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Prevention of steatohepatitis by pioglitazone: Implication of adiponectin-dependent inhibition of SREBP-1c and inflammation☆

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***Background/Aims*: Peroxisome proliferator-activated receptor gamma (PPARγ) agonist drugs, like pioglitazone (PGZ), are proposed as treatments for steatohepatitis. Their mechanisms of action remain ill-clarified.**

***Methods*: To test the hypothesis that PGZ improves steatohepatitis through adiponectin-dependent stimulation of AMPK and/or PPARα, mice lacking adiponectin (Adipo$^{-/-}$) or the AMPKα1 catalytic subunit (AMPKα1$^{-/-}$) or wild-type (Wt) mice were fed the methionine and choline deficient (MCD) diet, supplemented or not with PGZ.**

***Results*: In Wt mice, PGZ increased circulating levels of adiponectin and reduced the severity of MCD-induced steatohepatitis but there was no evidence of activation of AMPK or PPARα and their downstream targets. By contrast, PGZ completely repressed nuclear translocation of SREBP-1c, a key transcription factor for *de novo* lipogenesis. This effect was lacking in Adipo$^{-/-}$ mice in which PGZ failed to prevent steatohepatitis. Surprisingly, AMPKα1$^{-/-}$ mice were resistant to MCD-induced steatohepatitis, a status also associated with repression of SREBP-1c.**

***Conclusions*: The preventive effect of PGZ on MCD-induced steatohepatitis depends on adiponectin upregulation but apparently does not involve AMPK or PPARα activation. The inhibition of SREBP-1c and dependent repression of lipogenesis are likely to participate in this effect. The mechanisms by which PGZ and adiponectin control SREBP-1c and inflammation remain to be elucidated.**

***Keywords*: MCD diet; AMPK; PPARalpha; SREBP-1c; Mouse**

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Abbreviations: Adipo, adiponectin; AdipoR, adiponectin receptor; AMPK, AMP-activated protein kinase; ACC, acetyl-CoA carboxylase; ACO, acyl CoA oxidase; cDNA, complementary deoxyribonucleic acid; FA, fatty acid; FAS, fatty acid synthase; HSP-90, heat shock protein 90; IκBα, inhibitor of NF-κB alpha; MCD, methionine and choline deficient; mTOR, mammalian target of rapamycin; NASH, non-alcoholic steatohepatitis; NF-κB, nuclear factor-kappa B; p70S6K, p70 *Ribosomal* S6 Kinase, PGC-1α, peroxisome proliferator-activated receptor-gamma coactivator 1 alpha; PGZ, pioglitazone; PPAR, peroxisome proliferator-activated receptor; RPL19, ribosomal protein L19; SCD-1, stearoyl-CoA desaturase 1; SREBP-1c, sterol regulatory element binding protein 1c; TNF, tumor necrosis factor; Wt, wild-type.

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1. Introduction

Non-alcoholic steatohepatitis (NASH), histologically characterised by steatosis, hepatocellular damage, inflammation and variable fibrosis, is often seen in patients with obesity and/or insulin resistance [1]. NASH is therefore now recognised as the hepatic complication of the metabolic syndrome [2]. To date, there is no consensus on effective therapy [3]. Clinical studies provide evidence that insulin-sensitizing thiazolidinedione (TZD) drugs may be beneficial [4–6]. The mechanism of action of TZD in NASH is however incompletely understood. Three strategies of action, possibly acting in synergy, could be proposed: (i) TZD stimulate adipocyte proliferation and fat mass expansion favouring the storage of fat in adipose tissue, sparing peripheral tissues, such as liver, from fat accumulation and lipotoxicity [7], (ii) TZD modulate adipocytokine release, in particular they induce the production of adiponectin, favouring increased fatty acid (FA) oxidation, decreased lipogenesis [8] and decreased inflammation and/or (iii) TZD may modulate lipid metabolism, hepatic inflammation and fibrosis by activation of peroxisome proliferator-activated receptor gamma (PPARγ), PPARα, adiponectin or other cytokines [9–11].

TZD induces the expression of adiponectin by the adipose tissue, and adiponectin is believed to mediate some of the TZD's metabolic effects in target tissues, in particular, insulin sensitization [12]. Adiponectin increases FA β-oxidation in muscle [13] and decreases hepatic lipid content in *ob*/*ob* mice [14]. Also, adiponectin has direct anti-fibrotic [15] and anti-inflammatory properties [16].

