

RESEARCH PAPER

Metformin induces PGC-1 α expression and selectively affects hepatic PGC-1 α functions

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BACKGROUND AND PURPOSE

The objective of this study was to determine how the AMPK activating antidiabetic drug metformin affects the major activator of hepatic gluconeogenesis, PPAR γ coactivator 1 α (PGC-1 α) and liver functions regulated by PGC-1 α .

EXPERIMENTAL APPROACH

Mouse and human primary hepatocytes and mice *in vivo* were treated with metformin. Adenoviral overexpression, siRNA and reporter gene constructs were used for mechanistic studies.

KEY RESULTS

Metformin increased PGC-1 α mRNA and protein expression in mouse primary hepatocytes. 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) (another AMPK activator) had the opposite effect. Metformin also increased PGC-1 α in human primary hepatocytes; this effect of metformin was abolished by AMPK inhibitor compound C and sirtuin 1 siRNA. AMPK overexpression by AMPK-Ad also increased PGC-1 α . Whereas metformin increased PGC-1 α , it down-regulated gluconeogenic genes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase). Furthermore, metformin attenuated the increase in PEPCK and G6Pase mRNAs induced by PGC-1 α overexpression, but did not affect PGC-1 α -mediated induction of mitochondrial genes. Metformin down-regulated several key transcription factors that mediate the effect of PGC-1 α on gluconeogenic genes including Krüppel-like factor 15, forkhead box protein O1 and hepatocyte NF 4 α , whereas it increased nuclear respiratory factor 1, which is involved in PGC-1 α -mediated regulation of mitochondrial proteins.

CONCLUSIONS AND IMPLICATIONS

Down-regulation of PGC-1 α is not necessary for suppression of gluconeogenic genes by metformin. Importantly, metformin selectively affects hepatic PGC-1 α -mediated gene regulation and prevents activation of gluconeogenesis, but does not influence its regulation of mitochondrial genes. These results identify selective modulation of hepatic PGC-1 α functions as a novel mechanism involved in the therapeutic action of metformin.

Abbreviations

ACC, acetyl-CoA; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; ATP5 β , ATP synthase subunit β ; Cox, cytochrome c oxidase; Cox4i1, cytochrome c oxidase IV isoform 1; CRTc, cAMP response element-binding protein coactivator; Cys, cytochrome c; db-cAMP, dibutyryl-cAMP; FOXO1, forkhead box protein O1; G6Pase, glucose-6-phosphatase; HNF-4 α , hepatocyte NF 4 α ; KLF15, Krüppel-like factor 15; LKB1, liver kinase B1; NAD, nicotinamide adenine dinucleotide; NRF1, nuclear respiratory factor 1; PEPCK, phosphoenolpyruvate carboxykinase; PGC-1 α , PPAR γ coactivator 1 α ; SHP, small heterodimer partner NR0B2; SIRT1, sirtuin 1

Introduction

Metformin is a biguanide class drug widely used in the treatment of type 2 diabetes (Krentz and Bailey, 2005). Although metformin has been in clinical use for decades, its mechanism of action has only been partly solved. The major action of metformin is thought to be the suppression of hepatic glucose output mediated through the down-regulation of the expression of the rate-limiting gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) (Minassian *et al.*, 1998; Yuan *et al.*, 2002).

PPAR γ coactivator 1 α (PGC-1 α) is an inducible transcriptional coactivator involved in number of metabolic functions in high-energy tissues (Lin, 2009). PGC-1 α is an important regulator of mitochondrial biogenesis and function (Handschin, 2009). In addition it has many tissue specific functions. In liver, PGC-1 α stimulates gluconeogenesis, fatty acid oxidation and haeme biosynthesis (Handschin, 2009). Hepatic PGC-1 α is induced by fasting and subsequently up-regulates gluconeogenesis and the key enzymes involved, PEPCK and G6Pase. As PGC-1 α has been shown to play an important role in regulation of gluconeogenesis, it has been suggested that it is involved in the hepatic action of metformin (Viollet *et al.*, 2009). Shaw *et al.* reported that metformin, by affecting LKB1/AMPK activity, phosphorylates and translocates cAMP-responsive element-binding protein (CREB) coactivator (CRTC2; also known as TORC2) out of the nucleus (Shaw *et al.*, 2005). In turn, CRTC2 up-regulates the levels of PGC-1 α expression, which may have a role in the antihyperglycaemic mechanism of action of metformin (Viollet *et al.*, 2009). However, according to a recent study, neither AMPK nor LKB1 is needed for the suppression of hepatic glucose output by metformin (Foretz *et al.*, 2010). Interestingly, although PGC-1 α is a key regulator of energy metabolism, the effects of metformin on hepatic PGC-1 α expression and function have not been specifically studied.

In the current study, we have assessed the effects of metformin on PGC-1 α . In contrast to previous assumptions, we showed that metformin induces PGC-1 α expression in primary hepatocytes. Moreover, metformin selectively modified PGC-1 α -regulated liver functions and impaired the ability of PGC-1 α to up-regulate gluconeogenic genes.

Methods

Reagents

DMSO, dibutyryl-cAMP (db-cAMP), 8-bromo-cAMP, metformin, 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), compound C, esiGFP, esiAMPK α 1, esiAMPK α 2, siSIRT, qPCR oligos, FLAG immunoprecipitation kit and mouse anti- β -actin IgG (A1978), were purchased from Sigma-Aldrich (St. Louis, MO, USA). A769662 was from Tocris Bioscience (Bristol, UK). PEPCK, AMPK α 1, AMPK α 2 gene expression assays and negative control siRNAs used with siSIRT1 were from Life Technologies (Carlsbad, CA, USA). Rabbit anti-PGC-1 α IgG (sc-13067), rabbit anti-hepatocyte NF 4 α (HNF-4 α) IgG (sc-8987) and HRP-conjugated rabbit IgG (sc-2004) were from Santa Cruz Biotechnology (Santa Cruz,

CA, USA). Mouse anti-acetylated lysine (Ac-Lys) IgG (#9681) and rabbit anti-phospho-acetyl-CoA carboxylase (pACC) IgG (#3661) were from Cell Signaling Technology (Danvers, MA, USA). HRP-conjugated anti-mouse IgG was from GE Healthcare (Little Chalfont, UK). Mouse anti-PGC-1 α IgG (ST1202), used in the immunodetection of immunoprecipitated PGC-1 α was from Calbiochem (Darmstadt, Germany).

Cell cultures

Fresh, human primary hepatocytes from one donor were from Biopredic international (Rennes, France). Cells were incubated with 0.5, 1 and 2 mM metformin for 48 h in standard William's E medium, which was replaced after 24 h. In another experiment with human primary hepatocytes from three donors, cells were prepared as described previously (Krausova *et al.*, 2011). Hepatocytes were treated with 0.5, 1 and 2 mM metformin for 24 h in standard cultivation medium. Mouse primary hepatocytes were isolated from male DBA/2 or C57BL/6 mice (Center for Experimental Animals, University of Oulu, Finland) aged 8–10 weeks, as described previously (Arpiainen *et al.*, 2008), and cultivated in William's E medium containing 2.0 g·L⁻¹ D-glucose and ITS (insulin 5 mg·L⁻¹, transferrin 5 mg·L⁻¹, sodium selenate 5 μ g·L⁻¹, Sigma-Aldrich). The cultures were maintained for an additional 12–24 h before being infected with an adenovirus or subjected to various treatments.

HepG2 cells (ATCC, Manassas, VA, USA) were cultured in DMEM medium [Invitrogen (Carlsbad, CA, USA), Life Technologies] containing 4.5 g·L⁻¹ D-glucose, 10% (v v⁻¹) FBS, 100 U·mL⁻¹ of penicillin and 100 U·mL⁻¹ streptomycin (Invitrogen, Life Technologies).

