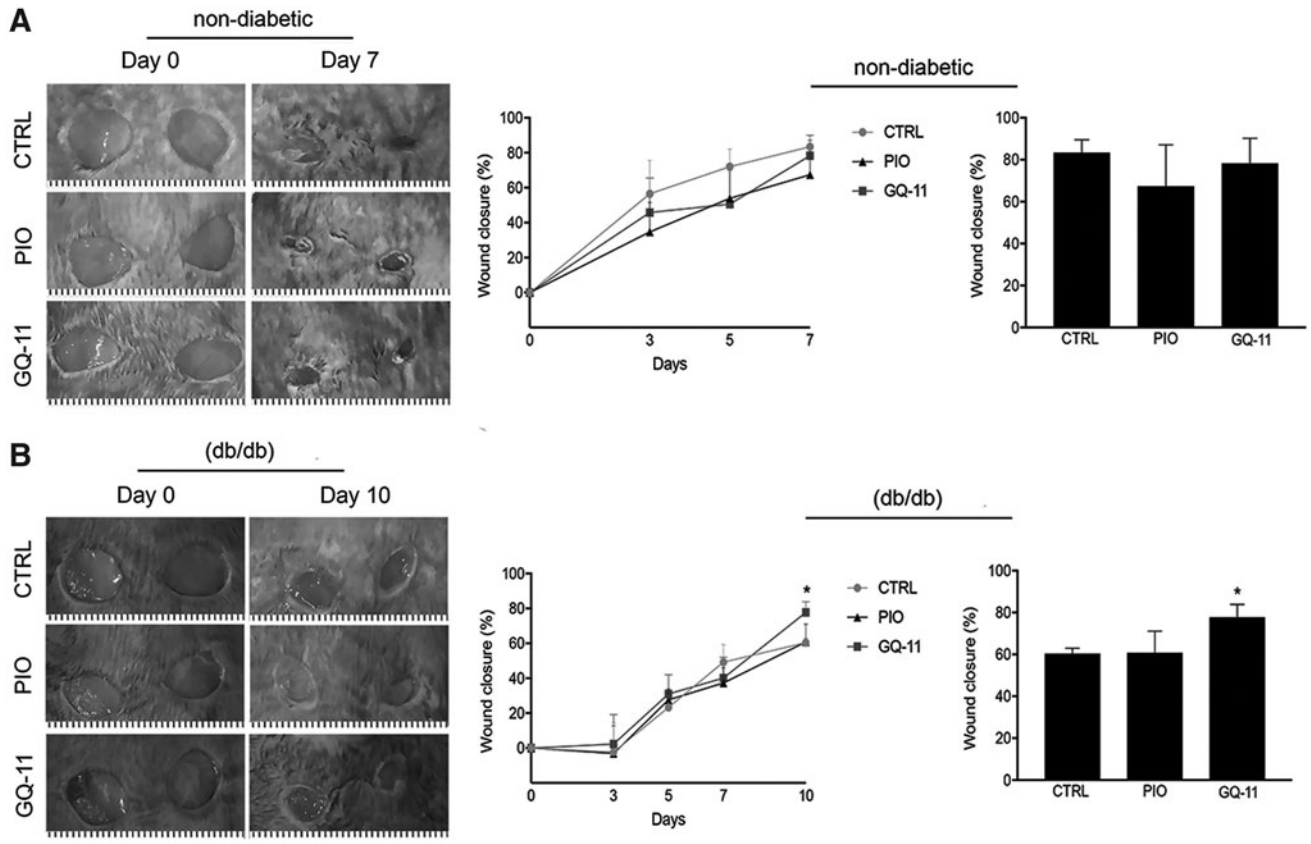

Figure 4. GQ-11 improves wound closure in db/db mice. **(A)** Representative lesions at scapula of nondiabetic mice (postsurgery and after 7 days), decrease of wound area along 7 days postsurgery and total wound closure. **(B)** Representative lesions at scapula of diabetic db/db mice (postsurgery and after 10 days), decrease of wound area along 10 days postsurgery and total wound closure. Mice were submitted to surgical procedure to induce scapula lesions and were treated at the 3rd day postsurgery with vehicle (CTRL—Pluronic Gel®), pioglitazone (PIO—2 mM), or GQ-11 (2 mM) for 4 days (nondiabetic mice) and 7 days (db/db mice). Positive controls [CTRL (+)] are represented by wounds of nondiabetic mice at the 3rd day postsurgery without treatment. Data are expressed as the mean ± SD of six mice per group. Statistical analyses were performed using ANOVA/Tukey's multiple comparison tests. $*p < 0.05$.

GQ-11 may induce resolution of inflammation in diabetic wounds.

DISCUSSION

Our previous studies indicated beneficial properties of a novel PPAR agonist (GQ-11) that may represent a promising candidate for the therapy of diabetes complications.[15] In this study, our major findings are that GQ-11 induces a pro-healing phenotype both in cultured macrophages and when applied topically to wounds of diabetic mice, which resulted in accelerated re-epithelization and collagen deposition.

GQ-11 is a dual, partial PPAR-γ/α agonist, and may influence wound healing through effects on inflammation. PPAR-γ/α can affect inflammation direct or indirectly.[22] First, PPAR-γ might modulate the expression/activity of G protein-coupled receptors (GPCRs) and transcriptional regulation of GPCRs kinase-2 activity, inducing anti-inflammatory responses.[23] Second, PPAR-α, whose activation stimulates the expression of genes encoding cytochrome P450 and β-oxidation enzymes, contributes to resolution of inflammation, through leukotriene B4 activity limitation.[24] Importantly, the inhibition of PPAR-γ activity is related to sustained production of IL-1β, whereas activation of PPAR-γ induces a protective anti-inflammatory effect.[25] Interestingly, it has been shown that the inflammation repression by PPAR-γ is dependent on PPAR-α, through IκBα induction, both *in vitro* and *in vivo*.[26] Thus, a dual/partial PPAR-γ/α agonist may represent a promising approach to dampen inflammation and improve wound healing, particularly in the setting of diabetes. In this study, GQ-11 increased anti-inflammatory and pro-healing factors expression, including *IL-10*, *Arg-1*, *Tgf*-β, and *Vegf*, both in cultured macrophages and *in vivo* in wounds (Figs. 1–3). GQ-11 also downregulated pro-inflammatory cytokines *Il-1*β and *Tnf*-α expression in strongly pro-inflammatory cultured macrophages, but did not

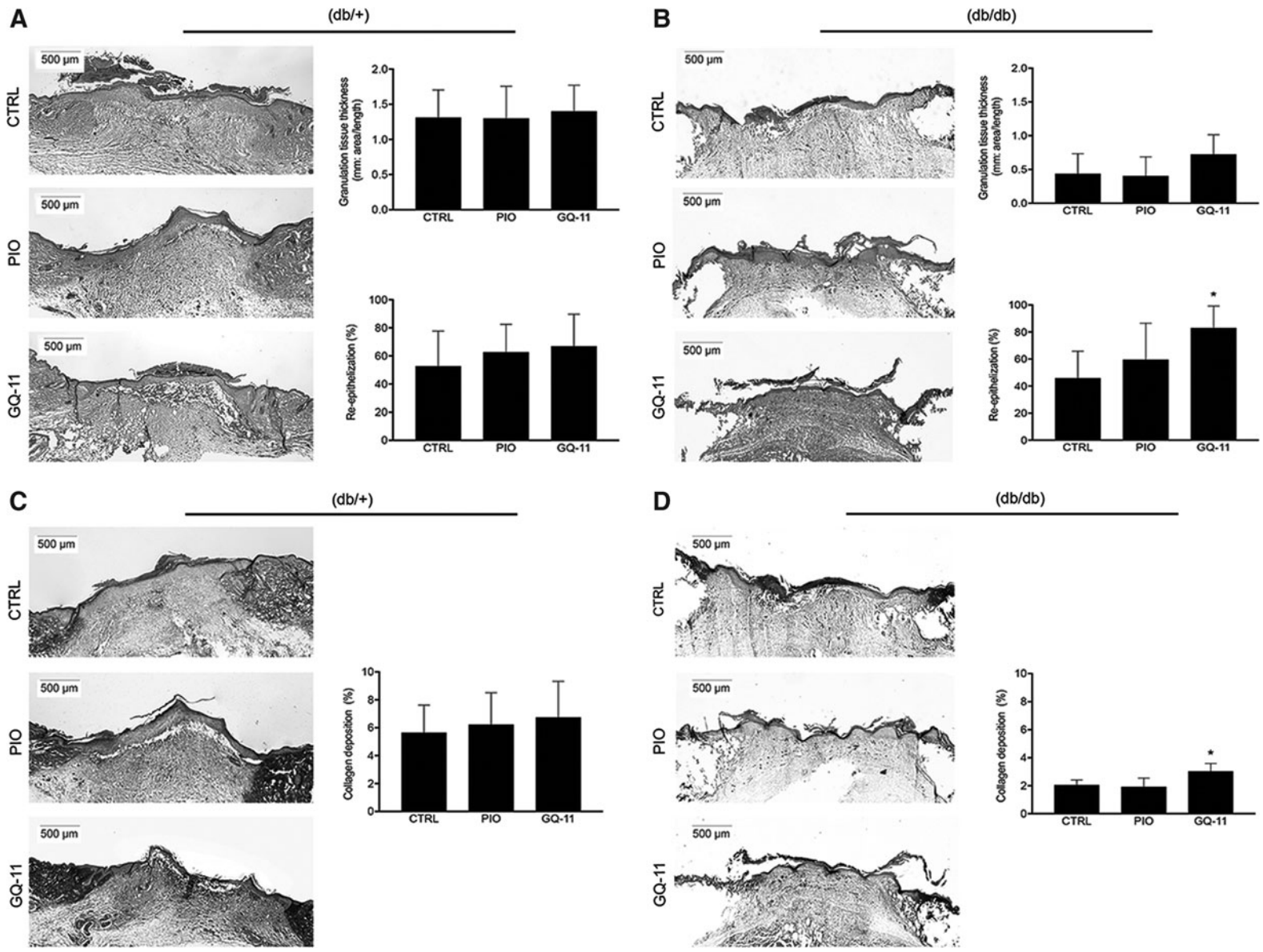

Figure 5. GQ-11 increases re-epithelization and collagen deposition in wounds of db/db mice. **(A)** Representative sections of wounds edge of nondiabetic mice, HE stained, and the respective thickness measurement and re-epithelization calculation. **(B)** Representative sections of wounds edge of db/db mice, HE stained and the respective thickness measurement and re-epithelization calculation. **(C)** Representative sections of wounds edge of nondiabetic mice, Trichrome stained, and respective collagen deposition. **(D)** Representative sections of wounds edge of db/db mice, Trichrome stained, and respective collagen deposition. Mice were submitted to surgical procedure to induce scapula lesion and were treated at the 3rd day postsurgery with vehicle (CTRL—Pluronic Gel), pioglitazone (PIO—2 mM), or GQ-11 (2 mM) for 4 days (nondiabetic mice) and 7 days (db/db mice). Positive controls [CTRL (+)] are represented by wounds of db/+ mice at the 3rd day postsurgery without treatment. Histological sections were collected with frozen samples with cryostat. Magnification 2×. Data are expressed as the mean ± SD of six mice per group. Statistical analyses were performed using ANOVA/Tukey's multiple comparison tests. $*p < 0.05$. HE, hematoxylin-eosin.

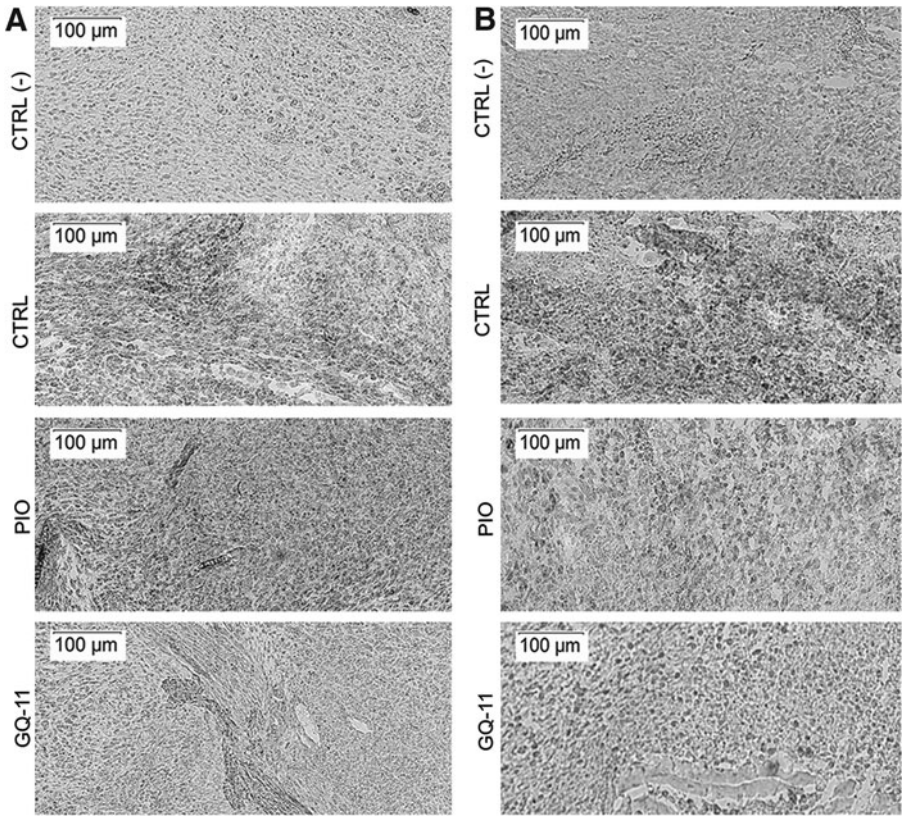

Figure 6. GQ-11 decreases macrophage infiltration in wounds of db/db mice. **(A)** Representative sections of Mac-3 staining in wounds of nondiabetic mice. **(B)** Representative sections of Mac-3 staining in wounds of db/db mice. Mice were submitted to surgical procedure to induce scapula lesion and were treated at the 3rd day postsurgery with vehicle (CTRL—Pluronic Gel), pioglitazone (PIO—2 mM), or GQ-11 (2 mM) for 4 days (nondiabetic mice) or 7 days (db/db mice). Positive controls [CTRL (+)] are represented by wounds of db/+ mice at the 3rd day postsurgery without treatment. Histological sections were collected with frozen samples with cryostat and staining was performed by immunohistochemistry with specific antibodies against Mac-3 (1:50). Magnification 10×.

affect expression of these cytokines in less inflammatory macrophages and during later stages of wound healing. These findings corroborate previous studies reporting anti-inflammatory effects of PPAR-γ agonists.[11,27,28]

In the context of normal wound healing, pro-inflammatory cytokines likely play important roles in the early inflammatory stage of wound healing.[29] We reported that loss of the NLRP3 inflammasome resulted in reduced IL-1β in wounds of nondiabetic mice and delayed healing.[30] *In vivo* studies report that macrophages are the main producers of IL-1β in wounds, both in nondiabetic and diabetic animals, especially in the early inflammatory stage, when macrophages are polarized to a pro-inflammatory phenotype.[9,27,31] This pro-inflammatory phenotype is also associated with macrophage phagocytosis of apoptotic cells,[32] which in turn is thought to induce transition to a noninflammatory healing-associated phenotype.[8,33] The transition from pro-inflammatory to noninflammatory macrophage phenotypes is also important for normal wound healing, where macrophages with the latter phenotype release growth factors important for the proliferative and remodeling stages of wound healing.[34] One such growth factor, TGF-β binds to TGF-β receptor I and induces phosphorylation of Smad4 resulting in further downregulation of pro-inflammatory genes and induction of fibroblast migration and proliferation.[35] In addition, the binding of Smad4 transcription factor induces the *Il-10* gene promoter, stimulating further *Il-10* expression in macrophages.[36] Thus, induction of such a phenotype switch in wounds of diabetic mice by GQ-11 may help to restore normal wound healing.