In the liver, adiponectin signals through two receptors, AdipoR1 and AdipoR2, which activate two distinct pathways. AdipoR1 is ubiquitously expressed, while AdipoR2 is most abundantly expressed in the liver [17]. Downstream of AdipoR1, adiponectin activates the AMP-activated protein kinase (AMPK), a pivotal kinase for the regulation of cellular energy homeostasis. In particular, activation of AMPK stimulates FA β-oxidation and inhibits hepatic *de novo* lipogenesis [18,19]. Activated, AMPK also negatively regulates transcription and activation of sterol regulatory element binding protein-1c (SREBP-1c), which controls the expression of nearly all genes required for *de novo* synthesis of FAs and triglyceride synthesis [18]. AdipoR2 preferentially recruits PPARα, to stimulate oxidation of lipids and prevent inflammation through inhibition of NF-κB [16].

We previously demonstrated that pioglitazone (PGZ), a synthetic PPARγ activator, prevents steatohepatitis in mice [20]. In this work, we used adiponectin-deficient mice (Adipo$^{-/-}$) to determine whether adiponectin is required for the preventive effect of PGZ. In order to assess the pathway recruited by adiponectin, we analysed the PPARα and AMPK pathways and used mice with impaired AMPK activity.

2. Materials and methods

2.1. Animals studies

AMPKα1 null-mice (AMPKα1$^{-/-}$) and AMPKα1$^{+/+}$ littermates were kindly provided by B. Viollet, Institut Cochin, Paris [21]. Adiponectin-knockout mice (Adipo$^{-/-}$) [22] were bred in our animal facility. C57BL6/J (Wt) mice were used as controls. All animals were kept in a temperature and humidity-controlled environment in a 12 h light–dark cycle. At all times, they were allowed free access to water and diet. The animals were handled according to the guidelines for humane care for laboratory animals in accordance with EU Regulation and the study protocol was approved by the local ethics committee.

Female (10 weeks old) Adipo$^{-/-}$, AMPKα1$^{-/-}$ and control mice were randomly assigned to one of the 3 dietary groups and received (a) a standard powdered diet (Pavan Service Carfil Quality, Belgium) (control group), (b) a methionine–choline deficient diet (cat. no 960439, ICN, USA) (MCD group) or (c) a MCD diet supplemented with PGZ 0.01% (wt/wt) (MCD + PGZ group) for 5 weeks ($n = 5$ mice/group). At the time of sacrifice (between 8:00 and 10:00 AM), blood was collected, the liver rapidly excised and weighed. A part of the liver was fixed in 4% formalin, the other immediately snap-frozen and kept at −80 °C until use.

In a separate experiment, C57BL6/J mice received a single dose of PGZ (100 mg/kg body weight) by gavage and were sacrificed after 0, 30 min, 12 h, 24 h, and 48 h ($n = 5$–6 mice/time point).

2.2. Total RNA extraction, Reverse Transcription and Quantitative PCR

Total RNA was extracted using TRIpure Isolation Reagent (Roche Diagnostics, Belgium). cDNA were synthesized and served as template to quantitate hepatic mRNA expression using real time PCR as previously described [23]. Primer pairs for transcripts of interest were designed using the Primer Express design software (Applied Biosystems) and listed in Table 1. RPL19 RNA was chosen as an invariant standard. Results were expressed as fold expression relative to expression in the control group (value set at 1) using the delta Ct method [23].

2.3. Western blot analyses

Liver homogenates, nuclear extracts and cytosolic fractions were prepared and protein concentrations determined as described elsewhere [24]. Proteins were separated by SDS–PAGE and transferred onto PVDF membrane (PolyScreen, NEN Life Science Products, USA). The membranes were then exposed to antibodies raised against: AMPKα1, AMPKα2, phospho-AMPKα (Thr 172) (Cell Signaling, USA), SREBP-1c (Santa-Cruz, USA), phospho-mTOR (Thr 2446) (Cell Signaling, USA), mTOR (Cell Signaling, USA), phospho-p70S6K (Thr 389) (Cell Signaling, USA), p70S6K (Cell Signaling, USA), Iκbα (Santa-Cruz, USA). The quantification of immune-reactive proteins was obtained by densitometry using the Gel Doc™ XR System 170-8170 device and software (Bio-Rad, USA). Expression of Heat Shock Protein (HSP)-90 (BD Transduction Laboratories, USA) or β-actin (Sigma, Germany) was used as a loading control.

2.4. AMPK assay

Total AMPK activity was assayed on liver homogenates after precipitation with 10% (wt/v) poly(ethylene glycol) 6000 (Merck, Belgium) [25]. 100 μg protein from liver homogenates were immunoprecipitated with protein-G-sepharose and isoform specific antibodies to the α1 or α2 catalytic subunits of AMPK (Cell Signaling) measure the AMPKα1 and α2 activities [26]. One unit of AMPK activity corresponds to 1 nmol of product formed per min under the assay conditions.