Adenoviruses and treatments of cells

Recombinant adenoviruses expressing green fluorescent protein (GFP-Ad) and PGC-1 α were generated as described previously (Arpiainen *et al.*, 2008). Adenovirus expressing human constitutively active AMPK α 1 (AMPK-Ad) subunit containing amino acids 1–321 was a gift from Prof. Risto Kerkelä (University of Oulu). PGC-1 α phosphorylation mutant (T177A S538A) plasmid was from Addgene (<http://www.addgene.org> plasmid 18093) and adenovirus was generated as described previously (Arpiainen *et al.*, 2008). Mouse primary hepatocytes were either infected or treated with chemicals in William's E growth medium (Sigma-Aldrich) without serum for the indicated time periods before RNA or protein extractions. A769662, Compound C and db-cAMP were dissolved in DMSO and all other chemicals were dissolved in water. Control samples were either treated with equal amount of vehicle or left untreated.

siRNA experiments

Mouse primary hepatocytes were transfected with X-tremeGENE siRNA transfection reagent (Roche, Basel, Switzerland) using 150 pmol per well (in six-well plates) or 45 pmol per well (in 12-well plates) of siRNAs and 2000 ng per well (in six-well plates) of esiRNAs. Three pooled negative control siRNAs were used with siSIRT1 and esiGFP was used with esiAMPK as a negative control (Table 1). After an initial 4 h incubation period siSIRT1 transfection medium was replaced by fresh medium containing 0.25 mM metformin.

Table 1

Sequences of the PCR primers and siRNA oligos

Gene	Forward	Reverse
mPGC-1 α	GCAGGTGGAACGAACTGAC CTGCTCTGGTTGGTGAGGA ^a	CTCAGCCTGGGAACACGTTA GCAGGCTCATTGTTGACTG ^a
mPEPCK	GGTGTCTACTGGGAAGGCATC	CAATAATGGGGCACTGGCTG
mSHP	GTACCTGAAGGGCAGCATCC	ACCAGGGCTCCAAGACTTCAC
mG6Pase	CATCAATCTCCTCTGGGTGG	TGCTGTAGTAGTCGGTGTC
hPGC-1 α	CCCAGATCTTCTGAACTTG	GCTAGCAAGTTTGCCTCATTC
18S	CGCCGCTAGAGGTGAAATTC	CCAGTCGGCATCGTTTATGG
mCycs	CCAAATCTCCACGGTCTGTTC	ATCAGGGTATCCTCTCCCCAG
mATP5b	GGTTCATCCTGCCAGAGACTA	AATCCCTCATCGAACTGGACG
mCox4i1	ATTGGCAAGAGAGCCATTCTAC	CACGCCGATCAGCGTAAGT
mCox1	ATTCCTTCATGTCCGACGAG	ACTGAGAAGCCCCCTCAAAT
mCox2	ACGAAATCAACAACCCCGTA	GGCAGAACGACTCGGTTATC
mNRF1	AGCACGGAGTGACCCAAAC	TGTACGTGGCTACATGGACCT
mKLF15	GAGACCTTCTCGTCACCGAAA	GCTGGAGACATCGTGTCAT
mFOXO1	CCCAGGCCGGAGTTTAACC	GTTGCTCATAAAGTCGGTGCT
mHNF-4 α	GTGGCGAGTCCTTATGACACG	GCTGTTGGATGAATTGAGGTTGG
hPEPCK	Hs00159918_m1 (Life Technologies)	
mAMPK α 1(Prkaa1)	Mm01296700_m1 (Life Technologies)	
mAMPK α 2(Prkaa2)	Mm01264789_m1 (Life Technologies)	
siRNA SIRT1	AAATGTCTCCACGAACAGC	GCTGTTCTGGAGACATTT
siRNA neg control	AM4635, AM4637, AM4390844 (Life Technologies)	
esiRNA EGFP	EHUEGFP (Sigma-Aldrich)	
esiRNA mouse Prkaa1	EMU190491 (Sigma-Aldrich)	
esiRNA mouse Prkaa2	EMU052501 (Sigma-Aldrich)	

^aFor adenoviral PGC-1 α detection.

esiAMPK samples were treated with 0.25 mM metformin 30 h post-transfection. After 42–48 h, cells were collected for RNA isolation.

Metformin in vivo experiment

The *in vivo* experiments were approved by the Finnish Animal Experiment Board (Permit ESAVI-2010-06188). C57/BL6NHsd mice (Harlan Laboratories, NL) were housed in animal room at 21 $\pm$ 3°C with a 12h:12h dark:light cycle. Mice were on a chow diet (69 kcal% carbohydrates, 22 kcal% protein and 9 kcal% fat; CRM(E), SDS, UK) and food and water were available *ad libitum*. Female, C57BL/6, pregnant mice were administered metformin 300 mg·kg⁻¹ day⁻¹, i.g. (n = 3), or vehicle being either saline or tap-water (n = 5) for 18 days (E0.5–17.5) as described previously (Salomaki *et al.*, 2013). The animals were killed 24 h after the last metformin dose and the livers were collected. Alternatively, 16–17-week-old male C57BL/6 mice were given metformin (300 mg·kg⁻¹) i.g. for 7 days between 07:00 and 10:00 h. On the seventh day of the treatment, metformin (n = 8) or saline (n = 8) was given 3 h prior to killing. Mixed medetomidine and ketamine anaesthesia combined with decapitation were used to kill the mice in all the experiments (Salomaki *et al.*, 2013). All studies involving

animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). According to an allometric body surface area method (Reagan-Shaw *et al.*, 2008), 300 mg·kg⁻¹ for mice corresponds to a dose of 1.7 g metformin day⁻¹ for a 70 kg man.

RNA preparation and quantitative RT-PCR

Total RNA was isolated using TRI-Reagent (Molecular Research Center, Cincinnati, OH, USA) or RNeasy mini kit for *in vivo* mice (Qiagen, Hilden, Germany) and cDNA generated as described previously (Buler *et al.*, 2012). Quantitative PCR reactions were performed with Applied Biosystems 7300 real time PCR system (Applied Biosystems, Carlsbad, CA, USA) using FastStart Universal SYBRGreen Master Mix or Probe Master Mix (Roche). Sequences of the primers and probes are listed in Table 1. Fluorescence values of the qPCR products were corrected with the fluorescence signals of the passive reference dye (ROX). qPCR results are presented as relative mRNA levels normalized against reference control 18S values (typically between 13 and 17 Ct) using the comparative Ct method (2^{- $\Delta\Delta$ Ct} method).

Western blot

Total and nuclear protein fractions were prepared as described previously (Schreiber *et al.*, 1989; Lamsa *et al.*, 2010) and were subjected to SDS-PAGE (10–12% in polyacrylamide) and transferred to a PVDF membrane (Millipore, Billerica, MA, USA). Membranes were incubated with an appropriate primary antibody in 5% skimmed milk in Tris-buffered saline with 0.1% Tween usually overnight followed by incubation with a secondary HRP-conjugated antibody. The immunoreactive bands were visualized by use of a chemiluminescent peroxidase substrate 1 reaction (Sigma-Aldrich).

Plasmids, transient transfection assays and immunoprecipitation

The *PGC-1 α* 5'-Luc and *PGC-1 α* 5'- Δ CRE-Luc reporter constructs were from Addgene (<http://www.addgene.org> plasmids 8887 and 8888) (Handschin *et al.*, 2003). Of the reporter plasmid, 0.5 μ g were transfected per well (12-well plates at a density of 3×10^5 mouse primary hepatocytes) using Tfx-20 reagent (Promega, Madison, WI, USA). Twenty-four hours post transfection, cells were treated with chemicals as indicated earlier. After 24 h, cells were assayed for luciferase activity with the Dual-Luciferase Reporter Assay System (Promega).