In the diabetic environment, a number of factors may promote a chronic inflammatory response that impairs wound healing. The persistent hyperglycemia observed in diabetes causes a set of glycation reactions resulting in generation of advanced glycation end-products (AGEs).[37] In turn, AGEs have direct and indirect actions in many cell types, including the induction of oxidative stress through the AGE Receptor (RAGE). Sustained activity of a reactive oxygen species-mediated pathway—induced either by RAGEs activation and/or im-

paired phagocytosis of apoptotic cells—may activate the NLRP3 inflammasome/IL-1β pathway inducing a pro-inflammatory positive feedback loop and contributing to the persistent inflammatory response related to impaired healing.[9,10] In addition, the activation of many factors, such as RAGE and inflammation mediators as MCP-1, contributes to upregulation of nuclear factor-κB and its target genes, including TNF-α,[38] which may also be involved in impaired wound healing. TNF-α can induce cell cycle arrest and apoptosis through forkhead box protein O1-dependent process[39] resulting in increased expression of pro-inflammatory cytokines, setting up another inflammatory positive feedback loop that impairs healing. GQ-11 may help to break these pro-inflammatory positive feedback loops and induce resolution of inflammation in diabetic wounds. When compared with pioglitazone, the downregulation of *Il-6* and *Tnf-*α observed in wounds treated with GQ-11 might also be related to decreased macrophage infiltration in the same groups, since the animals treated with pioglitazone did not show the same effect even upregulating *Il-10* and *Vegf*.

Previous studies by Sakai *et al.*[40] showed some different results when analyzing the effects of pioglitazone on wound healing. When comparing both studies, we can observe a different pattern in wound closure with greater re-epithelization on the first 7 days in Sakai *et al.*'s study,[40] whereas our model showed greater re-epithelization from 7th to 10th days. This difference could be related to distinct drug solubilization and local delivery on wounds as different vehicles were used in both studies. For sure, new formulations to improve drug delivery and efficacy are necessary for the future development of a new wound healing therapeutic agent for clinical use, especially with new structures, such as GQ-11.

KEY FINDINGS

- Greater re-epithelization when compared with pioglitazone and control groups.
- Increased collagen deposition when compared with pioglitazone and control groups.
- Inflammation modulation when compared with pioglitazone and control groups.

INNOVATION

Based on this, our findings suggest that wounds in diabetic mice treated with GQ-11 were in later stages of healing compared with controls, exhibiting greater re-epithelization and increased collagen deposition, likely transitioning into remodeling stage of tissue repair. The effects on healing can be attributed to the partial PPAR-γ/α agonism of GQ-11, which promoted cytokines modulation, leading to healing improvement. The search for a new PPAR agonist that shares classic TZDs therapeutic effects with less adverse effects is a key to a new approach in diabetic wound healing clinical trials to prevent its complications, such as amputations.

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AUTHOR DISCLOSURE AND GHOSTWRITING

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Abbreviations and Acronyms

AGEs = advanced glycation end-products
ANOVA = analysis of variance
RAGE = AGE receptor
BMDM = bone marrow-derived macrophages
CBA = cytometric bead array
cDNA = complementary DNA
DMSO = dimethyl sulfoxide
GPCRs = G protein-coupled receptors
HE = hematoxylin-eosin
IL = interleukin
LPS = lipopolysaccharide
MCP-1 = monocyte chemoattractant protein-1
Mo/Mp = monocytes/macrophages
mRNA = messenger RNA
NIH = National Institutes of Health
PBS = phosphate-buffered saline
PPARs = peroxisome proliferator-activated receptors
qPCR = quantitative PCR
SD = standard deviation
TGF = transforming growth factor
TNF = tumor necrosis factor
TZDs = thiazolidinediones
VEGF = vascular endothelial growth factor

6. CHAPTER III: “Dual PPAR agonist induces cell proliferation and differentiation in reconstructed human skin”.

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"Dual PPAR agonist induces cell proliferation and differentiation in reconstructed human skin"

"Dual PPAR agonist modulates cell behavior in RHE"

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Abstract

Chronic wounds associated with diabetes show an important health and social concern, becoming a topic of interest worldwide. Recent advances in human-based *in vitro* models represent new tools for signaling investigations and drug screening to new therapeutic approaches. Local administration of a new dual PPAR agonist (GQ-11) showed to promote anti-inflammatory effects, ECM deposition and re-epithelization induction in diabetic mice, suggesting an interesting approach to improve chronic wounds management and induce healing. In this study we proposed to evaluate GQ-11 effects in Reconstructed Human Skin (RHE), in order to prioritize the use of a relevant human model. Our results showed that GQ-11 modulated proliferation (Ki-67 and Ck-14), differentiation (α-SMA) and cell adhesion (Desmoglein-1) markers, confirming the promising and key approach to prevent diabetic wound complications, such as amputations.

Introduction

Diabetes Mellitus (DM) – insulin-dependent (type 1) or independent (type 2) – is a chronic non-communicable disease (NCD) with 8.8% prevalence among adults over 18 years of age worldwide. As widely reported, chronic wounds associated with DM affects up to 25% of diabetic patients with 14-24% incidence of amputation (1, 2), representing an important health problem.

Complications associated to diabetes are strictly related to persistent hyperglycemia (3, 4), contributing to a wide range of diabetic wound characteristics such as persistent inflammatory response with increased pro-inflammatory cytokine release and impaired growth factors and angiogenesis as well as extracellular matrix (ECM) depletion (5, 6).

It is reported that reactions of non-enzymatic glycation and glucose autoxidation promoted in persistent hyperglycemia lead to Reactive Oxygen Species (ROS) and Advanced Glycation End-Products (AGEs) formation. The AGEs constitute a variety of substances composed between reducing sugars or oxidized lipids with lysine and arginine residues, resulting in structural and functional alterations in important receptors (7). These alterations will contribute to many DM complications, such as cardiopathies, nephropathies, retinopathies and neuropathies pathogenesis (8, 9). Nevertheless, the accumulation of AGEs has been considered one of the factors responsible for healing impairment through loss of elasticity, affected cells growth, differentiation and motility as well as cytokines response, enzymatic activity (as metalloproteinases) and hemostasis (10).

Recent advances in human-based *in vitro* models represent new tools for drug screening and research to new therapeutic agents. The complexity of this strategy is relevant when considering to integrate immune cells and microfluidic platforms to study cell-type interactions and immune responses. Recently, our group reported the characterization of *in vitro* Reconstructed Human Epidermis (RHE) based of glycated collagen showing flattened epidermal

layers, reduced ECM deposition on dermis, altered ECM fibers pattern, poor keratinocytes stratification and differentiation as well as fibroblasts morphology alteration (11), representing an important new tool for research in aging and diabetic skin- related disturbances and diseases.

Thiazolidinediones (TZDs), a class of PPARγ agonists, are commonly used to treat insulin resistance and despite of hypoglycemic and pleiotropic anti-inflammatory properties, prolonged use shows important adverse effects – such as weight gain, bone loss and cardiovascular events – encouraging the search for new TZDs with better efficacy and reduced side effects (12-15). GQ-11 is a new thiazolidine compound with dual PPARα/γ agonism with classical TZD hypoglycemic effect besides lipid profile improve (14), reduce in obesity-induced metabolic alterations (14), macrophages modulation, re-epithelization promotion as well as collagen deposition increase in murine model of diabetic wound healing (16).

Thus, local administration of GQ-11 in wounds of diabetic mice showed to promote anti-inflammatory effects, ECM deposition and re-epithelization recovery, suggesting a promising approach to improve healing in diabetic wounds (16, 17).

In order to prioritize the complexity of human epidermis and the dissimilarity between human and rodent models, we here propose to use RHE based of glycated collagen as a tool to observe GQ-11 effects in human cells proliferation, differentiation, as well as cell adhesion, aiming to better understand its general effects in a relevant human model.

Results

GQ-11 induces cell migration and proliferation

The treatment with GQ-11 in incisions of RHE based on glycated collagen increased keratinocytes proliferation through the incision, as observed in Hematoxylin-Eosin staining (Fig.1A). Cell migration induce by GQ-11 was confirmed with HUVECs when compared to the control and Pioglitazone treatment (Fig.1B).

The treatment with Pioglitazone - the full PPARγ agonist - showed to upregulate Pparγ, while GQ-11 upregulated both Pparα and Pparγ in RHE, when comparing to negative or positive controls (Fig.1C).

In order to investigate the mechanistic behind increased keratinocytes proliferation induced by the treatment, expression of Metalloproteinase-9 (MMP-9) and Forkhead box O1 (FOXO1) were quantified. Consistently, incisions of RHE treated with GQ-11 showed both Mmp-9 and Foxo-1 downregulation when compared to negative and positive controls (Fig.1C). Pioglitazone treatment also downregulated Mmp-9 expression.

GQ-11 induces epidermis keratinocytes proliferation

In order to confirm proliferation grade of RHE keratinocytes treated with PPAR agonists, Cytokeratin 14 (Ck-14) and Ki-67 markers were labeled in histology samples. Both Pioglitazone and GQ-11 showed Ki-67 accumulation in basal epidermis, as well as Ck-14 throughout all epidermis (Fig.2A).

Total Ki-67 and Ck-14 protein were upregulated and increased in RHE treated with either GQ-11 or Pioglitazone when comparing to controls (Fig.2B/C).

GQ-11 induces fibroblasts and keratinocytes differentiation

Immunohistochemistry in RHE samples also indicated presence of alpha-Smooth Muscle Actin (α-SMA) in dermis fibroblasts (Fig.3A). Despite of observation in histology samples, both total protein and expression did not differ from controls (Fig.3B/C).

GQ-11 improves cell adhesion in basal epidermis

To assess cell adhesion modulation in histology samples, it was evaluated the presence of Desmonglein-1 though immunohistochemistry. It was possible to observe presence of the protein only in basal epidermis of RHE treated with GQ-11 (Fig.4A). In addition, the treatment showed to upregulate the expression and also total protein of the marker when comparing to either Pioglitazone or controls, indicating cell adhesion improve (Fig.4B/C).

Discussion

Epidermis is the most important barrier against external environment and after injury its barrier function recovery is important to prevent chronic wounds and possible infections. Wound healing response is a wide process involving many cell types and is commonly described in stages of hemostasis, early/late inflammation, proliferation and remodeling. Cytokines and growth factors play synchronized and complimentary roles to regulate these stages, thus, any alterations in these factors caused by systemic conditions, such as diabetes/hyperglycemia, may impair healing process (18). Migration and proliferation of epidermal keratinocytes in the wound site is a key aspect of re-epithelization and is in part regulated by Matrix Metalloproteinases 9 (MMP9) and Forkhead box O1 (FOXO1) signaling (19, 20). Keratinocytes migration is facilitated through ECM degradation by MMPs, but MMPs exacerbated activity leads to wound healing delay, as observed in diabetic wounds (19, 21). At the same time, it was demonstrated that FOXO1 promotes wound healing through TGF-β upregulation in normal skin wounds, but has the

opposite effect in diabetic wounds (22, 23). Nevertheless, the inhibition of MMP9 in diabetes rescues keratinocytes migration in a FOXO1-dependent manner due to its increased binding to MMP9 promoter and upregulation (20).

Other recent study also showed the relation of PPARγ and FOXO1 downregulation in diabetes and PPARα in FOXO1 inactivation (24). Consistently, our findings showed that treatment with GQ-11 - a dual PPARa/g agonist - and Pioglitazone - a classic full PPARy agonist - induced keratinocytes proliferation besides PPARy upregulation and MMP9/FOXO1 axis downregulation (Fig.1). The dual agonism of GQ-11 and observed PPARα upregulation might also be related to the stronger FOXO1 inhibition observed in keratinocytes when compared to Pioglitazone (Fig.1C). The dual agonist also showed to induce migration in endothelial cells (Fig.1B), suggesting the strong relation between the treatment and cell migration induce.

The proliferative grade of skin samples treated both with GQ-11 and Pioglitazone are also observed with Ck-14 expression (Fig 2A). While negative control exhibits a proliferative pattern in basal layers, glycated skin shows proliferative keratinocytes from basal to spinous layers, even when treated with PPAR agonists. In contrast, the expression of Ki-67 shows that the treatments induced proliferation of keratinocytes of basal layer (Fig. 2).

In wound healing process, fibroblasts differentiation to myofibroblasts is a key role in proliferation to remodeling stages transition. Myofibroblasts express α-SMA, under regulation of TGF-β, participating in ECM synthesis and force generation, resulting in ECM reorganization and wound contraction. Moreover, myofibroblasts profile showed to stimulate a more activated epidermis, which in turn, upregulates TGF-β, feeding a cycle for myofibroblasts differentiation (25-28). In our results, we demonstrated that GQ-11 induced fibroblasts differentiation to myofibroblasts through α-SMA presence in dermis fibroblasts (Fig.3A). In fact, previously reports showed increased TGF-β and collagen deposition in wounds of diabetic mice treated with GQ-11 (15), what might indicate the GQ-11 role to the α-SMA mark found in human skin.

Besides cells proliferation and differentiation modulation, GQ-11 also showed to modulate cell-to-cell adhesion structures, as observed in Desmoglein-1 upregulation in dermis and epidermis junction (Fig.4). Desmogleins play a role in cell junction formation, maintaining the integrity of cell-to-cell structures. Several studies suggest that depleted Desmoglein-1 could aggravate inflammation in epidermis causing barrier defects and fragility (29-31). It was not observed Desmoglein-1 expression increase in glycated skin samples when compared to the negative control, suggesting that GQ-11 could be related to its upregulation.

Altogether, previous studies showed GQ-11 modulation in macrophages polarization and cytokines profile (16), indicating anti-inflammatory effects and homeostasis induction in diabetic wound healing in mice. Here we demonstrate - considering both animals and human skin models - that GQ-11 role in impaired wound healing process might exert modulation in different cell types, such as macrophages, keratinocytes and fibroblasts probably attributed to its partial PPARα/γ agonism, regulating imbalanced growth factors and cytokines release.