HNF-4 α response element in front of TATA box was cloned into pGL3-Basic reporter vector. Of the reporter vector, 0.5 μ g was transfected per well (24-well plates at 80% confluence of HepG2 cells) using Eugene HD transfection reagent (Roche). Twenty-four hours post transfection cells were treated with metformin and 24 h later assayed for luciferase as mentioned earlier.

Of the *PGC-1 α* -FLAG, 15 μ g was transfected per plate (10 cm plates of HepG2 cells) using Eugene HD transfection reagent (Roche). Twenty-four hours post transfection, cells were treated with metformin and collected after another 72 h. *PGC-1 α* -FLAG was immunoprecipitated using a FLAG immunoprecipitation kit and immunoblotted against acetylated lysine (anti-Ac-lys), mouse *PGC-1 α* antibody was used as loading control.

Statistical analysis

Statistical analysis of the data was performed using GraphPad Prism Software (La Jolla, CA, USA). Unless stated otherwise, the comparison of means of two groups was done by Student's *t*-test, whereas multiple groups or two variables were compared by one-way and two-way ANOVAs followed by Dunnett's *post hoc* test. Statistically significant values are marked **P* < 0.05, ***P* < 0.01 or ****P* < 0.001 compared with untreated control or #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001 compared with the indicated treatment.

Results

Metformin down-regulates gluconeogenic genes, but induces *PGC-1 α* expression in hepatocytes

The effect of metformin on the expression of PEPCK, G6Pase and *PGC-1 α* was studied in mouse primary hepatocytes. After 48 h of treatment, the expressions of PEPCK and G6Pase

mRNAs were significantly down-regulated. In contrast, *PGC-1 α* mRNA was increased almost threefold by 1 mM metformin (Figure 1A). To determine the time course of *PGC-1 α* induction by metformin, 1 mM metformin was used as this concentration induced a maximal increase in *PGC-1 α* after a 48 h. The metformin concentrations used are higher than the observed blood concentrations in clinical use. However, because of hepatic accumulation, these experimental concentrations may reflect the *in vivo* intrahepatic concentrations as discussed by Foretz *et al.* (2010). An increase in *PGC-1 α* was detected after 12 h, but the highest level of *PGC-1 α* was observed at the last time point of 72 h. As expected, PEPCK and G6Pase were down-regulated (Figure 1B). The functional significance of the induction of *PGC-1 α* mRNA by metformin was also confirmed at the protein level; *PGC-1 α* protein level was increased fourfold after 48 h (Figure 1C).

We next studied whether metformin also induces *PGC-1 α* expression in human hepatocytes. Human hepatocytes were treated for 48 h with 0.5, 1 and 2 mM metformin. Similar to the mouse, human *PGC-1 α* mRNA was also up-regulated by metformin (Figure 1D). However, compared with mouse hepatocytes, a higher concentration of metformin was required to produce this effect. Furthermore, PEPCK was significantly down-regulated by metformin in the hepatocytes (Figure 1D). Essentially similar results were also observed after 24 h of metformin treatment of a separate batch of human hepatocytes (data not shown).

We also measured the effect of metformin *in vivo*. Female pregnant mice were treated with 300 mg·kg⁻¹ day⁻¹ metformin, i.e., for 18 days. The livers were collected 24 h after the last drug dose and analysed for *PGC-1 α* and PEPCK. *PGC-1 α* mRNA was not affected by metformin, instead, PEPCK was down-regulated by 40% (Figure 1E). We further studied the *in vivo* effects by treating male mice for 7 days with 300 mg·kg⁻¹ day⁻¹ metformin, i.e.; however, the samples were collected only 3 h after the last dose. In this experiment, *PGC-1 α* mRNA was increased 1.62-fold by metformin, but PEPCK was not affected (Figure 1F).

Metformin induces *PGC-1 α* through an AMPK and SIRT1 mediated mechanism

Metformin is a known activator of the energy sensor AMPK. We next investigated the involvement of AMPK in the *PGC-1 α* induction of metformin. Treatment of hepatocytes with two pharmacological activators of AMPK; AICAR and metformin resulted in opposite effects on *PGC-1 α* expression. While metformin time-dependently increased *PGC-1 α* , AICAR effectively down-regulated *PGC-1 α* expression (Figure 2A). Nevertheless, both compounds activated AMPK as assessed by phosphorylation of the AMPK target pACC (Figure 2B). The role of AMPK was further studied by using compound C, a pharmacological inhibitor of AMPK. Compound C abolished the induction of *PGC-1 α* by metformin, even reducing the expression level below that of the untreated control. In contrast, compound C potentiated the effect of AICAR (Figure 2C). A769662 is a small-molecular compound that directly activates AMPK. A769662 0.1 mM slightly increased the expression of *PGC-1 α* , but this was not statistically significant (Figure 2D). To study the effect of AMPK directly, hepatocytes were transduced with an

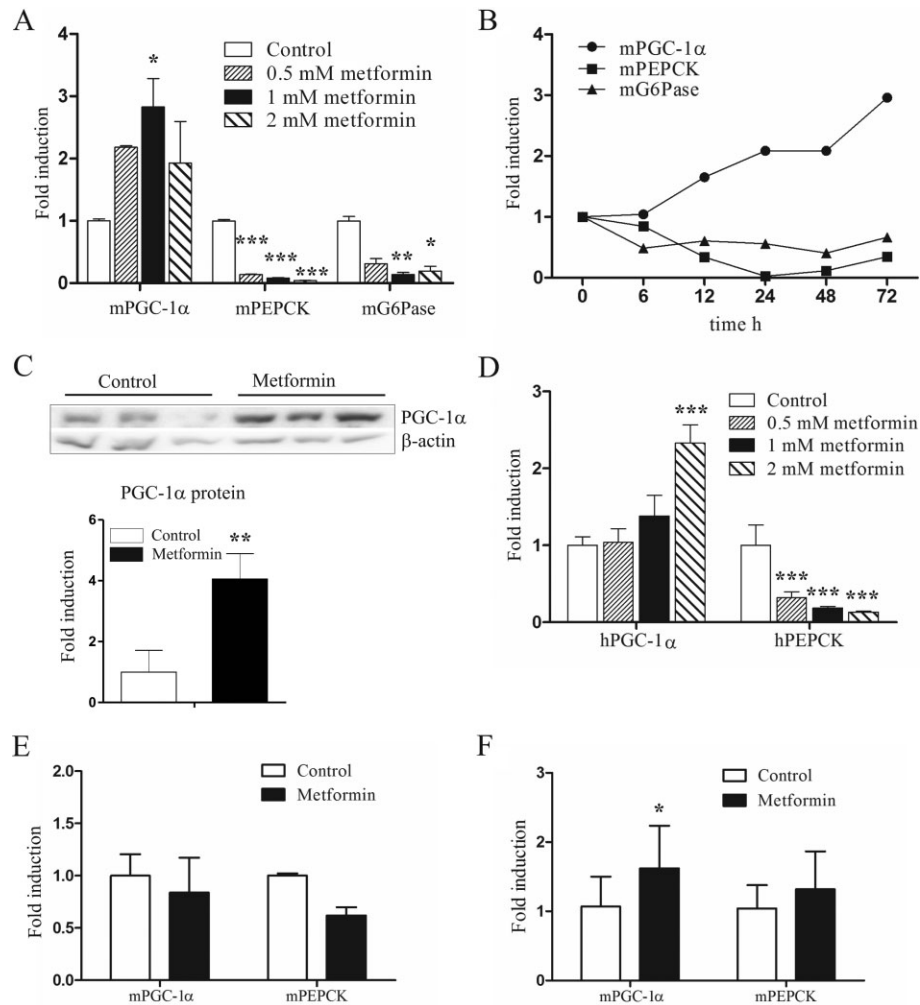


Figure 1

Effect of metformin treatment on PGC-1 α and gluconeogenic gene expression. (A) Mouse primary hepatocytes were treated with metformin for 48 h after which mRNAs were measured by RT-qPCR ($n = 3$). (B) Mouse primary hepatocytes were treated with 1 mM metformin for the indicated time periods after which mRNAs were measured by RT-qPCR ($n = 3$, but combined before analysis). (C) PGC-1 α protein was detected by immunoblotting in total protein fractions of mouse primary hepatocytes treated with metformin for 48 h. β -Actin was measured as a loading control. ($n = 3$) (D) Human primary hepatocytes were treated with metformin for 48 h and measured by RT-qPCR ($n = 3$). (E) Mice were administered metformin i.g. 300 mg·kg⁻¹ daily for 18 days. Livers were collected 24 h after the last dose after which mRNA was isolated from the vehicle ($n = 5$) or metformin ($n = 3$) treated individuals and measured by RT-qPCR. Statistical significance was not calculated. (F) Mice were administered metformin i.g. 300 mg·kg⁻¹ daily for 7 days. Livers were collected 3 h after the last dose after which mRNA was isolated from the vehicle ($n = 8$) or metformin ($n = 8$) treated individuals and measured by RT-qPCR. qPCR and Western blot results represent means of fold change + SD.

adenovirus coding for the constitutively active mutant of AMPK α 1 (AMPK-Ad). After 24 h, the expression of PGC-1 α was increased dose-dependently up to fivefold (Figure 2E). AMPK-Ad slightly down-regulated the expression of PEPCK. We also performed AMPK knockdown experiments with siRNAs against AMPK α 1 and AMPK α 2. In particular, AMPK α 2 was effectively knocked down by the siRNA used, whereas the effect of the AMPK α 1 siRNA was less noticeable (Figure 2F). The basal level of PGC-1 α was significantly reduced by the combination of AMPK α 1 and AMPK α 2 siRNAs. The effect of 0.25 mM metformin on PGC-1 α was modest in this particular experiment and did not reach statistical significance. However, the combination of AMPK α 1

and AMPK α 2 siRNAs also reduced PGC-1 α expression in metformin-treated samples. Altogether, these results suggest that AMPK is involved in the up-regulation of PGC-1 α induced by metformin, whereas the down-regulation of PGC-1 α induced by AICAR is mediated by an AMPK-independent mechanism.

AMPK activates SIRT1 by increasing cellular nicotinamide adenine dinucleotide (NAD)⁺ levels; also, metformin induces SIRT1 expression (Canto *et al.*, 2009; Caton *et al.*, 2010). Furthermore, in skeletal muscle, SIRT1 activation increases PGC-1 α mRNA expression (Amat *et al.*, 2009). We, therefore, tested whether SIRT1 is involved in the increase in PGC-1 α induced by metformin in hepatocytes. Indeed, SIRT1 siRNA

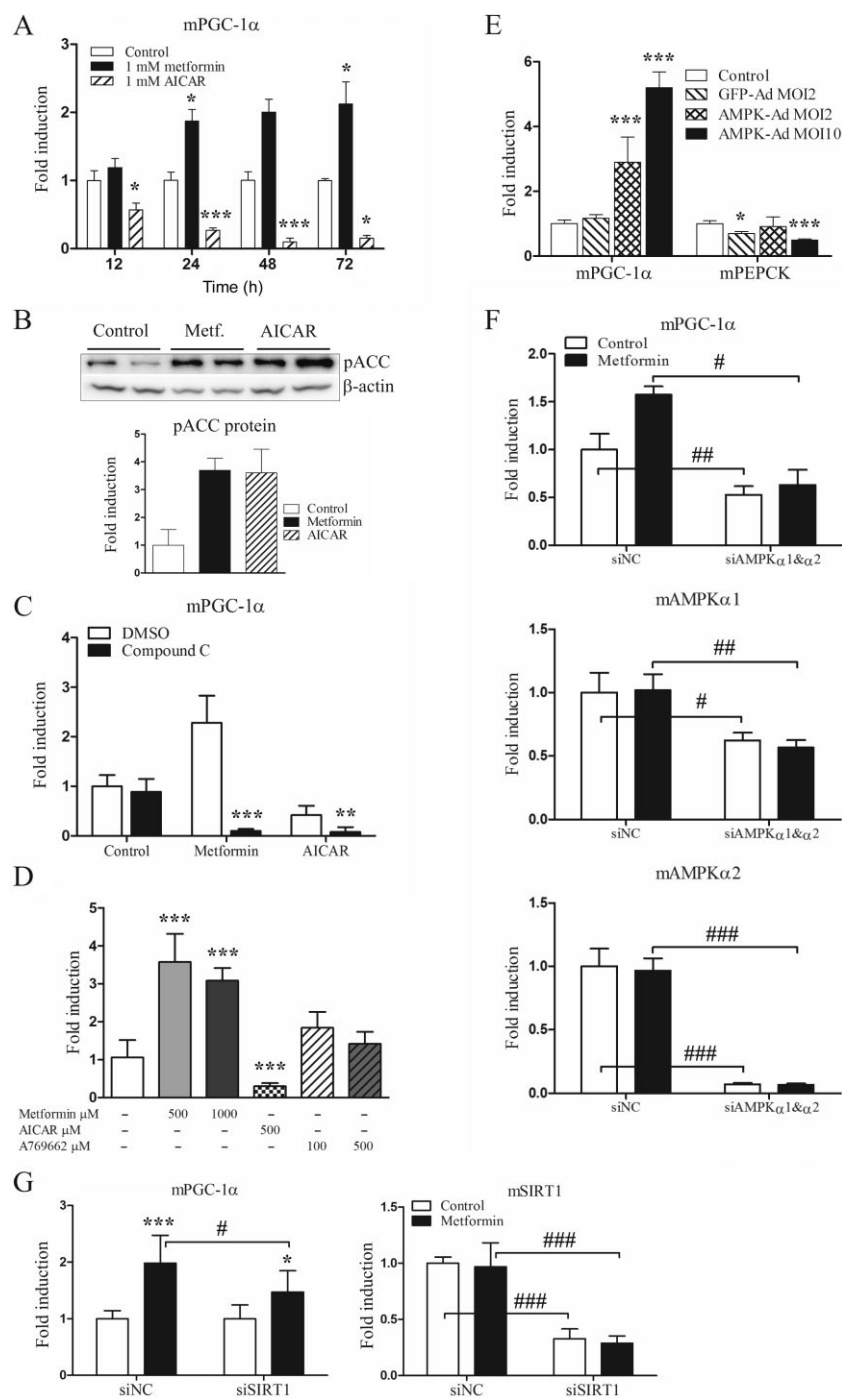


Figure 2

AMPK modulators and AMPK overexpression affect mouse PGC-1 α expression level. (A) Mouse primary hepatocytes were treated with 1 mM metformin or 1 mM AICAR for 12, 24, 48 and 72 h after which mRNAs were analysed by RT-qPCR ($n = 4$). (B) Mouse primary hepatocytes were treated with 1 mM metformin or 1 mM AICAR for 8 h after which cells were sonicated and centrifuged at 13 000 $\times g$, supernatant was collected and immunoblotted against pACC antibody. β -Actin was measured as a loading control ($n = 2$, statistical significance was not calculated). (C) Mouse primary hepatocytes were treated with either 1 mM metformin or 1 mM AICAR alone or combined with 20 μ M compound C in DMSO for 24 h after which mRNAs were analysed by RT-qPCR. ($n = 4$) (D) Mouse primary hepatocytes were treated with metformin, AICAR or A769662 for 48 h after which mRNAs were analysed by RT-qPCR ($n = 3$). (E) Mouse primary hepatocytes were infected with GFP-Ad (MOI 2) or AMPK-Ad (MOI 2 and 10) for 24 h after which mRNAs were measured using RT-qPCR ($n = 3$). (F) Mouse primary hepatocytes were transfected with siRNAs; negative control siGFP (siNC) or siAMPK α 1 combined with siAMPK α 2. Metformin 0.25 mM was added 30 h post-transfection and incubated for 42 h after which samples were analysed by RT-qPCR ($n = 3$). (G) Mouse primary hepatocytes were transfected with siRNAs; negative control (siNC) or siSIRT1. Metformin 0.25 mM was added 5 h post-transfection and incubated for 48 h after which samples were analysed by RT-qPCR ($n = 9$). qPCR results represent means of fold change $\pm$ SD and Western blot results means of fold change $\pm$ range.

attenuated the increase in PGC-1 α induced by metformin (Figure 2G).