Finally, wound healing is a complex process involving several inter-related biological and molecular activities for achieving tissue regeneration and the biggest challenge is to prevent its complications and morbidities, such as amputations and in severe cases, mortality.

Nowadays, chronic wounds show significant health, social and economic concerns on patients and society, becoming a topic of clinical interest worldwide. Improved management of wound healing is directly related to reduced risk of delayed healing and cost to health providers through hospital stays and nursing staff time spent with chronic wounds patients.

We then conclude that diabetic wound healing management with PPAR agonists is a key new approach to prevent its complications. The search for safe TZDs with less adverse effects and the dual agonism attributed to GQ-11 showed important features for a potential element in diabetic wound management. Next challenges include new formulations and drug delivery improves, necessary to develop safe therapeutic agents for clinical purposes.

Materials and Methods

Experimental Design

The aim of this study was to reproduce RHE based on glycated collagen, performing incisions and applying different treatments to observe cell behavior in proliferation and differentiation aspects. Cells were donated from University of Sao Paulo Hospital (HU-USP), isolated and cultivated in separate lots. The study was conducted in triplicate and reproduced with two different lots of cells (n=3).

GQ-11 synthesis

GQ-11 [(Z)-5-((1*H*-indol-3-yl)methylene)-3-(4-methylbenzyl)thiazolidine-2,4-dione] was synthesized as previously described (14), in the Laboratory of Drug Design and Synthesis of the Federal University of Pernambuco (Recife, Pernambuco, Brazil).

HUVECs culture and migration assay

Human Umbilical Vein Endothelial Cells (HUVECs) were maintained in RPMI medium (Gibco, Life Technologies, Grand Carlsbad, CA). The cells were cultured at 37°C in an atmosphere at 5% CO_2. Cells were seeded onto 50 mm imaging dishes and when in 100% confluence, a straight lesion was made in the center of the monolayer using a sterile 200 µL pipette tip, as previously described (32). The wells were subsequently washed twice with PBS to remove the dead cells and incubated with Vehicle (DMSO 0.01%, Sigma-Aldrich, St Louis, USA), Pioglitazone (2 mM, powder, DMSO diluted, Sigma-Aldrich, St Louis, USA) or GQ-11 (2 mM, powder, DMSO diluted). The lesions were photographed using phase contrast microscopy on an inverted microscope (Nikon, Tokyo, Japan) after 24 hours of treatment.

Reconstructed human skin in glycated collagen matrix

The reconstructed human skins were prepared in collaboration with Skin Lab of Clinical Analysis Department of University of Sao Paulo, according to previously described protocol (10).

Briefly, normal human skin cells obtained from donated foreskin samples from HU-USP were isolated through cleaning, fragmentation and digestion with dispase II (Roche Life Sciences, Branford, USA), overnight at 4ºC. Dermis and epidermis were mechanically separated to keratinocytes and fibroblasts isolation. Keratinocytes were cultured in KGM Gold Bullet Kit medium (Lonza KBM, Walkersville, USA) and fibroblasts were cultured in DMEM (Gibco, Life Technologies, Grand Carlsbad, CA), supplemented with 10% fetal bovine serum and antibiotics. Cells were maintained in a humidified incubator at 37ºC, 5 - 7.5% CO_2 and subcultured by treatment with 0.1% trypsin-EDTA when 80% confluency. Dermal compartment was prepared using commercial collagen glycated with sodium glyoxylate (Sigma-Aldrich, Sant Louis, MO) and sodium cyanoborohydride (Sigma-Aldrich, Sant Louis, MO) and human fibroblasts. After polymerization of collagen gel, human keratinocytes were seeded on the top of lattices and were kept submerged in an in-house prepared cultured medium for 24 hours. Subsequently, culture was raised and maintained at the air-liquid interface for 12 days to allow complete keratinocytes stratification and differentiation. Negative controls were cultured in non-glycated commercial collagen.

Skin incision and GQ-11 treatment

Incisions of reconstructed skins were induced after complete keratinocytes stratification and differentiation with a sterile 2 mm biopsy punch (Kolplast CI, Itupeva, SP, Brazil). Incisions were then treated with Vehicle (F-127® Pluronic Gel, Sigma-Aldrich, 25% in PBS, 0.1% DMSO), Pioglitazone (Sigma-Aldrich, powder, vehicle diluted 2 mM) or GQ-11 (powder, vehicle

diluted, 2 mM). For each group, 20 µL of gel was topically applied with a pipette on the surface and incubated for 24 hours. Negative controls were not treated.

Hematoxylin-Eosin staining and immunohistochemistry

After treatment period, skin samples were fixed in paraformaldehyde 4% for 1 hour, and dehydrated in increasing concentration of alcohols / xylol for paraffin inclusion in the Histopathology Unit ICB-USP. Paraffin sections (5 µm), were stained with hematoxylin-Eosin (HE).

For immunohistochemistry, all paraffin sections were incubated at 55ºC for 20 minutes for deparaffinization and then rehydrated. Antigen recovery was performed by incubation in citrate buffer pH 6.0 for 5 minutes at 95ºC. Immunolabeling assay was carried out using mouse monoclonal antibodies anti-cytokeratin 14 (Abcam, Cambridge, UK), anti-Ki-67 (Dako Agilent #51161-2, Santa Clara, USA), desmoglein-1 (Abcam #ab12077, Cambridge, USA) and α-SMA (Sigma-Aldrich, St Louis, USA).

A commercial kit with anti-mouse, -rabbit and -goat immunoglobulin secondary antibodies (LSAB+System HRP, Dako Agilent, Carpinteria, CA, USA) was used according to manufacturer's instructions and counterstained with hematoxylin.

All images were obtained by optical microscopy analyzed by NS-Elements software (Nikon Instruments, Melville, NY, USA).

Western Blotting

Skin samples were homogenized with a bead tissuelyser (Qiagen, Hilder, Germany) in RIPA buffer (Thermo Scientific, Waltham, USA) containing phosphatase and protease inhibitor cocktails (Roche Life Sciences, Branford, USA). Western blots were probed with antibodies against Ki-67 (Abcam #ab92742, Cambridge, USA), Ck-14 (Abcam #ab7800 Cambridge, USA),

desmoglein-1 (Abcam #ab12077, Cambridge, USA), α-SMA (Sigma-Aldrich, St Louis, USA) and β-actin (Abcam #ab8226, Cambridge, USA).

Quantitative PCR analysis

mRNA was isolated from skin samples using TRIzol reagent (Invitrogen, Cat# 15596026). 1 µg of RNA was reverse-transcribed into cDNA using the high-capacity cDNA SuperScript Vilo® kit, according to the manufacturer's instructions (Thermo Scientific, Cat# 11706050, Waltham, USA). qPCR was performed in an ABI 7500 Fast Real-Time PCR using SYBR green master mix (Thermo Scientific, Cat# 4385610) and primers for *Mmp-9, Foxo1, Ki-67, Ck-14, α-SMA* and *Desmoglein-1* (primer sequences available in Table 1). Expression levels of each target gene were normalized to *β-actin* and mRNA relative expression as internal efficiency controls. The mRNA fold change was calculated using the $2^{(-\text{Delta Delta C(t)})}$ method (33), and values expressed as fold increases relative to the negative control group.

Statistical analyses

Statistical analyses were performed using GraphPad Prism software, version 5.0. One-way analysis of variance (ANOVA) followed by Tukey's test was used to calculate statistical significance as appropriate. All data in this study are expressed as the mean ± standard deviation (S.D.). Values of $p < 0.05$ were considered significant.

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Author contributions: J.C.S. contributed to conception, design, acquirement, analysis and interpretation of all data, draft the manuscript and approve the final content. P.C.P. contributed with design, acquirement, analysis and interpretation of all data and draft the manuscript. S.R. contributed with data acquirement and technical support to in vitro methods. D.S.P.A. and S.S.M. contributed to conception, design, interpretation of all data, manuscript review, enhancement of intellectual content and final approve.

Competing interests: We have no competing or conflict of interests to declare.

Figures and Tables

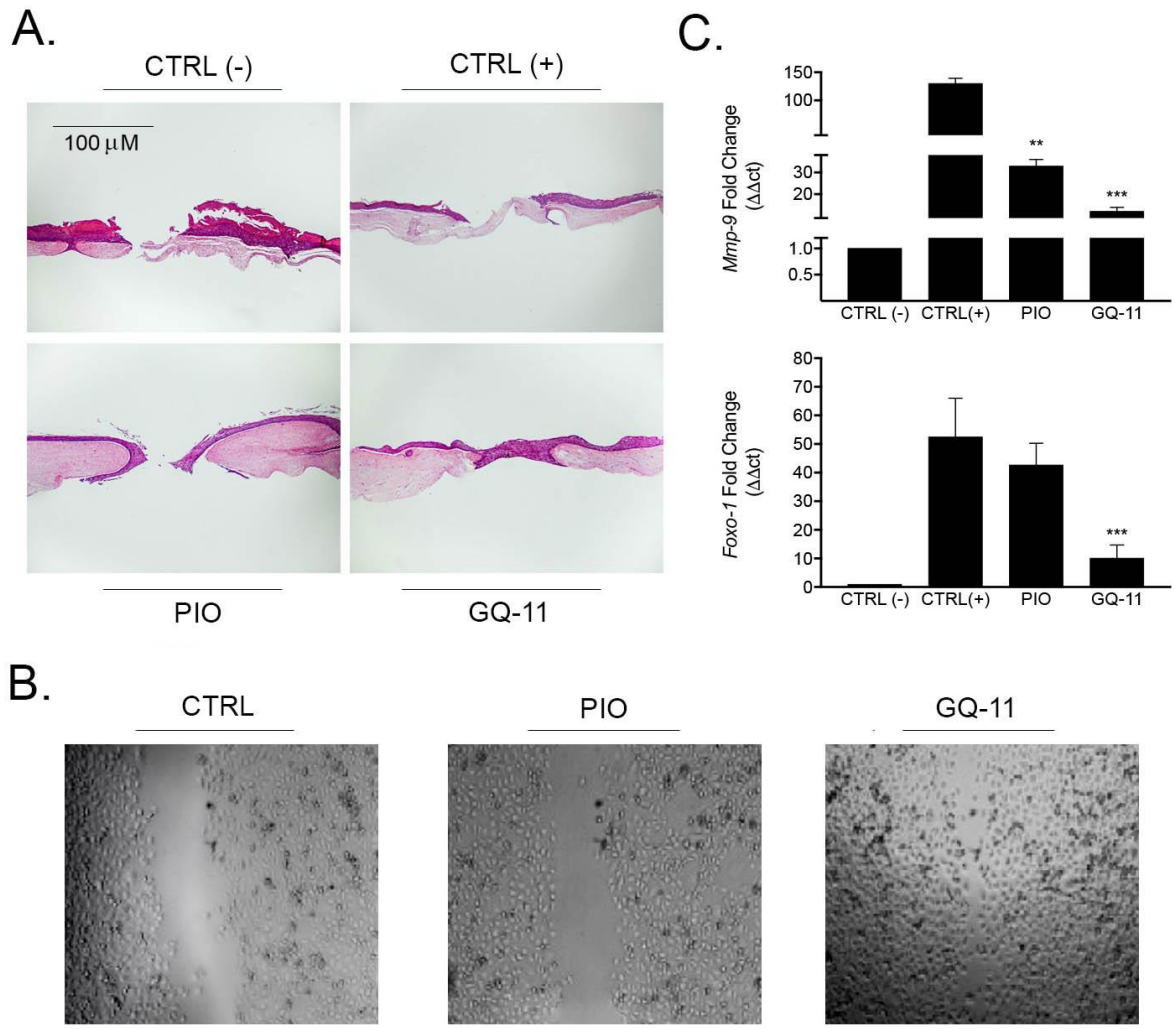

Figure 1. GQ-11 induces cells migration and proliferation. Representative Reconstructed Human Epidermis (RHE) based on glycated collagen, incisioned with 2 mm biopsy punch treated with DMSO (CTRL (+)), GQ-11 or Pio (2 mM) for 24 h, fixed and stained with H&E – magnification 4x (**A**). HUVECs were seeded and treated with DMSO (CTRL), Pio or GQ-11 (2 mM) after a straight lesion in the center of the plate wells – magnification 100x (**B**). Expression of *Pparα*, *Pparγ, Mmp-9* and *Foxo-1* (**C**). mRNA was quantified by qPCR. Negative controls (CTRL (-)) are represented by RHE based on control collagen, and were used in ddct calculations with *β-actin* as housekeeping gene (fold change). Data are expressed as the mean ± S.D. n=3. Statistical analyses were performed using ANOVA/Tukey's multiple comparison tests. **p < 0.01; ***p<0,001 *vs* CTRL(+).

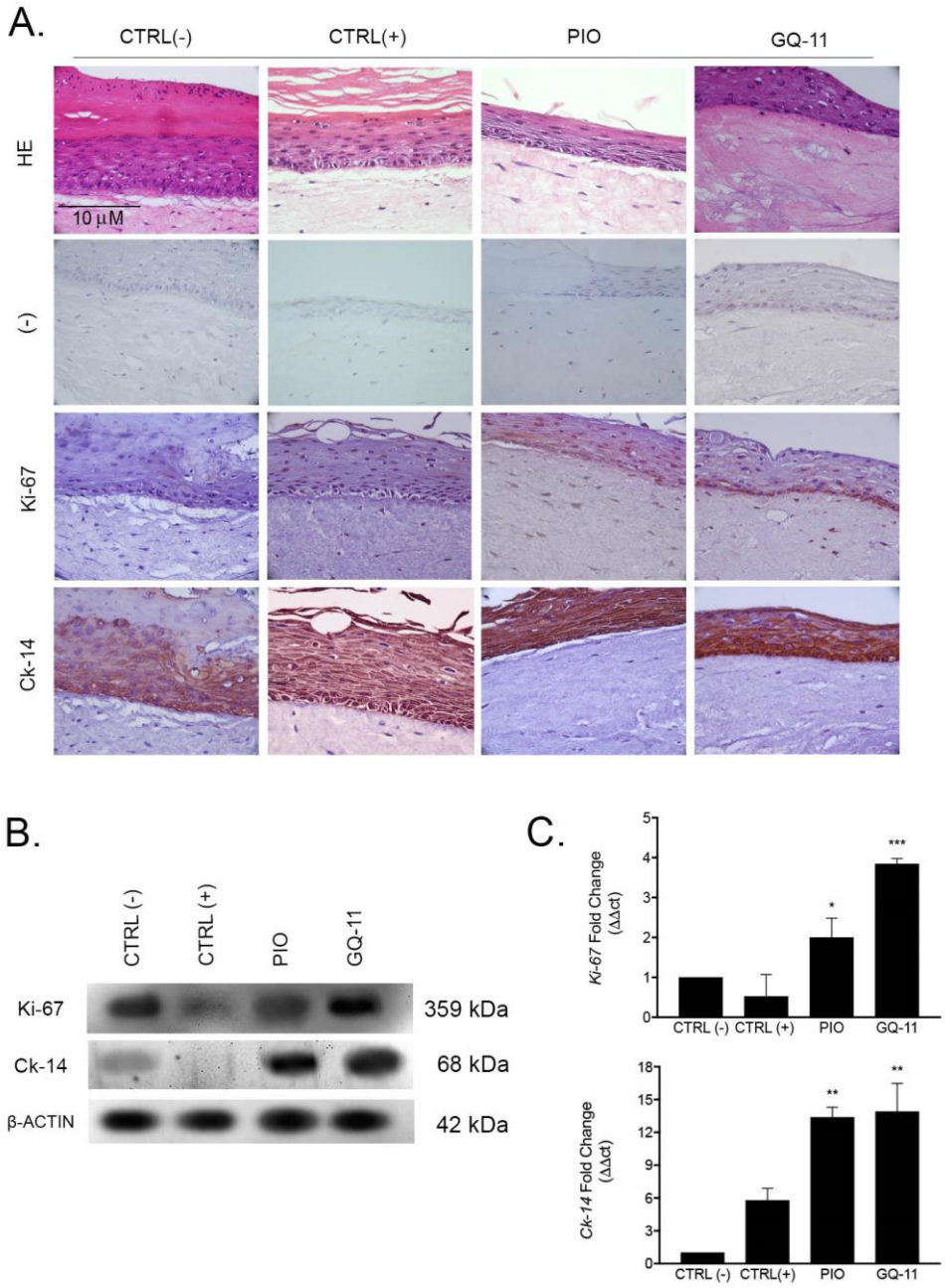

Figure 2. GQ-11 induces keratinocytes differentiation. Representative Reconstructed Human Epidermis (RHE) based on glycated collagen, incisioned with 2 mm biopsy punch treated with DMSO (CTRL (+)), GQ-11 or Pio (2 mM) for 24 h, fixed and and marked with *Ki-67* and *Ck-14* by immunohistochemistry – magnification 40x (**A**). Total Ki-67 and Ck-14 protein detected by Western Blot (**B**). Expression of Ki-67 and Ck-14 in RHE (**C**). mRNA was quantified by qPCR. Negative controls (CTRL (-)) are represented by RHE based on control collagen, and were used in ddct calculations with *β-actin* as housekeeping gene (fold change). Data are expressed as the mean ± S.D. n=3. Statistical analyses were performed using ANOVA/Tukey's multiple comparison tests. **p < 0.01; ***p<0,001 *vs* CTRL(+).

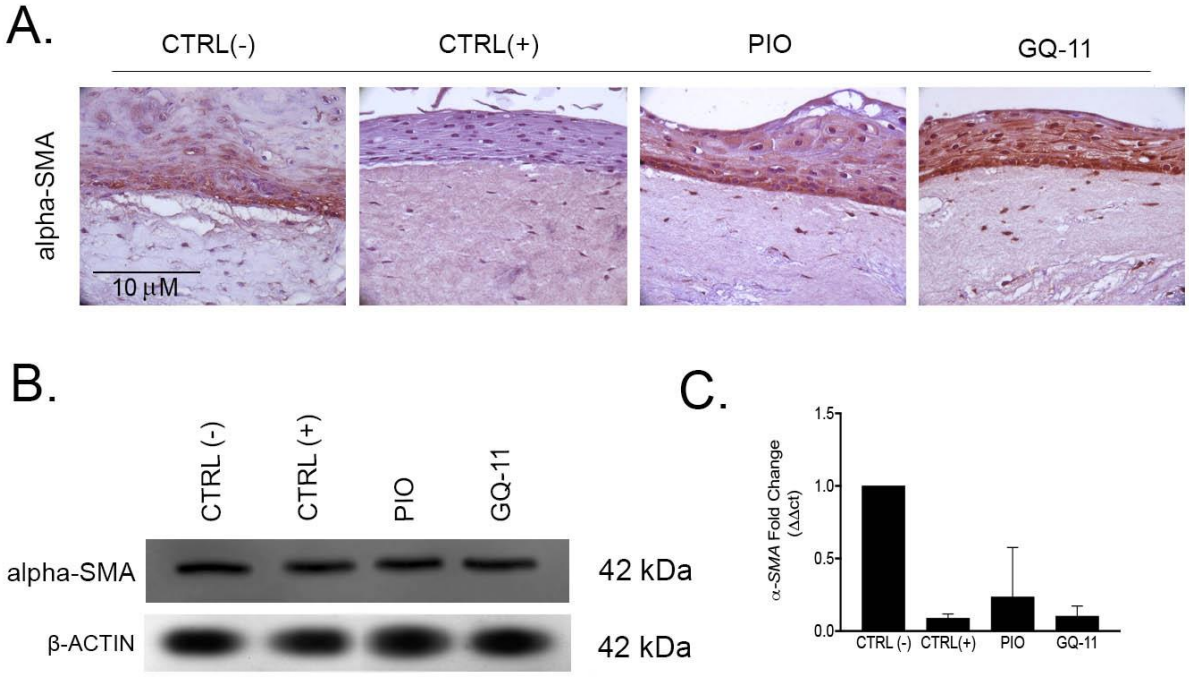

Figure 3. GQ-11 induces keratinocytes and fibroblasts differentiation. Representative Reconstructed Human Epidermis (RHE) based on glycated collagen, incisioned with 2 mm biopsy punch treated with DMSO (CTRL (+)), GQ-11 or Pio (2 mM) for 24 h, fixed and and marked with alpha-SMA by immunohistochemistry – magnification 40x (**A**). Total alpha-SMA protein detected by Western Blot (**B**). Expression of α-*SMA* in RHE (**C**) mRNA was quantified by qPCR. Negative controls (CTRL (-)) are represented by RHE based on control collagen, and were used in ddct calculations with β-*actin* as housekeeping gene (fold change). Data are expressed as the mean ± S.D. n=3. Statistical analyses were performed using ANOVA/Tukey's multiple comparison tests. **p < 0.01; ***p<0,001 *vs* CTRL(+).

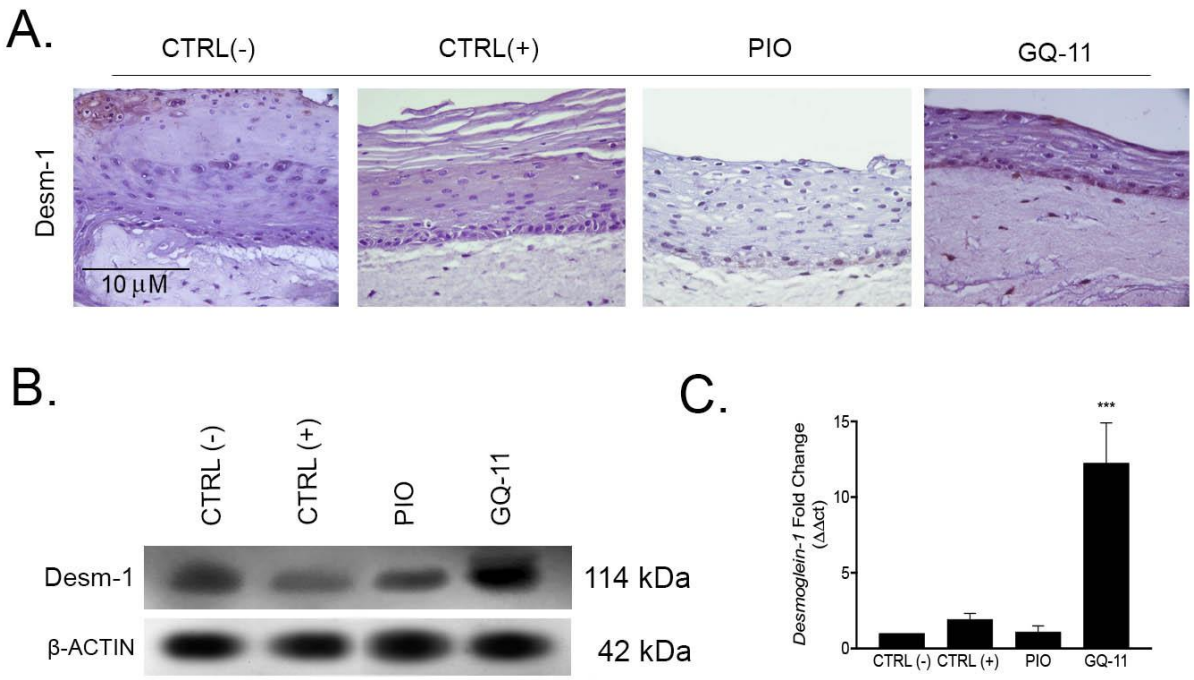

Figure 4. GQ-11 induces cell adhesion in basal epidermis. Representative Reconstructed Human Epidermis (RHE) based on glycated collagen, incisioned with 2 mm biopsy punch treated with DMSO (CTRL (+)), GQ-11 or Pio (2 mM) for 24 h, fixed and and marked with Desmoglein-1 by immunohistochemistry – magnification 40x (**A**). Total Desmoglein-1 protein detected by Western Blot (**B**). Expression of *Desmoglein-1* in RHE (**C**) mRNA was quantified by qPCR. Negative controls (CTRL (-)) are represented by RHE based on control collagen, and were used in ddct calculations with *β-actin* as housekeeping gene (fold change). Data are expressed as the mean ± S.D. n=3. Statistical analyses were performed using ANOVA/Tukey's multiple comparison tests. **p < 0.01; ***p<0,001 *vs* CTRL(+).

Table 1. Forward and reverse primers used for real-time PCR.

Gene	Forward primer (5′- 3’)	Reverse primer (5′- 3’)
α-SMA	ACTGGGACGACATGGAAAAG	TACATGGCTGGGACATTGAA
β-actin	GCCTTTGCCGATCCGC	GCCGTAGCCGTTGTCG
Ck-14	TCACAGCCACAGTGGACAAT	CCTTCAGGCTCTCAATCTGC
Desm-1	ACCGCATCTCTGGAGTAGGA	CCTGCAAATGTAGCCATTGA
FoxO-1	GATCCCGTAAGTCGGGCG	TCGATGGCCTTGGTGATGAG
Ki-67	AAAGAATTGAACCTGCGGAAGAGC	AGTATTATTTTGGCGTCTGGAGCG
Mmp-9	CCTTTGGACACGCACGAC	CCACCTGGGTTCAACTCACTC
Pparα	TCGTGCCGCTGAGCCTGG	GCCTCCGTCCGAGAGATGC
Pparγ	ATGCTGAAACCCTGAAGGTG	TGCTTGACCCTCAGAGACCT

7. CHAPTER IV: "A new dual PPARα/γ agonist (GQ-11), prevents ischemia-reperfusion damage in rats after supraceliac aorta clamping".

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International Journal of Cardiology

ORIGINAL ARTICLE: "A new dual-PPARα/γ agonist (GQ-11), prevents ischemia-reperfusion damage in rats after supraceliac aorta clamping".

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CONFLICT OF INTEREST

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INTRODUCTION

Ischemia-Reperfusion (I/R) is defined as a pathological condition characterized by restriction of blood supply followed by flow restoration and subsequent re-oxygenation, representing the major challenge during organ transplantation and general cardiothoracic surgery [1]. In its pathophysiology, not only the initial restriction of blood supply leads to a severe imbalance of metabolic demand but also the blood flow restoration exacerbates tissue injury and inflammation responses, contributing to a wide range of conditions [2, 3].

Ischemia condition is associated to the inhibition of oxygen-sensing prolylhyldroxylase (PHD) enzymes, once they require oxygen as a cofactor, which in turn leads to a post-translational activation of hypoxia and inflammatory signaling cascades, controlling transcription factors such as hypoxia-inducible factor (HIF) and nuclear factor-κB (NF-κb), sequentially inducing interleukyn-6 (IL-6) and tumor necrosis factor-α (TNF- α) [4, 5, 6]. Particularly, at this point it is reported increased permeability of capillaries and arterioles, following increased diffusion and fluid filtration across the tissues by activated endothelial cells, associated by itself to increased reactive oxygen species (ROS) and decreased nitric oxide (NO) production [7, 8]. In response to tissue damage, exacerbated free radicals release, oxidative stress induction as well as pro-inflammatory cytokines expression and activation of cell death programs are carried by reperfusion after ischemia. Therefore, the critical points of I/R are inflammation, headed by TNF-α and Il-6, and oxidative stress, headed by production of pro-oxidant mediators, depletion of superoxide dismutase (SOD), accumulation of free radicals and redox signaling disruption [9, 10].

PPARγ is a member of the nuclear receptor family, expressed throughout tissues and which primary controls adipocyte differentiation, glucose metabolism, and lipid homeostasis. However, apart from established metabolic actions, PPARγ also controls cell proliferation and has anti-inflammatory properties [11]. These PPARγ-related anti-inflammatory properties are provided by its expression in several immune cell types, such as activated macrophages, dendritic cells and activated T and B cells. Moreover, PPARγ is also able to regulate chemo-attraction and cell adhesion of these inflammatory cell types by inhibiting chemokine C-C ligand 2 (CCL-2), vascular cell adhesion protein (VCAM) and intracellular adhesion

molecule (ICAM) expression, improving endothelial function [12, 13]. Due to these regulatory properties, some reports have shown that the pre-treatment with a PPARγ agonist, pioglitazone, improved reperfusion injury in patients with myocardial infarction [14].

Nevertheless, the actual pharmacological approach to prevent I/R damage has so far been unsuccessful. In part because one unique drug cannot treat all metabolic disturbances caused with concecutive processes of I/R but also because in another hand, the use of these drugs implicates in important side effects. In the case of pioglitazone -and other thiazolidinediones (TZDs) use - fluid retention, body weight gain, peripheral edema, severe hepatotoxicity and cardiovascular risk are observed, encouraging the search for new derivatives with better therapeutic efficacy [15, 16, 17, 18, 19, 20].

GQ-11 is a new TZD derivative that, sharing a weak dual PPARα and PPARγ agonism, carries anti-inflammatory properties, modulating IL-6, MCP-1 and IL-10 expression and release, as previously reported [21]. Considering its properties, the aim of this study was to further investigate the effects of GQ-11 on inflammation and oxidative process in an I/R animal model.

METHODS

Animal Model and Treatment

Wistar rats, male, 60 days age, fed a standard diet, were hold at University of Genoa animal facilities. The study has been approved by the Animal Care Committee of University of Genoa (75/2018-PR: #22418.76) and followed guidance of Guide for the Care and the Use of Laboratory Animals [22] published by the US National Institutes of Health (NIH Publication No. 85-23, updated in 2011).

The animal protocol was designed to minimize pain or discomfort to the animals. The animals were acclimatized to animal facilities conditions (23 °C, 12 h/12 h light/dark, 50% humidity, ad libitum access to food and water) for 2 weeks prior to experimentation. Intragastric gavage administration was carried out with conscious animals, using straight gavage needles appropriate for the animal size (15-17g body weight: 22 gauge, 1 inch length, 1.25 mm ball diameter). All animals were euthanized by barbiturate overdose (intravenous injection, 150 mg/kg pentobarbital sodium) for tissue collection.