Rotenone is a complex I inhibitor that has been reported to induce PGC-1 α in hepatic cells through activation of CRTC3 (Than *et al.*, 2011). As metformin also inhibits complex I (El-Mir *et al.*, 2000), we studied the involvement of CRTC3 in the increase in PGC-1 α induced by metformin. CRTC3 siRNA had no effect on this increase in PGC-1 α , indicating that CRTC3 plays no role in this effect of metformin (data not shown).

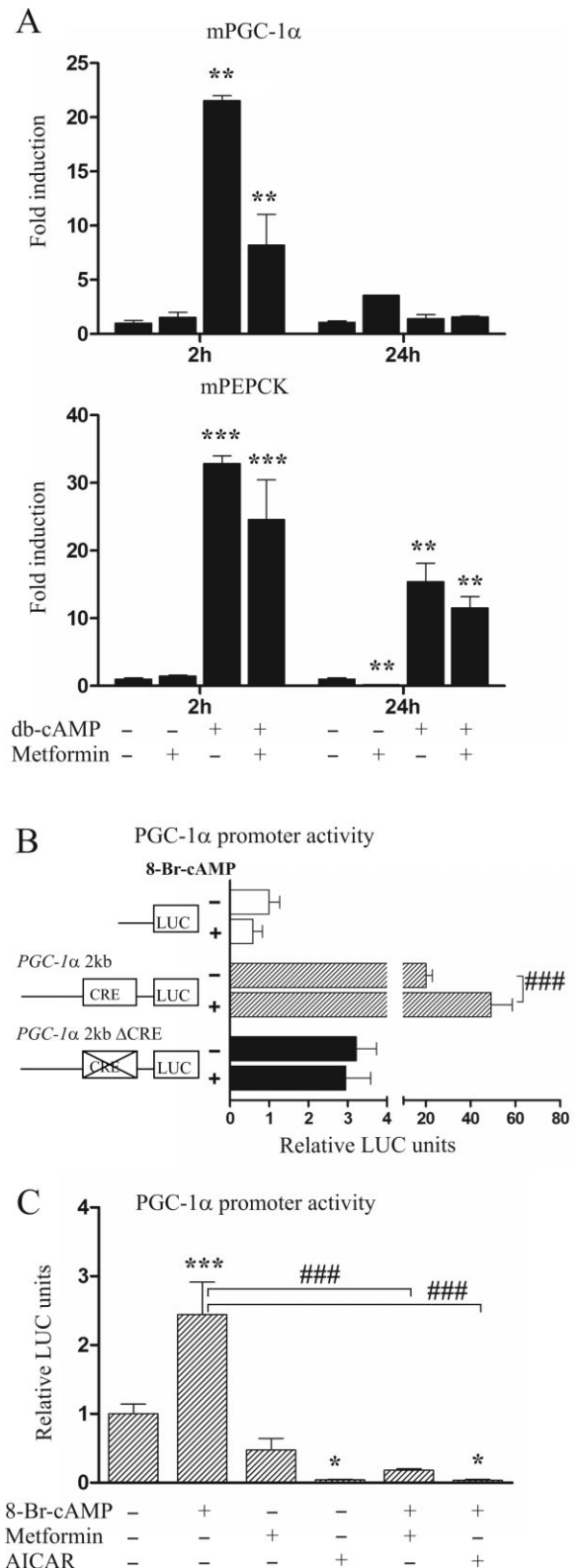
Metformin attenuates the increase in PGC-1 α induced by cAMP

Fasting is known to induce PGC-1 α expression through a mechanism involving CREB activation by cAMP (Herzig *et al.*, 2001). Furthermore, in a diabetic state, the altered balance of glucagon and insulin results in cAMP elevation in liver. Therefore, we studied the effect of metformin on the increase in PGC-1 α induced by cAMP. Treatment of primary hepatocytes with db-cAMP alone for 2 h increased PGC-1 α and PEPCK mRNAs 22- and 33-fold respectively (Figure 3A). After 24 h, this increase in PGC-1 α was no longer observed while PEPCK still remained up-regulated by 15-fold. Metformin treatment alone increased PGC-1 α 3.6-fold and down-regulated PEPCK by 0.2-fold compared with untreated controls after 24 h. The combination of db-cAMP with metformin attenuated the increase in PGC-1 α induced by cAMP to 37% of that induced by cAMP alone after 2 h. Similarly, although less efficiently, metformin also affected the increase in PEPCK induced by cAMP after 2 h. These results indicate that although metformin *per se* induces PGC-1 α expression, it has an antagonistic effect on cAMP-mediated up-regulation of PGC-1 α .

Induction of PGC-1 α by metformin is not mediated by the proximal PGC-1 α promoter

Next, we studied the involvement of the PGC-1 α gene proximal promoter in the regulation of PGC-1 α by metformin. We first transfected luciferase reporter construct regulated by -2533 to +78 bp of the PGC-1 α 5'-flanking region (pGL3-basic-PGC-1 α 2 kb) to mouse primary hepatocytes. cAMP has been shown to regulate PGC-1 α through a CREB-binding element located at -146 to -129 in the PGC-1 α 5'-flanking region. Therefore, we also transfected a PGC-1 α construct with mutated cyclic AMP-responsive element (CRE) site

(pGL3-Basic-PGC-1 α - Δ CRE). As expected, cAMP increased the wild-type construct, but not the CRE mutant construct. Interestingly, the CRE element also appeared to contribute to the constitutive expression of the PGC-1 α gene, and the CRE mutant construct was significantly less active than the wild type (Figure 3B).



Treatment of pGL3-Basic-PGC-1 α 2 kb transfected hepatocytes with metformin alone had no significant effect, while AICAR strongly down-regulated the luciferase activity. Both metformin and AICAR abolished the cAMP-mediated induction of PGC-1 α 2 kb-regulated luciferase activity (Figure 3C). Together, these results indicate that the induction of PGC-1 α by metformin is not mediated by the PGC-1 α 5'-flanking region studied. However, the metformin-induced suppression of the increase in PGC-1 α induced by cAMP is mediated by the previously identified CRE element.

Metformin selectively affects PGC-1 α regulated functions

PEPCK is an established target gene of PGC-1 α and overexpression of PGC-1 α strongly induces PEPCK expression (Yoon *et al.*, 2001). However, we found that metformin up-regulates PGC-1 α while simultaneously down-regulating the expression of PEPCK mRNA. This suggests that metformin affects the ability of PGC-1 α to induce PEPCK transcription. To test this hypothesis, we transduced mouse primary hepatocytes with PGC-1 α -Ad and treated the cells with metformin. Both PEPCK and G6Pase mRNA induction were reduced significantly by metformin indicating that metformin is able to reduce the co-activator function of PGC-1 α with regard to these genes (Figure 4A).

Mitochondrial biogenesis is another key cell function regulated by PGC-1 α (Wu *et al.*, 1999). Next, we studied if metformin also diminishes stimulation of mitochondrial biogenesis by PGC-1 α . PGC-1 α -Ad induced the expression of several genes coding mitochondrial proteins. Treatment of the cells with metformin did not affect the induction of these genes by PGC-1 α , indicating that metformin differentially affects the regulation of mitochondrial biogenesis and gluconeogenesis by PGC-1 α (Figure 4B).