In a pre-surgery period of 7 days, animals were separated in 4 groups (*n*=3): no treated/no surgical procedure (negative control), treated with vehicle (NaCl 0.9% Tween 0.25% - positive control), 10 mg/kg pioglitazone or GQ-11 (new derivative). The treatment was conducted by gavage in the animal facilities procedure room of the university, daily, at 5 pm. After treatment period, animals were conducted to the animal facilities surgery room, anesthetized with diazepam (subcutaneous – 5 mg/kg), ketamine (intraperitoneal – 75 mg/kg) and xylazine (intraperitoneal – 10 mg/kg) and shaved on abdominal area to be submitted to a surgical procedure of supraceliac aorta clamping for 30 minutes, inducing complete abdominal ischemia (except the negative controls). After 30 minutes of ischemia, the clamp was removed for a 3 hours reperfusion. During ischemia and reperfusion protocols, animals were maintained in their own cages, under anesthesia and warm light for body temperature maintenance. After procedure, animals were submitted to positron emission tomography (PET) or euthanized by barbiturate overdose (intravenous injection, 150 mg/kg pentobarbital sodium) for organ collection (liver and bowel) to investigate gene expression, protein profile and enzymes activity. Samples were collected in cryovials and quickly frozen on dry ice until storage at -80°C.

mRNA isolation and Quantitative Real-Time PCR

For gene expression analysis, we first performed RNA isolation of frozen tissue with Trizol® reagent (Thermo Scientific, Cat#15596026) and reverse-transcribed it using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Cat#K1631), following company instructions.

Quantitative Real-Time PCR (qPCR) was performed using SYBR Green Master Mix (BioRad Laboratories, Cat#170-8887) and specific primers (TIB MolBiol – UniGe) as listed (Table 1). mRNA fold change was calculated using ΔΔCt method [24] and the expression of *Pparα, Pparγ, Lxrα, Il-1β, Ccl-2, Catalase, SOD2, GPx, Metallothionein 1, Vcam and Vegf* were evaluated with *Rpl-4* as housekeeping gene.

Western Blot

Total protein samples were extracted with RIPA buffer (Sigma-Aldrich, St Loius, MO, USA), with a protease, proteasome and phosphatase inhibitor cocktail. Next, 50 μg of total protein was resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane using Bio-Rad transfer system (Bio-Rad, Richmond, CA, USA). The membranes were blocked

with 5% non-fat-milk in TBST for 2 hours at room temperature with gentle shaking and incubated overnight at 4°C with primary antibodies against β-actin (Sigma-Aldrich, St Louis, MO, USA; 1:40,000), VCAM-1 (Santa Cruz Biotechnology, Dallas, TX, USA; 1:1000), IL-10 (Abcam, Cambridge, UK; 1:1000), TGF-β (Abcam, Cambridge, UK; 0.5 μg/ μL), SOD2 (Novus, Centennial, CO, USA; 1:1000) and GPx (Novus, Centennial, CO, USA; 1:1000). After washing, membranes were incubated with a secondary antibody (Sheep anti-rabbit IgG, 1:3000, horseradish peroxidase-conjugated; Amersham Bioscences, Piscataway, NJ, USA). Membranes were developed using enhanced chemiluminescence reagents (Bio-Rad, Richmond, CA, USA).

TBARS quantification

TBARS were quantified as described by Ohkawa *et al*., 1979 [25] where homogenized frozen tissue was incubated with thiobarbituric acid reagent to be centrifuged and read in spectrophotometer at 532 nm wavelength. Samples were normalized by protein concentration using Bradford method and malondialdehyde (MDA, Sigma-Aldrich, Cat#100683-54-3) was used as a standard control, in the concentrations of 0.5, 1, 2, 4 and 8 μmol/mL.

Evaluation of Glutathione Peroxidase Activity

Glutathione peroxidase activity was evaluated [26] with frozen tissue centrifuged to be read in spectrophotometer at 340 nm wavelength with 0, 1, 3 and 5 minutes intervals in a reaction with 2. mM sodium azide, 7.5 U/mL glutathione reductase, 10 mM reduced glutathione, 5% mercaptopropionic acid, 1.2 mM β-NADPH, 4.8 mM tert-butyl and 200 mM phosphate buffer. Samples were normalized by protein concentration using Bradford method.

Evaluation of Total Superoxide Dismutase Activity

Total superoxide dismutase activity was evaluated [27] with homogenized frozen tissue centrifuged to be read in spectrophotometer at 560 nm wavelength with 0, 1, 3 and 5 minutes intervals in a reaction with 0.1 mM EDTA, 400 μM nitroblue tetrazolium chloride (NBT), 98 μM β-NADPH, 160 μM phenazine

methyl sulfate (PMS) and 50 mM phosphate buffer. Samples were normalized by protein concentration using Bradford method.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism software, version 5.0. One-way analysis of variance (ANOVA) followed by Tukey's/ Dunnett's post-test was used to calculate statistical significance as appropriate. All data in this study are expressed as the mean ± standard deviation (S.D.). Values of $P < 0.05$ were considered significant.

RESULTS

GQ-11 treatment prevents ischemia/reperfusion (I/R) damage in rats after supraceliac aorta clamping

To explore the main effects of GQ-11 in I/R we first submitted the animals to PET, observing ^{18}F-FDG uptake in liver (Figure 1A) and bowel (Figure 1B). When analyzing SUVmax, a marked difference was observed between the negative and positive controls, indicating ^{18}F-FDG uptake increase in the group submitted to aorta clamping. Importantly, GQ-11 treatment showed a more marked decrease of ^{18}F-FDG uptake than Pioglitazone in both organs (Figure 1C), even after I/R induction, when compared to positive control.

GQ-11 treatment decreases pro-inflammatory markers in liver and bowel

In order to better understand the effect of GQ-11 and pioglitazone on ^{18}F-FDG uptake, we investigated their PPAR agonism and inflammatory markers. In this sense, both drugs showed no influence in *Ppar*α expression (Figure 1D), but increased *Ppar*γ expression in the organs (Figure 1D), as wells as of *Lxr*α (Figure 1D). As previously reported, this PPAR agonism is correlated to an important anti-inflammatory potential. Indeed, GQ-11 treatment down-regulated *Il-1*β*, Il-6* and *Ccl-2* expression when compared to positive control (Figure 2A) besides to decrease Vcam-1 expression (Figure 2A) and release (Figure 2C). In addition, GQ-11 also promoted downregulation of IL-6 either in liver or bowel, and IFN-γ and TNF-α in the liver (Figure 2B). IFN-γ and TNF-α levels were not detectable in bowel samples.

GQ-11 treatment increases anti-inflammatory markers in liver and bowel.

Furthermore, the anti-inflammatory potential provided by GQ-11 treatment was also extended to upregulation of anti-inflammatory markers, such as *Il-10* expression and *Tgf-β* (Figure 3A) and release (Figure 3C) in both organs. Moreover, it was also reported upregulation of *Vegf* and *Mt1* expression, both in GQ-11 and Pioglitazone groups (Figure 3B).

GQ-11 treatment modulates enzymes activity, preventing TBARs formation and oxidative stress in liver and bowel.

Besides inflammation, oxidative stress markers were also evaluated. GQ-11 and pioglitazone increased *catalase* and *GPx* expression (Figure 4A) and release (Figure 4B) when compared to positive control. Both treatments also enhanced GPx activity and diminished TBARs (Figure 4C).

DISCUSSION

In this study we show that GQ-11, a new dual PPARα and PPARγ agonist prevents I/R-induced damage caused by inflammation and oxidative stress processes in rats.

First focusing in the evaluation of general abdominal inflammation on pre-treated rats submitted to I/R, we evaluated ^{18}F-FDG uptake using PET. ^{18}F-FDG is a radiopharmaceutical analog of glucose, developed as a PET radiopharmaceutical, used for tumor diagnosis, therapy monitoring and experimental cancer research. However, ^{18}F-FDG uptake is not specific to malignancies and accumulates in sites with acute inflammation [28]. Glucose is the primary metabolic substrate of macrophages at the same time that immune cells activation requires increased glucose uptake, primarily through Glucose Transporter 1 (GLUT1). Some other studies also report that PPARγ agonists affect GLUT1 expression in immune cells, oppositely to tumor cells, decreasing glucose analogs uptake [29, 30, 31].

Our PET images and analysis showed lower ^{18}F-FDG uptake both in liver and bowel of animals submitted to I/R and treated with pioglitazone or GQ-11, when compared to positive control (Figure **1**), suggesting improve of I/R-related inflammation in those animals treated with PPARγ agonists. These results corroborate with other studies showing that cytokine modulation and anti-inflammatory effects of PPARγ

agonists are directly related to decreased ^{18}F-FDG uptake, especially through Glut1 downregulation in macrophages [32].

In order to further investigate the regulation of I/R-related inflammation promoted by GQ-11 and pioglitazone, we analyzed the expression of inflammatory markers in liver and bowel. In general, PPARγ agonists have been related to I/R protective effects through inhibition of intracellular cell adhesion molecules expression, reduction of neutrophil and macrophages infiltration, modulating the inflammatory response and the oxidative stress in endothelium, thus, playing a main role in modulation of inflammatory process during reperfusion [33]. In fact, our major findings regarding inflammation profile showed increased expression of *Pparγ* in liver and bowel, promoted by pioglitazone and GQ-11 (Figure 1), and a strictly related downregulation of *Il-1β, Il-6* and *Ccl-2* in the same treatment groups and organs (Figure 2).

Consistently, previous studies also showed anti-inflammatory effects promoted by GQ-11, through TNF-α, MCP-1 and IL-1β modulation in chronic low grade inflammation [21]. Other evidence suggest that IL-1β plays a key role in pathophysiology of hepatic I/R injury, some of them attesting that neutrophils and macrophages can contribute to cytokine maturation, independently of inflammasome [34]. In turn, if inflammatory process induces marked neutrophils and macrophages recruitment, VCAM-mediated cell adhesion, also regulated by pro-inflammatory interleukins and TNF-α, may allow cells activation and release of proteolytic enzymes, aggravating endothelial damage [35]. Indeed, our findings also showed important downregulation of VCAM expression and release (Figure 2), probably due to downregulation of *Ccl-2* and other pro-inflammatory markers, suggesting potential endothelial protection.

In addition, this pro-inflammatory markers control may be related to upregulation of IL-10 (Figure 3), an anti-inflammatory cytokine expressed by alternatively activated macrophages, as well as TGF-β, involved in pro-inflammatory cytokines repression in macrophages through NF-κb inhibition [36].

Several other studies also have reported the role of angiogenesis in ischemic injury protection, important to promote the regeneration process in ischemic tissues [37, 38]. The formation of new blood vessels in ischemia is induced primarily by VEGF and involves a harmonized cellular interplay, including endothelial, hematopoietic and mesenchymal cells to increase local blood flux and supply nutrients, oxygen and other required factors, ensuring local homeostasis, promoting re-endothelization and improving the hemodynamic function, preventing hypoxia and apoptosis [39]. In this context, the upregulation of *Vegf*

combined with VCAM downregulation found in animals treated with GQ-11 (Figures 2 and 3), also seems to be important especially in restoration of endothelium function.

Besides inflammation, we also demonstrated that pioglitazone and GQ-11 were involved in oxidative stress regulation including the modulation of metallothioneins (MTs) expression and antioxidant enzymes activity. The upregulation of *Mt1* (Figure 3), involved in zinc and copper transportation, is also implicated in inflammation and oxidative stress handling. MTs neutralize cell stress and toxicity induced by increased cytosolic free zinc – in its turn induced by TNF-α and IL-6 through Zip transporters upregulation. Moreover, MTs also mediate ROS-scavenging effects through STAT3, preventing I/R-related cell stress and apoptosis [40, 41, 42].

Many studies support the idea of inflammation and oxidative stress as interdependent and connected processes, which co-exist at inflamed sites. Here we understand that ROS formation and pro-inflammatory cytokines production lead to exacerbated oxidative damage and enhanced pro-inflammatory responses in a dynamic process. Importantly, redox processes are involved in diminished bioavailability of endothelial-derived NO, which may result in impaired endothelial-dependent vascular reactivity, also activating MMPs, inducing degradation of ECM components and a persistent endothelial dysfunction, antagonizing PPARγ and aggravating I/R condition [43, 44, 45].

Investigating the effects of GQ-11 and pioglitazone on enzymatic antioxidant defense and an oxidative stress marker, we found increased catalase expression and GPx activity besides decreased TBARS levels (Figure 4). It was previously reported that pioglitazone binds to catalase with strong affinity, increasing significantly its activity [46]. It is also show that mutant PPARγ in endothelial cells during stress conditions has endothelium impairment restored by superoxide scavenger, suggesting that PPARγ function loss is caused by exacerbated oxidative stress (47). Furthermore, PPARγ mutation is also associated to upregulation of pro-oxidant genes as well as dowregulation of important anti-oxidant genes including those of catalase and SOD. This is consistent with catalase and SOD being PPARγ-target genes [47].

Likewise, products from lipid peroxidation are active compounds over free radical pathways from ROS interaction with polyunsaturated fatty acids and are associated to tissue injury. Many reports link lipid hydroperoxides to DNA damage and describe the importance of SOD, catalase and GPx-mediated protection [48, 49, 50]. In fact, our findings show that treatment with PPARγ agonists increased catalase

expression and GPx activity leading to H_2O_2 consumption and decreased oxidative stress what can attenuate I/R-related damage.

Briefly, the strong protective effect observed for GQ-11 treatment in animals submitted to I/R, seems to be based on a set of regulations promoted by its weak PPARγ agonism including attenuation of inflammation and oxidative stress.

CONCLUSIONS

In conclusion, regulation of both inflammation and oxidative stress seems to be important targets in the search for I/R management, suggesting that PPARγ agonists, including GQ-11, could be important mediators in these conditions. Nevertheless, the balance between therapeutic and side effects is determinant to safe and effective prevention strategies.

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FIGURE LEGENDS

Figure 1. Positron emission tomography images of liver (A) and bowel (B), respective SUVmax (C) and expression of *Ppar-α*, *Ppar-γ* and *Lxr-α* (D) mRNA in liver and bowel after ischemia/reperfusion in Wistar rats pre-treated with vehicle, GQ-11 or pioglitazone. Injection of ^{18}F-FDG was performed after 2h20 of reperfusion and images were acquired after 3 hours of reperfusion. mRNA was quantified by qPCR. Negative controls (CTRL (-)) are represented by animals not submitted to ischemia/reperfusion and/or treatment, and were used in ddct calculations with Rpl-4 as housekeeping gene (fold change). Positive controls (CTRL (+)) are represented by animals submitted to ischemia/reperfusion and treated with vehicle. Data are expressed as the mean ± S.D. n=3. Statistical analyses were performed using ANOVA/Tukey's multiple comparison tests. *p < 0.05; **p<0,01, ***p<0,001 *vs* CTRL+.

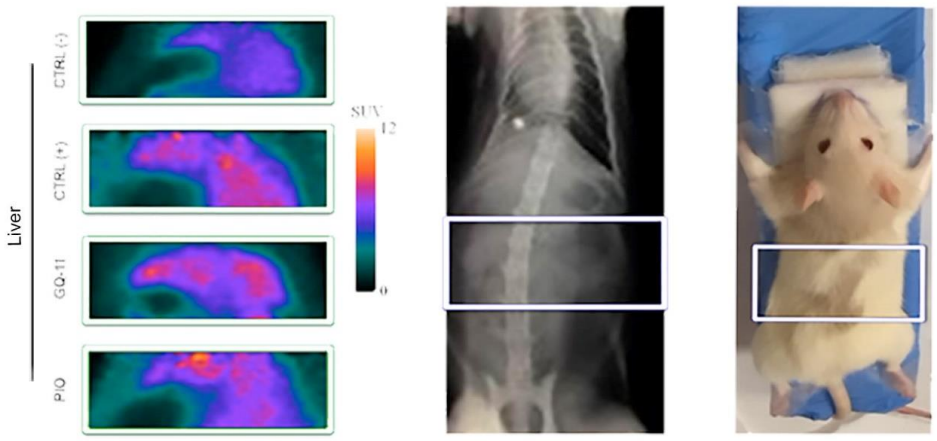
Figure 2. Expression of Il-1β, Il-6, Ccl-2 and Vcam-1 (A) in liver and bowel; total IL-6 content in liver and bowel, and IFN-γ and TNF-α content in liver (B); VCAM-1 protein (C) in liver and bowel of Wistar rats pre-treated with vehicle, GQ-11 or pioglitazone and submitted to ischemia/reperfusion. mRNA was quantified by qPCR. Negative controls (CTRL (-)) are represented by animals not submitted to ischemia/reperfusion and/or treatment, and were used in ddct calculations with Rpl-4 as housekeeping gene (fold change). Positive controls (CTRL (+)) are represented by animals submitted to ischemia/reperfusion and treated with vehicle. Total protein was quantified by cytometric bead array (CBA) and detected by western blot. Data are expressed as the mean ± S.D. n=3. Statistical analyses were performed using ANOVA/Tukey's multiple comparison tests. *p < 0.05; **p<0,01; ***p<0,001 *vs* CTRL+.

Figure 3. Il-10 and Tgf-β mRNA and IL-10 (A); Il-10 and Tgf-β protein (B); Vegf and Mt1 mRNA (C) in liver and bowel of Wistar rats pre-treated with vehicle, GQ-11 or pioglitazone and submitted to ischemia/reperfusion. mRNA was quantified by qPCR. Negative controls (CTRL (-)) are represented by animals not submitted to ischemia/reperfusion and/or treatment, and were used in ddct calculations with Rpl-4 as housekeeping gene (fold change). Positive controls (CTRL (+)) are represented by animals submitted to ischemia/reperfusion and treated with Vehicle. Total protein was quantified by cytometric bead array (CBA) and detected by western blot. Data are expressed as the mean ± S.D. n=3. Statistical analyses were performed using ANOVA/Tukey's multiple comparison tests. *p < 0.05; **p<0,01; ***p<0,001 *vs* CTRL+.

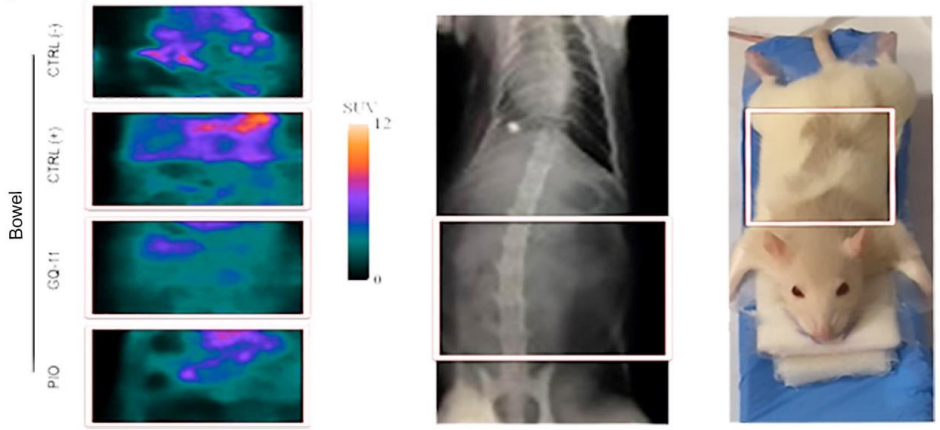
Figure 4. GPx, SOD2 and Catalase mRNA (A), GPx and SOD2 protein (B) and activity (C), and TBARs (C) in liver and bowel of Wistar rats pre-treated with vehicle, GQ-11 or pioglitazone and submitted to ischemia/reperfusion. mRNA was quantified by qPCR. Negative controls (CTRL (-)) are represented by animals not submitted to ischemia/reperfusion and/or treatment, and were used in ddct calculations with

Rpl-4 as housekeeping gene (fold change). Positive controls (CTRL (+)) are represented by animals submitted to ischemia/reperfusion and treated with Vehicle. Total protein detected by western blot. Data are expressed as the mean ± S.D. n=3. Statistical analyses were performed using ANOVA/Tukey's multiple comparison tests. *p < 0.05; **p<0,01; ***p<0,001 *vs* CTRL+.

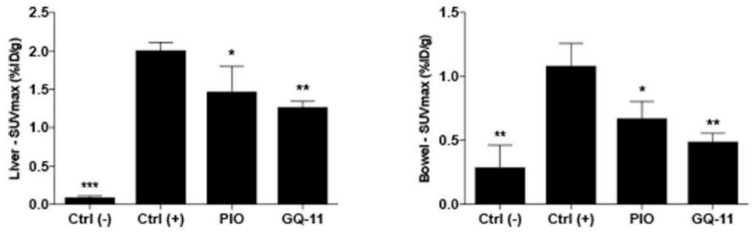
A.


Liver
CTRL (-)
CTRL (+)
GQ-11
PIO
SUV
12
0

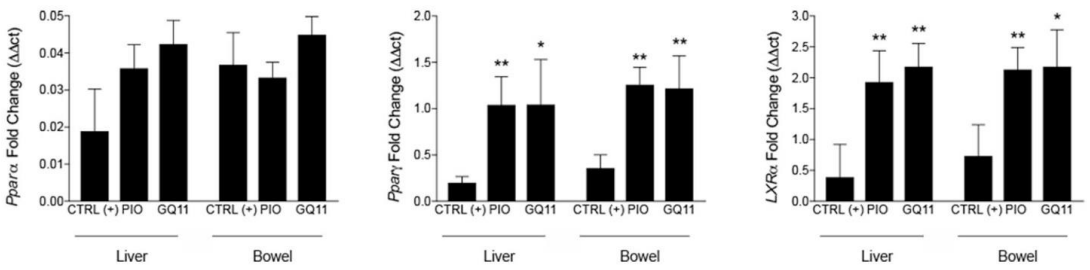
B.


Bowel
CTRL (-)
CTRL (+)
GQ-11
PIO
SUV
12
0

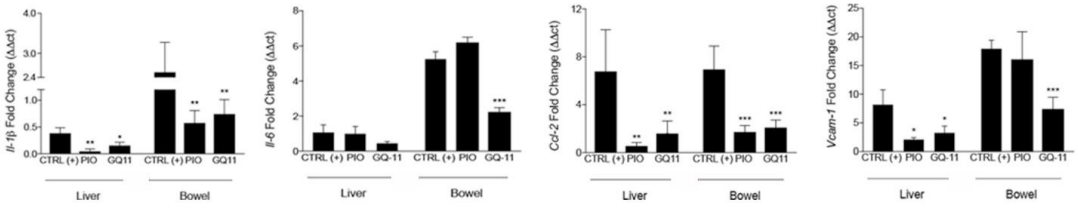
C.


Liver - SUVmax (%ID/g)
2.5
2.0
1.5
1.0
0.5
0.0
***
*
**
Ctrl (-)
Ctrl (+)
PIO
GQ-11
Bowel - SUVmax (%ID/g)
1.5
1.0
0.5
0.0
**
*
**
Ctrl (-)
Ctrl (+)
PIO
GQ-11

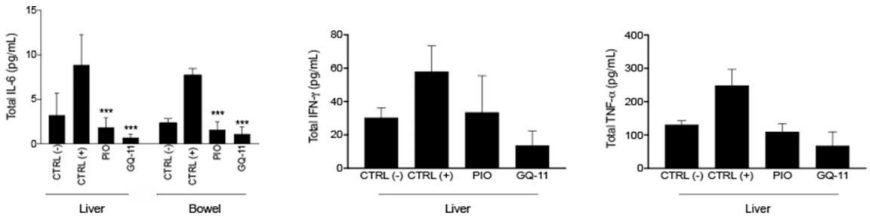
D.


Pparα Fold Change (ΔΔct)
0.05
0.04
0.03
0.02
0.01
0.00
CTRL (+) PIO GQ11 CTRL (+) PIO GQ11
Liver
Bowel
Pparγ Fold Change (ΔΔct)
2.0
1.5
1.0
0.5
0.0
**
*
**
**
CTRL (+) PIO GQ11 CTRL (+) PIO GQ11
Liver
Bowel
LXRα Fold Change (ΔΔct)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
**
**
*
CTRL (+) PIO GQ11 CTRL (+) PIO GQ11
Liver
Bowel

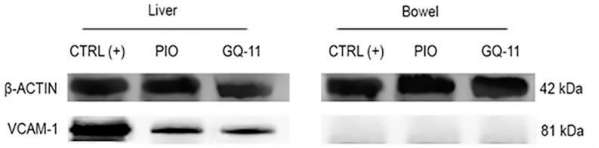
A.


Il-1β Fold Change (ΔΔct)
Il-6 Fold Change (ΔΔct)
Ccl-2 Fold Change (ΔΔct)
Vcam-1 Fold Change (ΔΔct)
CTRL (+) PIO GQ11
Liver
Bowel

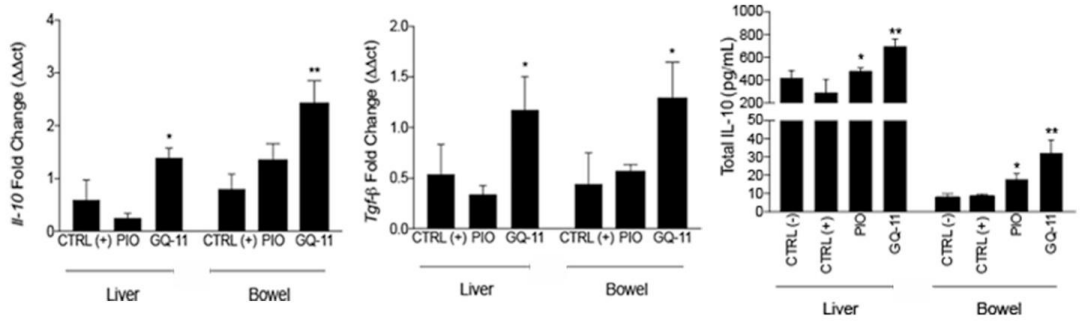
B.


Total IL-6 (pg/mL)
Total IFN-γ (pg/mL)
Total TNF-α (pg/mL)
CTRL (-) CTRL (+) PIO GQ-11
Liver
Bowel

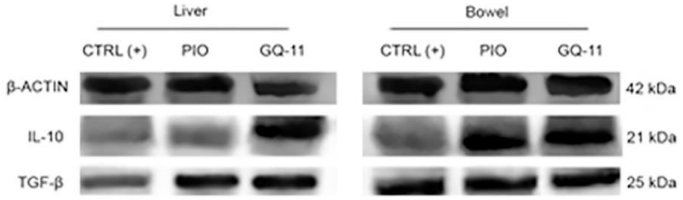
C.


Liver
Bowel
CTRL (+) PIO GQ-11
β-ACTIN
VCAM-1
42 kDa
81 kDa

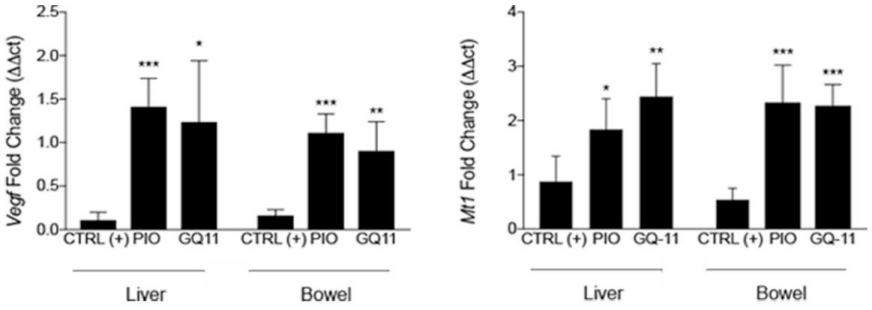
A.


Il-10 Fold Change (ΔΔct)
CTRL (+) PIO GQ-11 CTRL (+) PIO GQ-11
Liver
Bowel
Tgfβ Fold Change (ΔΔct)
CTRL (+) PIO GQ-11 CTRL (+) PIO GQ-11
Liver
Bowel
Total IL-10 (pg/mL)
CTRL (-) CTRL (+) PIO GQ-11 CTRL (-) CTRL (+) PIO GQ-11
Liver
Bowel

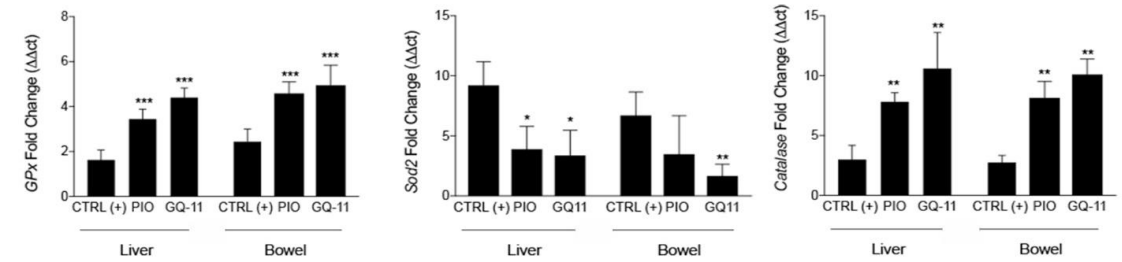
B.


Liver
Bowel
CTRL (+) PIO GQ-11 CTRL (+) PIO GQ-11
β-ACTIN
IL-10
TGF-β
42 kDa
21 kDa
25 kDa

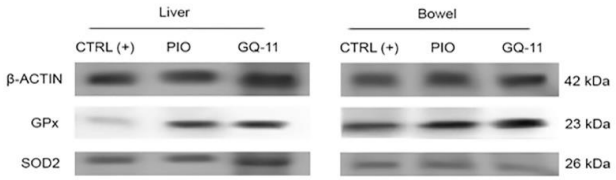
C.


Vegf Fold Change (ΔΔct)
CTRL (+) PIO GQ11 CTRL (+) PIO GQ11
Liver
Bowel
Mt1 Fold Change (ΔΔct)
CTRL (+) PIO GQ-11 CTRL (+) PIO GQ-11
Liver
Bowel

A.