Characterization of mechanisms by which metformin selectively prevents PGC-1 α -mediated up-regulation of gluconeogenesis

Next, we studied potential mechanisms that could prevent the induction of the gluconeogenic genes by PGC-1 α . PGC-1 α function is regulated by post-transcriptional modifications. Two modifications potentially relevant for metformin's action include phosphorylation by AMPK (Jager *et al.*, 2007) and deacetylation by SIRT1 (Rodgers *et al.*, 2005). Furthermore, a priming signal by AMPK-mediated phosphorylation appears to be necessary for SIRT1-mediated deacetylation of PGC-1 α (Canto *et al.*, 2009). To study the potential effect of PGC-1 α phosphorylation by AMPK, we prepared an adenovirus with mutated AMPK phosphorylation sites in the PGC-1 α expression vector (T177A, S538A). Primary hepatocytes were transduced with the phospho-mutant or the wild type PGC-1 α adenoviruses and treated with metformin. After 48 h, the cells were collected and mRNA expression of the PEPCK and G6Pase mRNA was measured. Mutation of the phosphorylation sites did not affect the induction of the genes studied by PGC-1 α . Furthermore, metformin similarly down-regulated the induction of some genes despite the mutation (data not shown). This indicates that the attenuation of the PGC-1 α -mediated gluconeogenic gene induced by metformin does not involve PGC-1 α phosphorylation by

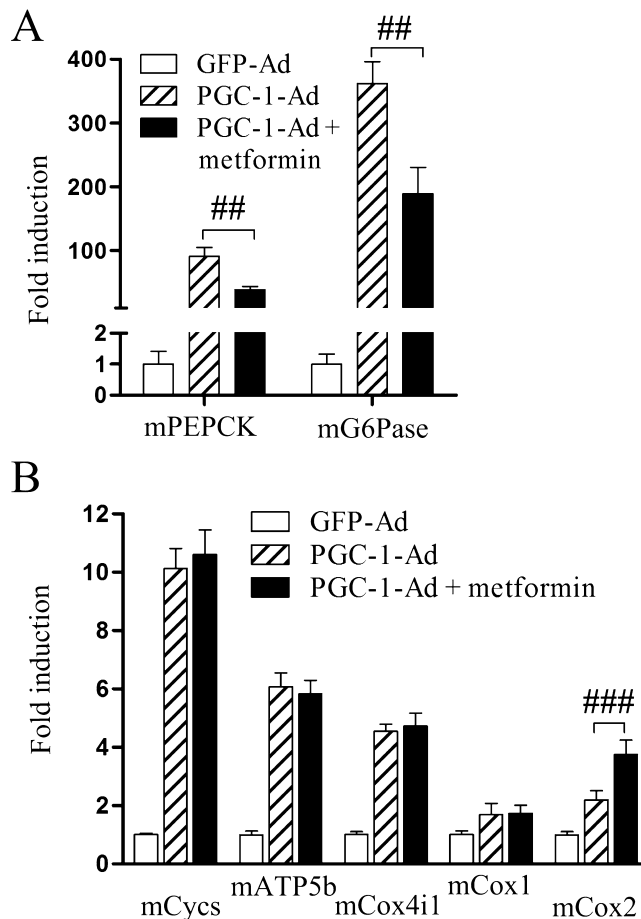


Figure 4

The functional effect of metformin on gluconeogenesis and selected mitochondrial-acting genes. Mouse primary hepatocytes were infected with PGC-1 α -Ad at MOI 1 for 24 h after which 1 mM metformin was added. After 48 h cells were collected and mRNAs analysed by RT-qPCR for measuring (A) PEPCK and G6Pase expression and (B) selected genes encoding mitochondrial proteins. Results are compared with GFP-Ad treatment, set as onefold and normalized to equate the levels of PGC-1 α expression between the treatments; ($n = 3$). qPCR results represent means of fold change + SD.

AMPK. Furthermore, we studied the effect of metformin on PGC-1 α acetylation status in HepG2 cells. Treatment with metformin resulted in 40% deacetylation of PGC-1 α compared with the control (Figure 5A) in agreement with SIRT1 activation by metformin.

We also considered other putative mechanisms for the suppression of gluconeogenic genes. The small heterodimer partner NROB2 (SHP) is supposedly induced by metformin through AMPK and inhibits HNF-4 α -mediated induction of PEPCK (Kim *et al.*, 2008). However, in contrast to the result expected, we were unable to detect any up-regulation of SHP expression in primary hepatocytes treated with metformin. In fact, SHP mRNA levels were down-regulated by both metformin and AICAR (Figure 5B).

Next, we hypothesized that metformin could modulate the expression of the PGC-1 α target genes by regulating the DNA binding transcription factors interacting with PGC-1 α .