GPx Fold Change (ΔΔct)
Sod2 Fold Change (ΔΔct)
Catalase Fold Change (ΔΔct)
CTRL (+) PIO GQ-11 CTRL (+) PIO GQ-11
CTRL (+) PIO GQ11 CTRL (+) PIO GQ11
CTRL (+) PIO GQ-11 CTRL (+) PIO GQ-11
Liver
Bowel

B.


Liver
Bowel
CTRL (+) PIO GQ-11 CTRL (+) PIO GQ-11
β-ACTIN
GPx
SOD2
42 kDa
23 kDa
26 kDa

C.

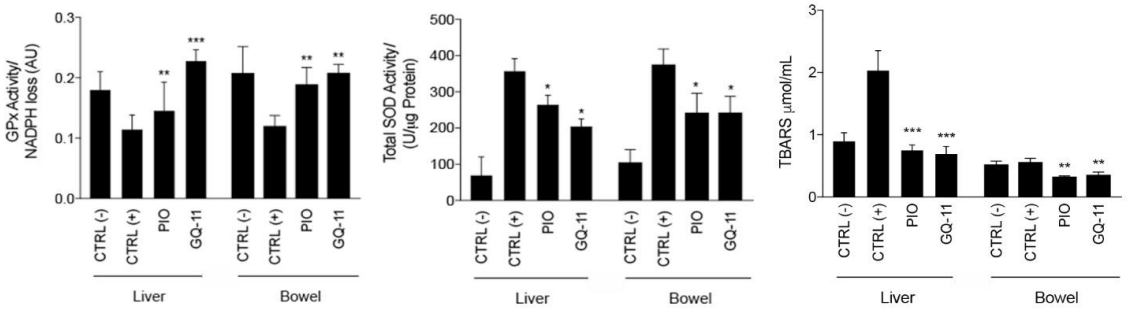

GPx Activity/ NADPH loss (AU)
Total SOD Activity/ (U/μg Protein)
TBARS μmol/mL
CTRL (-) CTRL (+) PIO GQ-11 CTRL (-) CTRL (+) PIO GQ-11
Liver
Bowel

TABLE 1. Forward and reverse primers used for real-time PCR.

Gene	Forward primer (5′- 3’)	Reverse primer (5′- 3’)
Pparα	CCCCACTTGAAGCAGATGACC	CCCTAAGTACTGGTAGTCCGC
Pparγ	CGGAGTCCTCCCAGCTGTTCGCC	GGCTCATATCTGTCTCCGTCTTC
Lxrα	TCAAGGGAGCACGCTACATT	CCTCTTCTTGACGCTTCAGTTT
Il-1β	AGGCATAACAAGCTCATCTGGG	CATCTGGACAGCCCAAGTCAAGG
Il-6	TACATATGTTCTCAGGGAGAT	GGTAGAAACGGAACTCCAG
Ccl-2	AGGGCTTGGGTTGGTTCATT	AGCTGCTATCTCTGGAAGCTG
Il-10	TAAGGGTTACTTGGGTTGCC	TATCCAGAGGGTCTTCAGC
Tgf-β	CCGCAACAACGCAATCTATG	AGCCCTGTATTCCGTCTCCTT
Vcam	AAGTGGAGGTCTACTCATTCC	GGTCAAAGGGGTACACATTAG
Vegf	TTTCGGGAACTAGACCTCTCACC	CTTCATGTCAGGCTTTCTGGATT
Mt1	CTGCTCCACCGGCGG	GCCCTGGGCACATTTGG
Cat	CCTGAGAGAGTGGTACATGC	CACTGCAAACCCACGAGGG
SOD2	CAGAAGGCAAGCGGTGAAC	TAGCAGGACAGCAGATGAGT
GPx	CATTGAGAATGTCGCGTCCC	TTGCCATTCTCCTGATGTCCG
Rpl-4	TCCGCCAGGCTAGGAATGTA	AGGTGGGATCTGTCTGCTAGT

RAW DATA

Figure 1C. SUVmax (%ID/g - Mean ± S.D.)

	Ctrl (-)	Ctrl (+)	PIO	GQ-11
Liver	0.09±0.02	2.01±0.10	1.47±0.33	1.27±0.08
Bowel	0.29±0.17	1.08±0.18	0.67±0.13	0.49±0.07

Figure 1D. *PPARα* fold change (Mean ± S.D.)

	Ctrl (+)	PIO	GQ-11
Liver	0.019±0.011	0.036±0.006	0.042±0.006
Bowel	0.037±0.009	0.033±0.004	0.045±0.005

Figure 1D. *PPARγ* fold change (Mean ± S.D.)

	Ctrl (+)	PIO	GQ-11
Liver	0.201±0.067	1.040±0.303	1.044±0.486
Bowel	0.360±0.143	1.258±0.187	1.219±0.352

Figure 1D. *LXRα* fold change (Mean ± S.D.)

	Ctrl (+)	PIO	GQ-11
Liver	0.392±0.528	1.930±0.507	2.81±0.377
Bowel	0.733±0.505	2.134±0.355	2.180±0.595

Figure 2A. *IL-1β* fold change (Mean ± S.D.)

	Ctrl (+)	PIO	GQ-11
Liver	0.384±0.103	0.045±0.046	0.155±0.061
Bowel	2.529±0.747	0.577±0.231	0.743±0.273

Figure 2A. *IL-6* fold change (Mean ± S.D.)

	Ctrl (+)	PIO	GQ-11
Liver	1.065±0.436	0.996±0.414	0.444±0.110
Bowel	5.247±0.428	6.203±0.298	2.237±0.246

Figure 2A. *Ccl-2* fold change (Mean ± S.D.)

	Ctrl (+)	PIO	GQ-11
Liver	6.788±3.475	0.559±0.281	1.595±1.047
Bowel	6.955±1.950	1.726±0.541	2.095±0.629

Figure 2A. *Vcam-1* fold change (Mean ± S.D.)

	Ctrl (+)	PIO	GQ-11
Liver	8.170±2.597	2.072±0.365	3.233±1.229
Bowel	17.940±1.476	16.084±4.823	7.438±2.044

Figure 2B. Total IL-6 (pg/mL - Mean ± S.D.)

	Ctrl (-)	Ctrl (+)	PIO	GQ-11
Liver	3.210±2.494	8.848±3.418	1.848±1.086	0.678±0.397
Bowel	2.313±0.526	7.688±0.794	1.578±0.888	1.090±0.835

Figure 2B. Total IFN-γ and TNFα in liver (pg/mL - Mean ± S.D.)

	Ctrl (-)	Ctrl (+)	PIO	GQ-11
IFN-γ	30.2±5.9	57.9±15.4	33.4±22.0	13.7±8.6
TNF-α	130.5±12.3	248.8±48.3	109.7±24.5	67.4±42.1

Figure 3A. *IL-10* fold change (Mean ± S.D.)

	Ctrl (+)	PIO	GQ-11
Liver	0.597±0.374	0.252±0.085	1.392±0.181
Bowel	0.798±0.285	1.364±0.295	2.440±0.410

Figure 3A. *Tgf-β* fold change (Mean ± S.D.)

	Ctrl (+)	PIO	GQ-11
Liver	0.539±0.294	0.339±0.089	1.174±0.330
Bowel	0.443±0.306	0.572±0.062	1.297±0.348

Figure 3A. Total IL-10 (pg/mL - Mean ± S.D.)

	Ctrl (-)	Ctrl (+)	PIO	GQ-11
Liver	419.0±67.6	291.5±116.2	478.8±28.6	695.5±65.4
Bowel	7.6±12.4	8.3±1.4	17.6±3.3	32.2±7.2

Figure 3C. *Vegf* fold change (Mean ± S.D.)

	Ctrl (+)	PIO	GQ-11
Liver	0.107±0.093	1.412±0.326	1.236±0.704
Bowel	0.162±0.065	1.112±0.217	0.903±0.335

Figure 3C. *Mt1* fold change (Mean ± S.D.)

	Ctrl (+)	PIO	GQ-11
Liver	0.873±0.428	1.832±0.522	2.439±0.557
Bowel	0.539±0.195	2.332±0.630	2.273±0.357

Figure 4A. *GPx* fold change (Mean ± S.D.)

	Ctrl (+)	PIO	GQ-11
Liver	1.624±0.442	3.440±0.449	4.398±0.431
Bowel	2.440±0.564	4.585±0.516	4.945±0.894

Figure 4A. *Sod2* fold change (Mean ± S.D.)

	Ctrl (+)	PIO	GQ-11
Liver	9.209±1.978	3.898±1.893	3.366±2.108
Bowel	6.708±1.944	3.485±3.193	1.667±0.983

Figure 4A. *Catalase* fold change (Mean ± S.D.)

	Ctrl (+)	PIO	GQ-11
Liver	2.995±1.193	7.821±0.752	10.606±3.005
Bowel	2.752±0.591	8.154±1.376	10.106±1.291

Figure 4C. GPx Activity – NADPH loss (AU - Mean ± S.D.)

	Ctrl (-)	Ctrl (+)	PIO	GQ-11
Liver	0.180±0.030	0.114±0.024	0.145±0.048	0.228±0.018
Bowel	0.208±0.044	0.120±0.017	0.189±0.028	0.208±0.014

Figure 4C. Total SOD Activity – NADPH loss (U/μg Protein - Mean ± S.D.)

	Ctrl (-)	Ctrl (+)	PIO	GQ-11
Liver	68.8±51.59	356.9±34.69	264.4±26.05	204.4±20.86
Bowel	105.3±34.92	375.6±42.33	242.6±53.38	242.9±44.42

Figure 4C. TBARS (μmol/mL - Mean ± S.D.)

	Ctrl (-)	Ctrl (+)	PIO	GQ-11
Liver	0.895±0.138	2.030±0.320	0.749±0.087	0.691±0.121
Bowel	0.524±0.053	0.563±0.059	0.328±0.011	0.359±0.042

8. CONCLUSIONS

The use of PPAR dual agonists, such as GQ-11, is a key new approach to prevent diabetes complications, such as delayed healing followed by amputations, and ischemia-reperfusion syndrome. Inflammation modulation – through cytokines balance promoted by GQ-11 treatment - showed to be the central regulator of imbalanced factors in both cases.

In addition, regulation of oxidative stress also showed important role along anti-inflammatory effects observed after aorta-clamping. Nevertheless, the balance between therapeutic and side effects is crucial for safe and effective strategies, besides drug delivery improves necessary for a better exploitation of the compound effects.

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10. APPENDIX

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

September 13, 2018

Timothy J. Koh
Kinesiology and Nutrition
M/C 994

Office of Animal Care and
Institutional Biosafety Committees (MC 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

Dear Dr. Koh:

The protocol indicated below was reviewed at a convened ACC meeting in accordance with the Animal Care Policies of the University of Illinois at Chicago on **08/21/2018**. *The protocol was not initiated until final clarifications were reviewed and approved on* **09/08/2018**. *The protocol is approved for a period of **3*** years with annual continuation.

Title of Application: Mechanisms of Wound Inflammation and Healing

ACC Number: 18-129

Initial Approval Period: 09-08-2018 to 08-21-2019

Current Funding: ***Portions of this protocol are supported by the funding sources indicated in the table below.***
Number of funding sources: 1

Funding Agency	**Funding Title**			**Portion of Proposal Matched**
NIH	*Macrophage Phenotype And Impaired Wound Healing*			*All matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
RO1GM092850-05	*Funded*		*UIC*	*Timothy Koh*

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare (OLAW), NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the funding proposal are matched to this ACC protocol.**

In addition, all investigators are responsible for ensuring compliance with all federal and institutional policies and regulations related to use of animals under this protocol and the funding sources listed on this protocol. Please use OLAW's "*What Investigators Need to Know about the Use of Animals*" (http://grants.nih.gov/grants/olaw/InvestigatorsNeed2Know.pdf) as a reference guide. Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,

Amy Lasek

Amy Lasek, PhD
Chair, Animal Care Committee
AL /*ss*
cc: BRL, ACC File, Jingbo Pang, Pijus Barman

Ministero della Salute

Direzione Generale della Sanità Animale e dei Farmaci Veterinari
Ufficio 6

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OGGETTO: D.lgs. 26/2014 in materia di protezione degli animali utilizzati a fini scientifici. Trasmissione autorizzazione ai sensi dell'art. 31.
Autorizzazione n° 75/2018-PR (Risposta a **prot. 22418.76 del 11/10/2017**)

Si trasmette l'autorizzazione **n° 75/2018-PR** rilasciata in data **5/2/2018**, ai sensi dell'art. 31 del D.lgs. 26/2014.

IL DIRETTORE DELL'UFFICIO 6
Dr. Vincenzo Ugo SANTUCCI

Responsabile del procedimento: Dr. Vincenzo Ugo SANTUCCI
Referenti: G. Aleandri - g.aleandri-esterno@sanita.it
SAN MARTINO Palombo 02MMXVIII

Ministero della Salute

DIREZIONE GENERALE DELLA SANITÀ ANIMALE E DEI FARMACI VETERINARI
UFFICIO 6

Autorizzazione n. 75 **/2018-PR**

IL DIRETTORE GENERALE

Vista la domanda di autorizzazione del progetto di ricerca **"Modulazione della risposta infiammatoria mediante somministrazione di agonisti PPARγ (pioglitazone) in corso di ischemia viscerale chirurgicamente indotta in modello animale"**, ex articolo 31 del decreto legislativo 4 marzo 2014, n. 26, acquisita con prot. 22418.76 del 11/10/2017 ed integrazione del 25/01/2018, inoltrata dall'**Ospedale Policlinico San Martino – Sistema Sanitario Regione Liguria – Istituto di Ricovero e Cura a Carattere Scientifico per l'Oncologia, sede legale in Genova, Largo R. Benzi, 10**, per il tramite dell'Organismo preposto al benessere degli animali di cui all'articolo 25 del menzionato d.lgs. n. 26/2014, e finalizzata all'esecuzione del progetto di ricerca come descritto nella documentazione allegata alla domanda;

Visto l'articolo 31, comma 1, del d.lgs. n. 26/2014, nel quale il Ministero della salute è individuato quale autorità competente al rilascio dell'autorizzazione all'esecuzione di progetti di ricerca che prevedono l'utilizzo di animali a fini scientifici secondo le finalità di cui all'articolo 5, comma 1, in continuità con la precedente normativa di cui al decreto legislativo 27 gennaio 1992, n. 116;

Visti gli articoli 12, 13, 14, 15, 16 e 17 del succitato d.lgs. n. 26/2014, che stabiliscono le modalità di utilizzazione degli animali nelle procedure condotte a fini scientifici;

Visti gli articoli 31, 32, 34 e 35, nonché gli Allegati IV, VI, VII e IX del d.lgs. n. 26/2014, che fissano i requisiti generali per il rilascio di autorizzazione per progetti di ricerca;

Vista la nota n. 2767 del 20/01/2018, con cui l'Istituto Superiore di Sanità ha comunicato l'esito positivo della valutazione tecnico-scientifica sul progetto di ricerca;