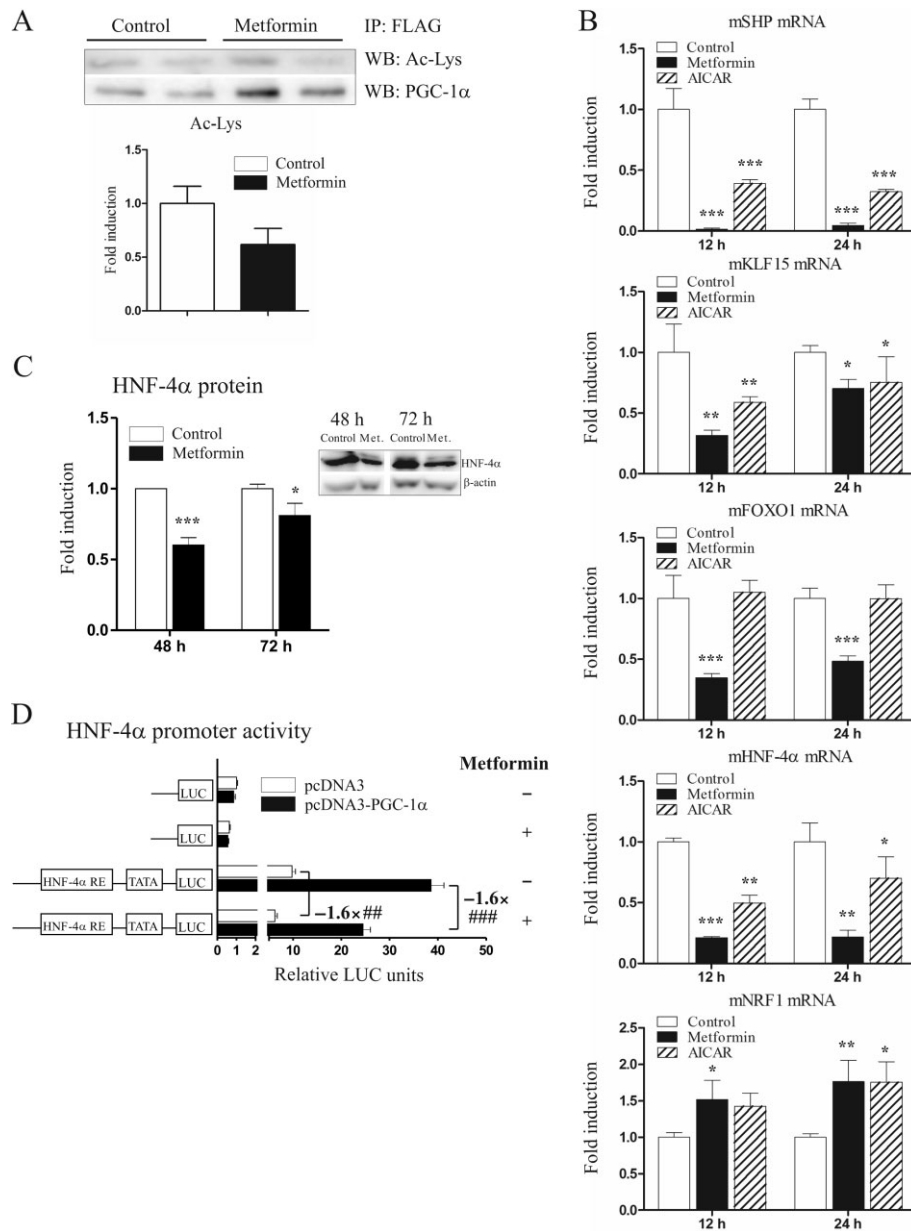


Figure 5

Effect of metformin on acetylation of PGC-1 α and genes regulating PEPCK and G6Pase. (A) HepG2 cells were transfected with PGC-1 α -FLAG plasmid and 24 h post transfection treated with 2 mM metformin for 72 h after which FLAG fusion protein was isolated by immunoprecipitation. The protein fractions were immunoblotted against acetylated lysine antibody (Ac-Lys) and PGC-1 α antibody was used as a loading control ($n = 2$, statistical significance was not calculated). (B) Mouse primary hepatocytes were treated with 1 mM metformin or 1 mM AICAR for 12 and 24 h after which mRNAs were analysed by RT-qPCR ($n = 4$). (C) Mouse primary hepatocytes were treated with 1 mM metformin for 48 and 72 h after which nuclear protein fractions were immunoblotted against HNF-4 α antibody and β -actin was used as a loading control ($n = 2$ for 48 h/ $n = 3$ for 72 h, two/three parallel experiments combined). Immunoblot is representative of one experiment. (D) HepG2 cells were transfected with HNF-4 α -TATA-LUC or promoterless control plasmid pGL3-Basic (LUC), and after 24 h were treated with metformin for 24 h ($n = 6$). Results are relative luciferase units normalized to control LUC set as onefold. qPCR and Western blot results represent means of fold change + range and luciferase results means of relative LUC units + SD.

Krüppel-like factor 15 (KLF15), forkhead box protein O1 (FOXO1) and HNF-4 α have all been shown to regulate PEPCK and G6Pase genes, and are co-activated by PGC-1 α (Puigserver *et al.*, 2003; Rhee *et al.*, 2003; Takashima *et al.*, 2010). We, therefore, investigated the effect of metformin on

the expression of these transcription factors in primary hepatocytes. Metformin down-regulated the mRNAs of all three transcription factors after 12 and 24 h treatment (Figure 5B). Also AICAR down-regulated KLF15 and HNF-4 α , but had no effect on FOXO1 (Figure 5B). Furthermore, treat-

ment with 1 mM metformin down-regulated HNF-4 α protein levels after 48 and 72 h (Figure 5C).

PGC-1 α induces the expression of many nuclear- and also indirectly mitochondrial-encoded mitochondrial proteins through a mechanism involving nuclear respiratory factor 1 (NRF1) (Wu *et al.*, 1999). We therefore analysed the effect of metformin treatment on NRF1 expression. In contrast to the transcription factors involved in the regulation of the gluconeogenic genes, NRF1 was increased by metformin (and also AICAR) treatment suggesting that the availability of the DNA binding transcription factors co-activated by PGC-1 α influences the functional selectivity of PGC-1 α .

Furthermore, we investigated the effect of metformin on the co-activation of HNF-4 α by PGC-1 α . We transfected HepG2 cells with a reporter construct under the control of the HNF-4 α site. HepG2 cells express constitutively high levels of HNF-4 α , but little PGC-1 α . Cotransfection of the cells with a PGC-1 α plasmid further activated luciferase activity indicating co-activation of HNF-4 α by PGC-1 α . Treatment of the cells with metformin down-regulated the luciferase activity 1.6-fold both with and without PGC-1 α compared with untreated cells (Figure 5C). These results suggest that metformin does not affect the co-activation function of the PGC-1 α or its interaction with HNF-4 α . Instead, the diminished gene expression is mediated by a down-regulation of the DNA binding transcription factors, in this case HNF-4 α .

Discussion and conclusions

PGC-1 α has an important role in regulating hepatic energy balance and has been associated with the pharmacodynamic effects of metformin. Shaw *et al.* suggested that transcriptional regulation of PGC-1 α through co-activator CRTC2 is a key step in the mechanism of action of metformin (Shaw *et al.*, 2005). Our current study, however, shows that transcriptional regulation of the *PGC-1 α* gene is not a major mechanism by which metformin inhibits gluconeogenesis. Indeed, the ability of metformin to down-regulate gluconeogenic genes is not dependent on the expression level of PGC-1 α . However, PGC-1 α -mediated gene regulation is affected selectively by metformin and activation of the mitochondrial biogenesis by PGC-1 α is not disrupted by metformin.

We showed for the first time that the primary effect of metformin is mediated by the induction of both mRNA and protein levels of hepatic PGC-1 α . The mRNA induction was confirmed in both mouse and human primary hepatocytes. However, the 18 day treatment of mice with 300 mg·kg⁻¹ metformin *in vivo* did not result in changes in PGC-1 α expression in the liver. The reasons for this lack of PGC-1 α induction in liver *in vivo* are unclear. One possibility is an insufficient hepatic metformin concentration (average blood concentration 174 nmol·L⁻¹ at the time of sample collection). Indeed, the induction of PGC-1 α by metformin in skeletal muscle was demonstrated with a much higher dose (631 mg·kg⁻¹) (Suwa *et al.*, 2006). Furthermore, it is possible that PGC-1 α is induced only transiently during the intrahepatic peak concentrations of metformin. In support of this hypothesis, when we collected the liver samples 3 h after the last metformin dose (altogether 7 days, 300 mg·kg⁻¹) we detected a modest, but statistically significant, increase in PGC-1 α . Similar to

skeletal muscle (Suwa *et al.*, 2006; Jager *et al.*, 2007), we demonstrated that the effect of metformin on PGC-1 α expression is mediated by AMPK in hepatocytes. This is supported by several lines of evidence. Firstly, compound C completely abolished the induction of PGC-1 α by metformin; and secondly, AMPK-Ad induced PGC-1 α mRNA expression. In addition, the knocking down of AMPK resulted in diminished PGC-1 α levels in both untreated and metformin-treated hepatocytes, further suggesting a role for AMPK in the regulation of PGC-1 α levels. Interestingly, another established AMPK modulator, AICAR had an opposite effect on PGC-1 α expression and inhibited the transcription of PGC-1 α very efficiently. AICAR and metformin have distinct ways to activate AMPK. While metformin targets mitochondrial respiratory complex I (El-Mir *et al.*, 2000) causing inhibition of ATP production (Owen *et al.*, 2000), AICAR, in contrast, is quickly converted to 5-aminoimidazole-4-carboxamide-1 (ZMP) in cells mimicking 5'-AMP (Sullivan *et al.*, 1994). Indeed, both compounds also activated AMPK in our current study. Nevertheless, AICAR must regulate PGC-1 α in an AMPK-independent fashion. Interestingly, several such functions of AICAR have been described (Viollet *et al.*, 2009; Santidrian *et al.*, 2010). In support of the AMPK-independent repression of PGC-1 α by AICAR, A769662, a direct, small molecular activator of AMPK (Oakhill *et al.*, 2009) did not suppress PGC-1 α expression, but rather had a tendency to increase PGC-1 α . AICAR is a popular experimental compound to study AMPK function, but our observations indicate that the results obtained using AICAR should be interpreted with caution.