Considerato che ricorrono i requisiti stabiliti dal d.lgs. n. 26/2014 per il progetto da autorizzare;

Preso atto che il responsabile del progetto di ricerca, ai sensi dell'articolo 3, comma 1, lettera g) del d.lgs. n. 26/2014, è il **Prof. Domenico PALOMBO**;

Considerato che lo **stabilimento utilizzatore dell'Ospedale Policlinico San Martino – Sistema Sanitario Regione Liguria – Istituto di Ricovero e Cura a Carattere Scientifico per l'Oncologia, sito in Genova, Largo R. Benzi, 10, è regolarmente autorizzato con n° 14/2017-UT del 31/05/2017, ai sensi del D.lgs. 26/2014**;

Visto l'articolo 4, comma 2 e l'articolo 16 del decreto legislativo 30 marzo 2001, n. 165 e successive modifiche, recanti le funzioni dei dirigenti di uffici dirigenziali;

Responsabile del procedimento: Dr. Vincenzo Ugo SANTUCCI
Referenti: Zappulla - f.zappulla-esterno@sanita.it; Aleandri - g.aleandri-esterno@sanita.it

AUTORIZZA

1. L'**Ospedale Policlinico San Martino – Sistema Sanitario Regione Liguria – Istituto di Ricovero e Cura a Carattere Scientifico per l'Oncologia, sede legale in Genova, Largo R. Benzi, 10,** all'esecuzione del progetto di ricerca ex articolo 31 del decreto legislativo 4 marzo 2014, n. 26, in conformità a quanto indicato nella richiesta di autorizzazione citata in premessa ed, in particolare, con riferimento a:

"Modulazione della risposta infiammatoria mediante somministrazione di agonisti PPARγ (pioglitazone) in corso di ischemia viscerale chirurgicamente indotta in modello animale"

2. Il **Prof. Domenico PALOMBO** quale responsabile del progetto di ricerca, ai sensi dell'articolo 3, comma 1, lettera g) del d.lgs. n. 26/2014;

3. L'**Ospedale Policlinico San Martino – Sistema Sanitario Regione Liguria – Istituto di Ricovero e Cura a Carattere Scientifico per l'Oncologia, sede legale in Genova, Largo R. Benzi, 10,** all'esecuzione del progetto di ricerca di cui al punto 1 nello **stabilimento utilizzatore dell'Ospedale Policlinico San Martino – Sistema Sanitario Regione Liguria – Istituto di Ricovero e Cura a Carattere Scientifico per l'Oncologia, sito in Genova, Largo R. Benzi, 10, regolarmente autorizzato con n° 14/2017-UT del 31/05/2017, ai sensi del D.lgs. 26/2014.**

Alla conclusione del progetto di ricerca il responsabile di cui all'articolo 3, comma 1, lettera g) del d.lgs. n. 26/2014 dovrà inviare alla scrivente Amministrazione la documentazione necessaria ai fini della valutazione retrospettiva come previsto dall'articolo 32 del citato decreto.

La presente autorizzazione ha una durata di sessanta mesi e può essere revocata secondo quanto previsto dall'articolo 31, comma 15 del d.lgs. n. 26/2014.

IL DIRETTORE GENERALE
(Dott. Silvio BORRELLO)

05 FEB 2018

Responsabile del procedimento: Dr. Vincenzo Ugo SANTUCCI
Referenti: Zappulla - f.zappulla-esterno@sanita.it; Aleandri - g.aleandri-esterno@sanita.it

OSPEDALE POLICLINICO SAN MARTINO
Sistema Sanitario Regione Liguria
Istituto di Ricovero e Cura a Carattere Scientifico per l'Oncologia

ANIMAL FACILITY

Genoa, March 22, 2019

To whom it may concern:

The Animal Facility of "Ospedale Policlinico San Martino" is responsible for the care, welfare and health of laboratory animals (rodents, lagomorphs, cephalopods), included immunologically compromised nude, SCID mice, humanized models NOD/SCID - NSG mice and several genetically modified models. The animal facility is designated as a Research Facility by the Italian Ministry of Health (N° 14/2017-UT – 31/05/2017).

This facility includes 5 animal rooms under exclusion barrier (ventilated caging systems) and 15 animal rooms under conventional conditions, a rodent quarantine, procedure rooms, imaging resources, a surgery suite, a large efficient cage wash area with a modern rack washer and two large autoclave units. This facility provides space for the housing of over 7000 mice, 500 rats, 30 rabbit and 12 octopus. Some rodents housed in this facility are housed in individually ventilated caging (IVC) systems.

In our facility we test for rodent pathogens every year using one or more of the following testing strategies: direct colony animal sampling or sentinel rodent testing. If using rodent sentinels, all sentinel animals should have a minimum of 8 weeks of exposure and preferably 12-16 weeks of exposure before testing to allow time for sero-conversion and to develop a detectable parasite infestation.

The well-being and state of health of experimental animals is regularly monitored by the veterinarian with a view to prevent the pain or avoidable suffering, distress or lasting harm. In the Animal Facility the "Three Rs" tenet is applied by scientists in the respect of the national current regulations regarding the protection of animals used for scientific purpose (D. Lvo 4 marzo 2014, n. 26, legislative transposition of Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes).

The "Three Rs" tenet - Replacement, Reduction and Refinement - is grounded in the premise that animals should be used only if a scientist's best efforts to find a non-animal alternative have failed, and that when animals are needed, only the most humane methods should be used on the smallest number of animals required to obtain valid information.

Use of the Three Rs tenet assists in improving the welfare of animals used in science in several ways: it addresses a range of concerns about scientific animal use; it places a focus on individual animals; it adapts and responds to new information; it balances the needs of science and the needs of the animals; and it unites disparate groups with an interest in the welfare of animals used in science.

Research protocols presented by **Prof. Domenico Palombo** are included in those reviewed and approved by the OPBA (Institutional Animal Welfare Body) and authorized by the Italian Ministry of Health on **October 11 2017** with the **N. 75/2018-PR**.

Yours sincerely,

Michele Cilli DVM

Designated Veterinary

Animal Welfare Body Coordinator

Janus - Sistema Administrativo da Pós-Graduação

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas
Documento sem validade oficial

FICHA DO ALUNO

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Curso:	Doutorado
Programa:	Farmácia (Fisiopatologia e Toxicologia)
Área:	Fisiopatologia
Data de Matrícula:	14/04/2015
Início da Contagem de Prazo:	14/04/2015
Data Limite para o Depósito:	15/04/2019
Orientador na USP:	Prof(a). Dr(a). Dulcineia Saes Parra Abdalla - 10/08/2016 até o presente. Email: dspa@usp.br

Aluno USP em convênio de dupla titulação com instituição estrangeira

Instituição Conveniada:	Università degli Studi di Genova, Itália
Orientador na Instituição Conveniada:	Patrizia Perego
Proficiência em Línguas:	Inglês, Aprovado em 14/04/2015
Data de Aprovação no Exame de Qualificação:	Aprovado em 26/01/2017
Data do Depósito do Trabalho:	
Título do Trabalho:	
Data Máxima para Aprovação da Banca:	
Data de Aprovação da Banca:	
Data Máxima para Defesa:	
Data da Defesa:	
Resultado da Defesa:	
Histórico de Ocorrências:	Primeira Matrícula em 14/04/2015

Aluno matriculado no Regimento da Pós-Graduação USP (Resolução nº 6542 em vigor de 20/04/2013 até 28/03/2018).
Última ocorrência: Prorrogação em 26/03/2019
Impresso em: 27/03/2019 11:26:35

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Sigla	Nome da Disciplina	Início	Término	Carga Horária	Cred.	Freq.	Conc.	Exc.	Situação
FBC5766-4/2	Tópicos em Análises Clínicas IV	04/08/2015	16/11/2015	15	1	90	A	N	Concluída
QBQ5802-5/1	Metodologias em Bioquímica e Biologia Molecular: Conceitos e Aplicações (Instituto de Química - Universidade de São Paulo)	21/08/2015	01/10/2015	60	4	85	A	N	Concluída
FBC5700-6/2	Aterosclerose: Fisiopatologia, Diagnóstico e Terapêutica	03/11/2015	30/11/2015	60	4	100	A	N	Concluída
Atividade do Programa	Trabalho Publicado na revista Pharmacological Research, com o trabalho intitulado: "New PPARy partial agonist improves obesity-induced metabolic alterations and atherosclerosis in LDLr(-/-) mice", v. 104, p. 49 a 60, United States - 2015 (1)	17/12/2015	17/12/2015	-	2	-	-	-	-
Atividade do Programa	Participou da Etapa de Estágio Supervisionado em Docência do Programa de Aperfeiçoamento de Ensino junto à Disciplina FBC0416 Fisiopatologia II, ministrada aos alunos de graduação do curso de Farmácia e Bioquímica da Faculdade de Ciências Farmacêuticas da Universidade de São Paulo (2)	01/02/2016	30/06/2016	-	3	-	-	-	-
FBC5792-3/3	Tópicos em Análises Clínicas III	08/03/2016	20/06/2016	15	1	87	A	N	Concluída
BMA5887-2/6	Princípios de Tomografia Computadorizada e Ressonância Magnética Aplicados à Anatomia (Instituto de Ciências Biomédicas - Universidade de São Paulo)	15/03/2016	23/05/2016	30	2	100	A	N	Concluída
BTC5828-1/3	Biossegurança em Laboratórios (Curso Interunidades: Biotecnologia - Universidade de São Paulo)	04/04/2016	12/06/2016	60	4	90	A	N	Concluída
FBC5748-4/2	Trabalhos Científicos: da Elaboração à Publicação	05/04/2016	17/05/2016	60	4	80	A	N	Concluída

	Créditos mínimos exigidos		Créditos obtidos
	Para exame de qualificação	Para depósito de tese	
Disciplinas:	0	20	25
Estágios:			
Total:	0	20	25

Créditos Atribuídos à Tese: 167

Observações:

1) Créditos atribuídos de acordo com o Artigo 64 do Regimento de Pós-Graduação e aprovados pela Comissão de Pós-Graduação, em Sessão de 15/12/2016.

2) Créditos atribuídos de acordo com o disposto na Portaria GR-3588 e GR-4391 - PAE, de 31.08.09 e aprovados pela Comissão de Pós-Graduação, em Sessão de 15/12/2016.

Conceito a partir de 02/01/1997:

A - Excelente, com direito a crédito; B - Bom, com direito a crédito; C - Regular, com direito a crédito; R - Reprovado; T - Transferência.

Um(1) crédito equivale a 15 horas de atividade programada.

Jacqueline Cavalcante Silva

https://orcid.org/0000-0001-6838-2621

Also known as

SILVA JC

Country

Brazil

Education and qualifications (6)

University of Sao Paulo: São Paulo, São Paulo
2015-04-01 to 2019-06-01 | PhD in Pathophysiology (Clinical Analysis)
Education
Source: Jacqueline Cavalcante Silva

Universita degli Studi di Genova: Genova, Liguria
2015-04-01 to 2019-06-01 | PhD in Translational Medicine (DISC)
Education
Source: Jacqueline Cavalcante Silva

University of Illinois at Chicago: Chicago, IL
2017-02-01 to 2017-10-01 | PhD - Visitor Student (Kinesiology and Nutrition)
Qualification
Source: Jacqueline Cavalcante Silva

University of Sao Paulo: São Paulo
2012-06-01 to 2015-03-01 | Masters in Science / Biochemistry (Clinical Analysis)
Education
Source: Jacqueline Cavalcante Silva

University of Illinois at Chicago: Chicago, IL
2014-02-01 to 2014-09-01 | Masters - Visitor Student (Medicine, Pathology and Pharmacology)
Qualification
Source: Jacqueline Cavalcante Silva

Universidade Presbiteriana Mackenzie: Sao Paulo, SP
2007-01-01 to 2010-12-01 | Bachelor in Nutrition
Education
Source: Jacqueline Cavalcante Silva

Funding (5)

Modulation of inflammation and angiogenesis by a new thiazolidine compound (GQ-11) in visceral ischemia
Fundação de Amparo à Pesquisa do Estado de São Paulo
(São Paulo, São Paulo)
2017-09 to 2018-04 | Grant
GRANT_NUMBER: 16/19737-1
Source: Jacqueline Cavalcante Silva

Modulation of tissue repair by a new thiazolidine compound (GQ-11) on experimental models of insulin resistance and visceral ischemia
Fundação de Amparo à Pesquisa do Estado de São Paulo
(São Paulo, São Paulo)
2017-03 to 2017-09 | Grant
GRANT_NUMBER: 16/16850-1
Source: Jacqueline Cavalcante Silva

Action of a novel thiazolidinedione (GQ-11) in tissue repair process on experimental models of insulin resistance and vascular surgery
Fundação de Amparo à Pesquisa do Estado de São Paulo
(São Paulo, São Paulo)
2016-04 to 2019-05 | Grant
GRANT_NUMBER: 16/002333
URL: https://bv.fapesp.br/en/bolsas/163724/action-of-a-novel-thiazolidinedione-gq-11-in-tissue-repair-process-on-experimental-models-of-insu/ (https://bv.fapesp.br/en/bolsas/163724/action-of-a-novel-thiazolidinedione-gq-11-in-tissue-repair-process-on-experimental-models-of-insu/)
Source: Jacqueline Cavalcante Silva

Immunomodulation and angiogenic activity of new thiazolidine compounds in wound healing process on MKR mice

Fundação de Amparo à Pesquisa do Estado de São Paulo (São Paulo, São Paulo)
2014-02 to 2014-09 | Grant
GRANT_NUMBER: 13/23140-2
Source: Jacqueline Cavalcante Silva

Investigation of new thiazolidine compounds effects in animal model of metabolic syndrome

Fundação de Amparo à Pesquisa do Estado de São Paulo (São Paulo, São Paulo)
2012-09 to 2015-03 | Grant
GRANT_NUMBER: 12/14360-6
Source: Jacqueline Cavalcante Silva

Works (4 of 4)

New PPAR agonist (GQ-11) improves wound healing in diabetic mice

Advances in Wound Care
2019-02-01 | journal-article
AUTHENTICUSID: https://www.liebertpub.com/doi/abs/10.1089/wound.2018.0911?journalCode=wound
Source: Jacqueline Cavalcante Silva

Serum amyloid A links endotoxaemia to weight gain and insulin resistance in mice.

Diabetologia
2016-04 | journal-article
PMID: 27126803
DOI: 10.1007/s00125-016-3970-z
Source: Europe PubMed Central

New PPARγ partial agonist improves obesity-induced metabolic alterations and atherosclerosis in LDLr(-/-) mice.

Pharmacological Research

2016-02-01 | journal-article

ARK: 10.1016/j.phrs.2015.12.010

Source: Jacqueline Cavalcante Silva

GQ-11: A new PPAR agonist improves obesity-induced metabolic alterations in LDL -/- mice

International Journal of Obesity

journal-article

AUTHENTICUSID: https://www.ncbi.nlm.nih.gov/pubmed/29453462

Source: Jacqueline Cavalcante Silva

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