Furthermore, we showed that the induction of PGC-1 α by metformin involves SIRT1. This is in agreement with previous results obtained in skeletal muscle (Amat *et al.*, 2009). Metformin induces SIRT1 activity by increasing the NAD⁺/NADH ratio and protein level through an AMPK-mediated mechanism in hepatocytes (Caton *et al.*, 2010). Metformin also induces SIRT1 mRNA, but a relatively high concentration is needed (Buler *et al.*, 2012). With the low, 0.25 mM concentration of metformin, we did not detect any increase in SIRT1 mRNA. However, SIRT1 siRNA clearly attenuated the increase in PGC-1 α induced by metformin indicating that SIRT1 activation, presumably through an increased NAD⁺/NADH ratio, plays a role in this effect of metformin. In skeletal muscles, PGC-1 α T177/S538 phosphorylation by AMPK results in autoregulation through the proximal promoter (Jager *et al.*, 2007). However, in hepatocytes, the overexpression of PGC-1 α by an adenovirus had no effect on the expression of endogenous PGC-1 α suggesting that autoregulation plays no significant role (data not shown). Furthermore, metformin did not affect luciferase activity under the control of the 2 kb proximal 5'-flanking region of the PGC-1 α gene in primary hepatocytes. Thus the exact regulatory mechanisms mediating the increase in PGC-1 α induced by metformin in liver appear to be different from those in skeletal muscle.

While metformin primarily induced PGC-1 α expression, it was still able to attenuate the increase in PGC-1 α induced by cAMP. This is in agreement with the previous report showing that AMPK-mediated phosphorylation of CRTC2 reduces PGC-1 α expression (Shaw *et al.*, 2005). Indeed, this may play some role in the action of metformin in diabetic patients. However, a down-regulation in PGC-1 α expression is obviously not needed for the reduction in gluconeogenesis

induced by metformin. In fact, metformin attenuated the induction of PEPCK and G6Pase induced by PGC-1 α overexpression indicating that metformin controls the ability of PGC-1 α to regulate target gene activation. Besides stimulating gluconeogenesis, PGC-1 α is a critical regulator of mitochondrial biogenesis (Handschin, 2009). In particular, PGC-1 α -Ad induced nuclear-encoded cytochrome c (Cycs), ATP synthase subunit β (ATP5B) and cytochrome c subunit 4 (Cox4i1), and to a lesser extent mitochondrial encoded cytochrome c oxidase I and II (Cox1 and Cox2, respectively). While metformin reduced this PGC-1 α -stimulated induction of gluconeogenic genes, mitochondrial genes were equally induced with and without metformin indicating a selective effect of metformin on PGC-1 α -mediated gene regulation.

The selective effect of metformin on PGC-1 α function resembles that recently described for PGC-1 α phosphorylation by S6 kinase activated upon feeding (Lustig *et al.*, 2011). However, metformin has been reported to inhibit the mammalian target of rapamycin/S6 kinase pathway (Dowling *et al.*, 2007) suggesting that phosphorylation by S6 kinase may not be involved in this effect of metformin on PGC-1 α . We, therefore, investigated several mechanisms that could potentially prevent the activation of gluconeogenesis by PGC-1 α . It has been suggested that metformin up-regulates SHP, which then acts as a repressor of HNF-4 α - and FOXO1-mediated regulation of gluconeogenic gene transcription (Kim *et al.*, 2008). Unexpectedly, we were unable to corroborate these results. In fact, SHP was significantly down-regulated in primary hepatocytes by 12 h after metformin treatment. Also, Krausova *et al.* did not detect an increase in SHP induced by metformin in primary hepatocytes (Krausova *et al.*, 2011). The reason for these differences is currently unknown. We also investigated the possibility that posttranslational modification of PGC-1 α by AMPK or SIRT1 could modulate PGC-1 α function after metformin treatment, because both are activated by the drug. However, mutation of the PGC-1 α AMPK phosphorylation sites threonine-177/serine-538 did not abolish the effect of metformin on PGC-1 α function in the context of gluconeogenic genes (data not shown). However, we observed that metformin treatment resulted in deacetylation of PGC-1 α , probably mediated by activation of SIRT1. Nevertheless, PGC-1 α deacetylation should lead to increased gluconeogenic gene activation (Rodgers *et al.*, 2005) and, therefore, would not explain the observed down-regulation of the gluconeogenic genes.

KLF15, FOXO1 and HNF-4 α are the transcription factors that activate the gluconeogenic genes together with PGC-1 α (Puigserver *et al.*, 2003; Rhee *et al.*, 2003; Takashima *et al.*, 2010). We observed that metformin down-regulated all of these DNA binding transcription factors. While KLF15 and HNF-4 α were also reduced by AICAR, FOXO1 was not, again indicating differences in the response to these two AMPK activators. Recently, KLF15 was shown to contribute to suppression of hepatic glucose production induced by metformin. Administration of metformin to mice has been found to lower gluconeogenic gene levels as well as KLF15 and an upregulation in the expression of KLF15 restored the expression of PEPCK (Takashima *et al.*, 2010). Previously, FOXO1 has not been associated with the inhibition of gluconeogenesis by metformin, but metformin has been reported to reduce the expression of fatty acid-binding protein 4 by

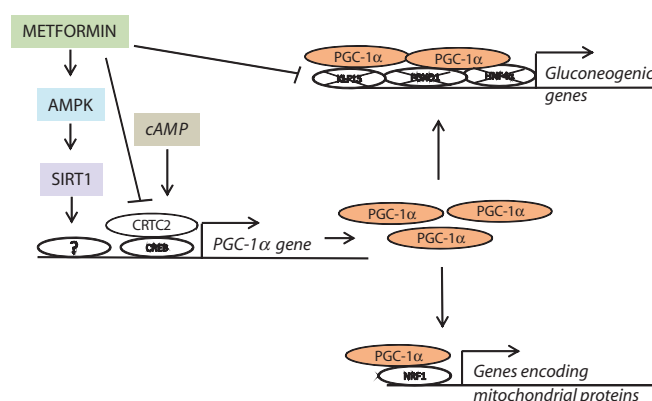


Figure 6

A proposed model for the effect of metformin on hepatic PGC-1 α regulation and function. We propose that metformin activates PGC-1 α gene transcription through AMPK and SIRT1. However, metformin represses the increase in PGC-1 α induced by cAMP. Furthermore, metformin prevents the ability of PGC-1 α to induce gluconeogenic genes.

inhibiting FOXO1 (Song *et al.*, 2010). The down-regulation of HNF-4 α by metformin has not been reported previously. However, several studies have shown that AMPK and AICAR down-regulate and destabilize HNF-4 α protein (Leclerc *et al.*, 2001; Hong *et al.*, 2003; Prieur *et al.*, 2005). We also hypothesized that metformin could decrease the expression of HNF-4 α . Indeed, we found that both HNF-4 α mRNA and protein level as well as HNF-4 α -mediated transcriptional activity were reduced by metformin. However, in the context of the reporter gene, PGC-1 α was able to activate HNF-4 α -mediated transcriptional activity regardless of the metformin treatment suggesting that metformin does not significantly inhibit the PGC-1 α -HNF-4 α interaction. Altogether, these results suggest that metformin down-regulates multiple key transcription factors and PGC-1 α interaction partners, which attenuates the ability of PGC-1 α to induce gluconeogenic genes. In contrast, the effect of PGC-1 α on mitochondrial biogenesis mediated by NRF1 is not down-regulated by metformin, which would explain its selective influence on different PGC-1 α -regulated cell functions. A proposed model of metformin's effects on PGC-1 α expression and function is presented in Figure 6.

In summary, our results indicate that the down-regulation of PGC-1 α is not necessary for suppression of gluconeogenic genes by metformin. However, metformin regulates PGC-1 α expression and function at multiple levels and by various mechanisms. Metformin increases PGC-1 α expression as such, but attenuates cAMP-mediated up-regulation of PGC-1 α . Importantly, metformin selectively affects hepatic PGC-1 α -mediated gene regulation, and prevents activation of gluconeogenesis, but does not influence its regulation of mitochondrial genes.

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Conflict of interest

None.

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